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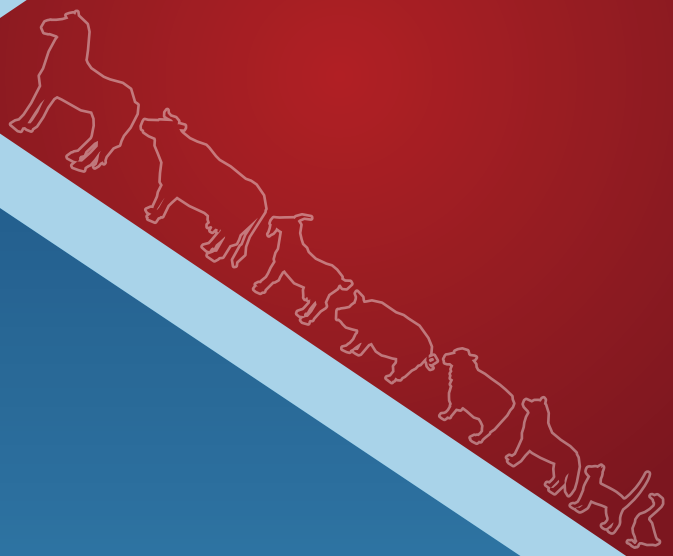

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RESEARCH ARTICLE

Comparison of the Efficacy of the Nictitating Membrane Flap, Conjunctival Pedicle Flap, and Platelet-Rich Fibrin Membrane Graft Techniques in the Surgical Management of Corneal Necrosis in Cats: A Retrospective Study (2016-2020)

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Abstract

The presented study was designed to compare the clinical features and the surgical outcomes of three techniques, such as the conjunctival pedicle flap (CPF), platelet-rich fibrin membrane graft (PRFMG), and nictitating membrane flap (NMF) applied after lamellar keratectomy to treat corneal sequestrum in cats. The postsurgical outcomes, complications, and recurrence rate of the lesions in 31 eyes of a total of 30 cats of different ages, breeds, and gender diagnosed with corneal necrosis were evaluated in the study. After partial keratectomy, patients were divided into three groups, each containing ten animals. Perioperative findings of all cases that underwent CPF, PRFMG, and NMF applications, including postsurgical complications, were evaluated. All patients were regularly monitored. Advantages and disadvantages were noted for all three techniques during and after the surgery. Despite long-term medical treatment with CPF (3-4 months), varying degrees of permanent fibrosis partially impairing vision developed in the related defect area, but no recurrence was observed in eyes that could not be fully keratectomy. In all cases with PRFMG, it was determined that the corneal granulation tissue disappeared completely within 5-6 weeks and the corneal transparency increased by 70% within 1.5-2 months, while recurrence developed in 2 cases. In eyes treated with NMF method, although tectonic support to the cornea and no additional tissue transplantation to accelerate the healing of the defect area was performed, the recovery period was fast and the duration of medical treatment applied to reduce or completely resolve the corneal fibrosis after surgery was very short. A rapid corneal restoration was determined in cats treated with the NMF method. Anatomical outcome was evaluated as positive in all methods. Both fast and satisfactory cosmetic appearance was achieved in PRFMG and NMF techniques. The results of this study proved that all three techniques are suitable for treating corneal necrosis in cats because of their practicality, relative effortlessness and non-invasiveness.

Keywords: *Cat, Corneal necrosis, Nictitating flap, Pedicle flap, PRF*

Kedilerde Kornea Nekrozunun Cerrahi Tedavisinde Nictitating Membran Flep, Konjonktival Pedikül Flep ve Trombositten Zengin Fibrin Membran Grefti Tekniklerinin Etkinliğinin Karşılaştırılması: Retrospektif Çalışma (2016-2020)

Öz

Sunulan çalışma, lamellar keratektomi sonrası uygulanan konjonktival pedikül flebi (CPF), trombositten zengin fibrin membran grefti (PRFMG) ve niktitans membran flebi (NMF) gibi üç tekniğin kedilerde kornea sekestrumunun tedavisinde klinik özelliklerini ve cerrahi sonuçlarını karşılaştırmak için tasarlanmıştır. Çalışmada kornea nekrozu tanısı konulan farklı yaş, cins ve cinsiyette toplam 30 kedinin 31 gözünde lezyonların cerrahi sonrası sonuçları, komplikasyonları ve nüks oranları değerlendirildi. Parsiyel keratektomiden sonra hastalar, her biri on hayvan içeren üç gruba ayrıldı. CPF, PRFMG ve NMF uygulanan tüm olguların ameliyat sonrası komplikasyonları da dahil olmak üzere perioperatif bulguları değerlendirildi. Tüm hastalar düzenli olarak izlendi. CPF ile uzun süreli medikal tedaviye rağmen (3-4 ay) ilgili defekt alanında görmeyi kısmen bozan değişen seviyede kalıcı fibrozis gelişti ancak tam keratektomi yapılamayan gözlerde dahi nüks görülmedi. PRFMG'li tüm olgularda 5-6 hafta içinde korneal granülasyon dokunun tamamen ortadan kalktığı ve 1.5-2 ay içinde korneal saydamlığın %70 arttığı belirlenirken, 2 olguda nüks gelişti. NMF yöntemi ile tedavi edilen gözlerde ise korneaya tektonik destek ve defekt alanına iyileşmeyi hızlandıracak herhangi bir ek doku nakli yapılmamasına rağmen iyileşme periyodu hızlıydı ve ameliyattan sonra kornea fibrozunun azaltılması veya tam olarak çözülmesi için uygulanan medikal tedavi süresi oldukça kısaydı. NMF yöntemi ile tedavi edilen kedilerde hızlı bir kornea restorasyonu belirlendi. Tüm yöntemlerde anatomik sonuç olumlu olarak değerlendirildi. PRFMG ve NMF tekniklerinde hem hızlı hem de tatmin edici kozmetik görünüm sağlandı. Bu çalışmanın sonuçları, pratiklikleri, göreceli zahmetsiz olmaları ve invaziv olmamaları nedeniyle kedilerde kornea nekrozunun tedavisi için her üç tekniğin de uygun olduğunu kanıtladı.

Anahtar sözcükler: *Kedi, Kornea nekrozu, Niktitans flep, Pedikül flep, PRF*

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Introduction

Corneal sequestration, also known as corneal mummification, corneal nigrum, and primary necrotizing keratitis, is a degenerative corneal disorder encountered in cats and horses, affecting all corneal layers from the epithelium to the Descemet membrane [1,2]. Although all cats are affected, Burmese, Persian, Siamese, and Himalayan cats are the most susceptible breeds reported [3].

The etiology of the disease is not fully understood. Various causes and predisposing factors, such as entropion, lagophthalmos, reduced corneal sensitivity, ulcerative keratitis, trichiasis, tear film abnormalities, genetics, feline herpesvirus-1 infection, have been reported [1,2,4,5]. Although there is no indication of age and gender-related predisposition, corneal sequestrum is most commonly seen between the ages of 2-7 years [6-8].

Corneal necrosis is a lesion located in the cornea's central or paracentral region as a unilateral, oval-to-round, black-to-brown, pigmented, and localized plaque [2]. In the standard approach to corneal necrosis, the treatment of choice is either surgery or medical therapy, depending on the lesion's size and the affected corneal layers' depth. Medical therapy is preferred when the lesion is restricted to the corneal epithelium, while the surgical approach is the best option if the lesion has advanced to the deep layers [1]. The most desired treatment in managing the Feline corneal sequestrum (FCS) targets recovering structural integrity with subtle alteration in corneal transparency. In most cases, veterinary ophthalmologists are inclined to treat the lesions either by applications providing tectonic support to the healthy cornea or a superficial keratectomy. The tectonic support might be achieved by conjunctival grafts, biomaterials, corneoscleral transposition, and keratoplasty [9]. Even though some authors proposed superficial keratectomy and NMF as a one-step combined method, it is feasible for the lesions in which less than 50% of the corneal stromal depth is involved. When the lesion affects deeper layers (50-70% of stromal depth), alternative methods such as conjunctival grafting, corneoconjunctival transposition (SKK), biomaterial grafting [using an amniotic membrane (AM), bovine pericardium, and porcine small intestinal submucosa (SIS)], and keratoplasty are required to reinforce corneal strength. More extensive lesions (>70% of stromal depth) and full-thickness FCS were previously fixed by AM grafting, SIS, and keratoplasty, which require expertise, sophisticated skills, and special equipment. Moreover, providing and storing donor tissue might be a hindrance. The relevant inconveniences necessitate an alternative practical, easily accessible, less costly, replicable, and sustainable technique, which involves an optically transparent biocompatible material with minimal health risk, yet with optimum biomechanical strength to properly suture ocular surfaces [9].

The PRFMG was efficiently and reliably utilized as a coadjuvant therapeutic agent, alternative to biomaterial grafting, to surgically treat ocular surface disorders in humans. The graft enhances tissue regeneration, minimizing inflammation and fibrosis. The fibrin membrane rich in growth factors is a standardized and optimized technology concerning tissue repair and regeneration, with highly qualified features, such as the ability to induce tissue regeneration, bactericidal and bacteriostatic activity, potential anti-inflammatory and antifibrotic properties, compatible with those of an biomaterial grafting. Multiple preclinical and clinical trials have well documented its success in human ophthalmology [10].

To the best of our knowledge, no reported data is available regarding the outcomes of the PRFMG technique in cats. Therefore, the presented retrospective study was designed, aiming to evaluate the effectiveness and outcomes of the NMF technique individually or in combination with either the CPF or a novel technique, the PRFMG, in the surgical treatment of deep corneal ulcers followed by lamellar keratectomy in cats with deep and full-thickness corneal necrosis of varying etiology. The study also aimed to provide insight into these techniques' reliability and efficacy regarding potential recurrence.

Material and Methods

Ethical Approval

This study carried out was approved by the Committee of Istanbul University-Cerrahpaşa, Animal Experiments Ethics with the decision numbered 2021/34.

Animal

The study material consisted of 30 cats of different breeds, ages, and gender diagnosed with corneal necrosis in Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Surgery between February 2016 and October 2020.

Ophthalmic Examination

All patients underwent a complete ophthalmic examination, including a series of tests such as pupillary light reflex, menace response, dazzle reflex, fluorescein staining test, Schirmer tear test, and intraocular pressure measurement, and fundus examination.

The cats suffering from corneal necrosis pertinent to the surgical intervention were elected by ophthalmological exam, yet the patients with eye conditions apt to hamper corneal healing, such as dry eye and loss to follow-up, were excluded from the study. Patients admitted for surgery were intraoperatively standardized as either deep stromal or full-thickness corneal necrosis and were randomly included in one of the three study groups, each containing ten cats.

Preparation for Surgery and Lamellar Keratectomy

Topical antibiotic ofloxacin (Exocin®, Ibrahim Abdi, Türkiye) and artificial tear drops sodium hyaluronate (Dryex®, Ibrahim Abdi, Türkiye) were applied three times a day, and ocular moisturizing gel carbomer (Thilo Tears®, Liba, Türkiye) was applied two times a day in all cases 1-7 days before the surgical procedure. Analgesia was provided with 0.1-0.2 mg/kg subcutaneous meloxicam and antibiotheraphy by 25-30 mg/kg intravenous ceftriaxone 15-20 min before surgery. Premedication with 1 mg/kg xylazine (IV) (Basilazin®, Bavet, Türkiye) was followed by induction with 5 mg/kg ketamine (IV) (Alfamine®, Atafen, Türkiye), and general anesthesia was maintained with 2-2.5% isoflurane (Forane®, Abbott, USA) and 100% oxygen. The necrotic corneal tissue was removed by cutting the stroma considering the lesion's depth using a 3.2 mm angled corneal knife. The keratectomy region was 1-2 mm larger than the corneal defect directed towards the healthy transparent cornea surrounding the necrosis *(Fig. 1-b, Fig. 5-b, Fig. 6-b, Fig. 7-b)*.

Application of Conjunctival Pedicle Flap (CPF)

After keratectomy, an incision perpendicular to the limbus was made on the eye's lateral bulbar conjunctiva. With the microsurgical scissors inserted under the incision, the conjunctive tissue was separated from the subjacent sclera by blunt dissection. The conjunctival graft's initial incision

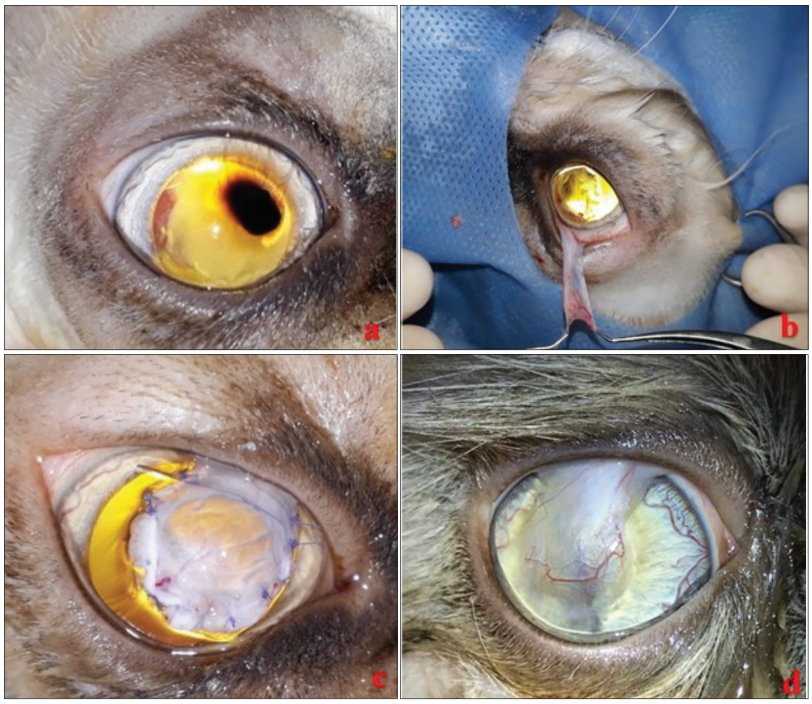


Fig 1. Case 2: Photos showing salient steps of corneal necrosis and conjunctival pedicle flap surgery in case 2. **(a)-** Central corneal necrosis in a 2-year-old Persian cat, **(b)-** Appearance of keratectomy site and adjacent bulbar conjunctival pedicle flap of appropriate size and shape, **(c)-** The conjunctival pedicle flap is sutured over the cornea using 8-0 polyglactin 910 sutures, **(d)-** The image before cutting the conjunctival pedicle flap

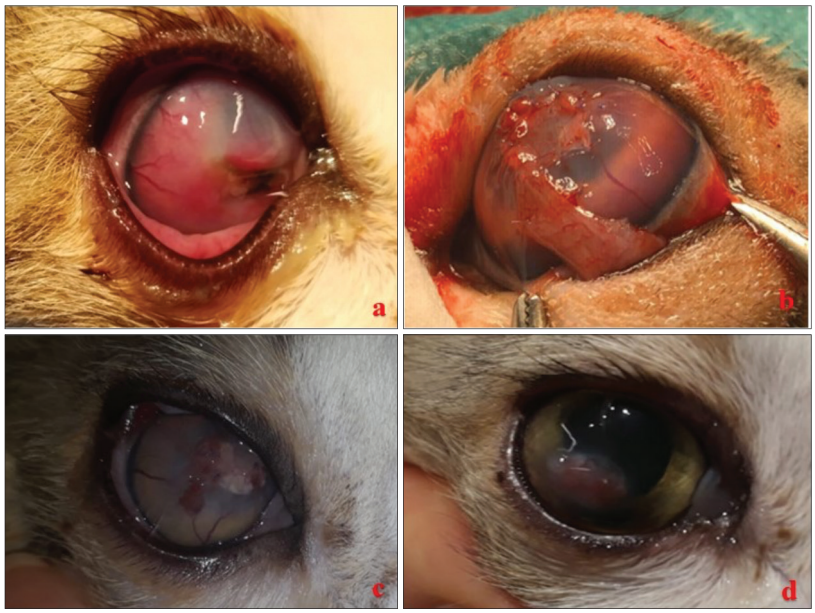


Fig 2. Case 24: After conjunctival pedicle flap surgery. **(a)-** A cat's eye with a conjunctival pedicle flap immediately after surgery, **(b)-** Edema and vascularization in flap and cornea 3 weeks after surgery, **(c,d)-** The appearance of the cornea and conjunctival pedicle flap remnants 45 and 75 days after the operation

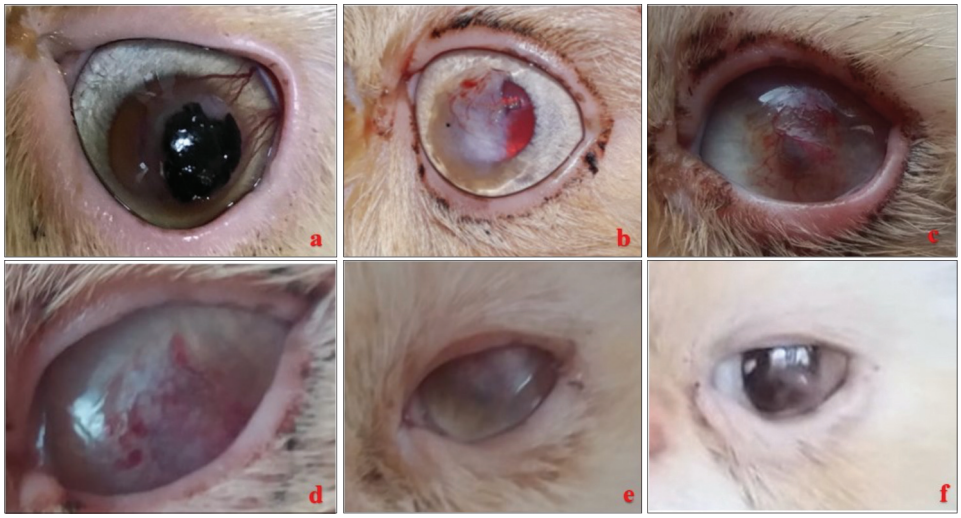
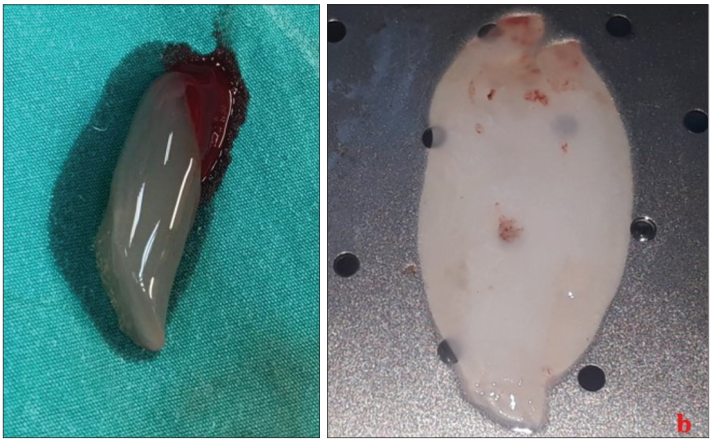


Fig 3. Case 9: (a)- The appearance of central corneal necrosis at presentation. The remnants of the conjunctival pedicle flap 6 months after surgery. 6 weeks, **(b)-** 3 months **(c),** 4 months, **(d)-** 5 months, **(e)-** and 6 months, **(f)-** postoperative view

Fig 4. (a)- The appearance of the PRF after it is removed from the tube, **(b)-** Conversion of the PRF to the membrane form to be transplanted into the cornea



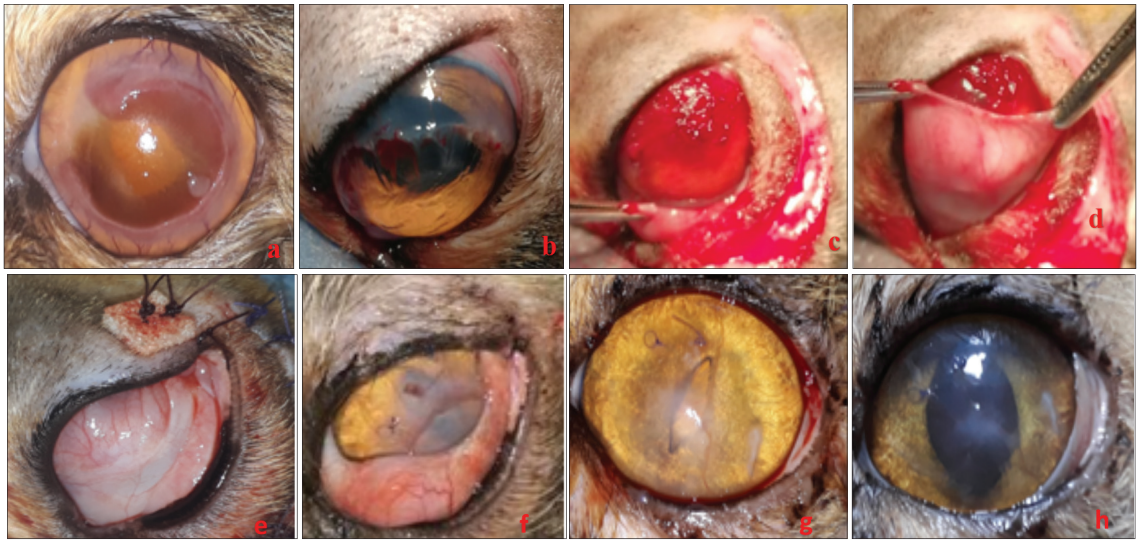


Fig 5. Case 17: (a)- Corneal necrosis concurrent with lower eyelid entropion, **(b)-** Removal of corneal necrosis by lamellar keratectomy, **(c,d,e)-** Treatment of cornea with PRFMG and NMF after lamellar keratectomy and eyelid with Holtz celcus method, **(f)-** The appearance of the cornea on the 30th day **(f),** 40th day **(g),** and 60th day **(h)**

was made with scissors parallel to and approximately 1-2 mm away from the limbus, the second incision cut was directed parallel to the initial wide enough to cover the corneal defect, and then the conjunctiva was released. The conjunctival graft's base was placed at 12 o'clock, extending vertically from the limbus to the defected area. The graft's length was determined based on the graft's distance to the site, and the graft was sutured to the

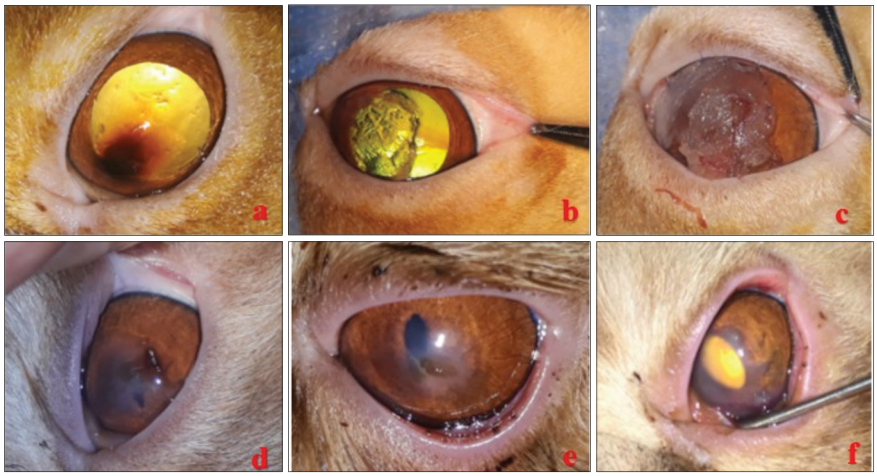


Fig 6. Case 19: Photos showing the PRFMG graft. **(a)**- Appearance of corneal necrosis, **(b)**- View of the cornea after lamellar keratectomy, **(c)**- Fixation of the PRFM graft to the cornea with 8/0 polyglactin 910. Recurrence of corneal necrosis was at the margin of keratectomy 6 **(d)** and 7 weeks **(e)** after the surgery, **(f)**- Removal of necrosis for the second time with total lamellar keratectomy and then appearance of the cornea 3 weeks after NMF application alone

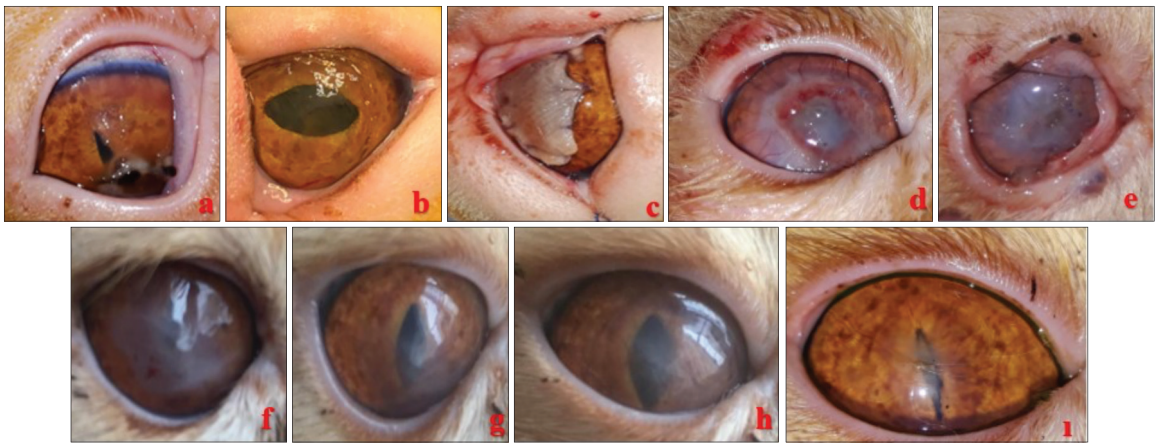


Fig 7. Case 16: (a)- Appearance of corneal necrosis, **(b)**- View of the cornea after lamellar keratectomy, **(c)**- Fixation of the PRFM graft to the cornea with 8/0 polyglactin 910, **(d)**- The dissolution of the PRFM graft in certain areas on the cornea and its replacement by marked vascularization and granulation tissue. The appearance of the cornea and PRFM graft 3 week after the surgery, **(e)**- Superficial vascularization and focal areas of pigment in the defect area and a few remaining sutures, **(f,g,h,ı)**- Clinical appearances of the cornea at intervals of 1 week.

keratectomy area on the cornea with simple interrupted sutures starting with the distal tip using an 8/0 polyglactin 910 (Vicryl®, Ethicon) suture material *(Fig. 1-c, Fig. 2-b)*.

Application of Platelet-Rich Fibrin Membrane Graft (PRFMG)

The procedure used in the study to prepare a platelet-rich fibrin membrane was modified from that previously described for human PRFMG application [11]. Three-5 mL of fresh blood was collected from the sedated cats' jugular vein into 10-mL anticoagulant-free tubes 10-15 min before the surgery. The tubes were centrifuged immediately at 2700 rpm for 12 min using a Hettich 200 EBA model centrifuge to separate platelet-poor plasma (PPP), platelet-rich fibrin (PRF), and red blood cells. The upper two layers consisting of PPP and PRF were gently removed from the tube with the aid of forceps, and the red blood cells collected at the bottom were cut with sterile scissors *(Fig. 4-a)*. The fibrin clot was converted into a membrane by being pressed gently by sterile gauze to be sutured to the corneal ulcer bed *(Fig. 4-b)*. The PRF membrane graft's length and breadth were adjusted to those of the corneal lesion, assuring that the graft was 1-2 mm larger than the corneal defect. Then, the membrane graft was sutured to the healthy corneal tissue at the lesion's margins with simple interrupted sutures using 8/0 polyglactin 910 (Vicryl®, Ethicon) *(Fig. 5-c, Fig. 6-c, Fig.7-c)*.

After the two techniques mentioned above (CPF and PRFMG) were performed, a nictitating membrane flap was applied for three weeks to protect the graft materials against drying *(Fig. 5-d,e)*.

Application of the Nictitating Membrane Flap (NMF)

The nictitating membrane flapping, a relatively simple technique, was performed to support and accelerate ulcer area healing after lamellar keratectomy. The flap was applied in a U-suture type using a 2/0 polyglycolic acid suture material and kept for three weeks.

Postoperative Management

Postoperatively, topical ofloxacin (Exocin®, Ibrahim Abdi, Türkiye) and sodium hyaluronate (Dryex®, Ibrahim Abdi,

Türkiye) drops were applied for two weeks as five times and three times a day, respectively and Elizabethan collar was prescribed for each patient during at least 4-6 weeks. The Nictitating membrane flap sutures were removed three weeks after the operation. Then carbomer-containing artificial tear gel (Thilo tears®, Liba, Türkiye) was applied as three times a day. Exocin and Dryex eye drops were continued for the following 1 week while prescribed five times a day in fluorescein stain-positive patients with incomplete corneal ulcer healing. At fourth weeks, topical ofloxacin was replaced by topical steroid eye drops (Tobramycin and dexamethasone 0.3% suspension -Tobradex®, Novartis, Türkiye) prescribed four times daily to alleviate corneal scarring.

Six to eight weeks after the surgery, the pedicle flaps were cut using corneal scissors under sedation and/or local anesthesia according to the patient's clinical status *(Fig. 3-b)*.

Results

A total of 30 cats (23 Persian, 5 British Shorthair, 1 Sphynx and 1 Domestic Shorthair cats) with corneal sequestrum were treated in the study. Fifteen were males, and fifteen were females, seven of which were spayed, and the other two were intact. The affected cats' average age was 3.5 years, ranging from 1.5 to 13 years of age. The findings regarding the breed, age, gender, clinical history, surgical methods applied, and the postsurgical outcomes of all patients were summarized in *Table 1*.

The corneal defect was caused by entropion *(Fig. 5-a)*, debridement of indolent corneal ulcer, topical steroid treatment followed by FHV-1 infection *(Fig. 2-a)*, and trauma in twelve, three, three, and other three patients, respectively. The lesion had developed due to miscellaneous causes of unknown origin in nine cats *(Fig. 1-a, Fig. 3-a)* mostly detected in Persian cats with central corneal localization *(Fig. 6-a, Fig. 7-a)*.

In the CPF technique, 3 weeks after the surgery, it was well-positioned with the neatly placed sutures, epithelialization was monitored in the conjunctival tissue's edges, and the preclinical appearance of corneal damage improved; however, the lower portion of the flap was not fully attached to the cornea. On the other hand, the conjunctival pedicle was well-vascularized with circumferential superficial corneal neovascularization, and the flap was well aligned and unnoticeable at the corneal margin *(Fig. 2-c)*. The flap was thicker and looked more hyperemic than the normal bulbar conjunctiva in all cases. Superficial neovascularization was regressed to 60% in the fourth week, 10% the sixth to eightieth weeks clinical inspections *(Fig. 1-d)*. The stromal corneal defect was filled up with the flap, but the stitches on some flap portions were loosened due to excessive stretching in one case (case no. 2, *Table 1)*. During the long-term follow-ups, neither pigmentation nor recurrence was monitored with the conjunctival pedicle flap technique. After 1.5-2 months, central corneal opacity diminished 20%. The components of the anterior segment of the bulbus oculi became distinguishable, of varying degrees, at the lesion site 2.5-3 months after the procedure *(Fig. 2-d, Fig. 3-c,d)*. Despite the long-term medical treatment (3-4 months), the conjunctivalization process was prolonged in the area of interest. Abundant fibrosis developed in large and centrally located defect areas, which partially impaired the vision. Scarring was mild, moderate *(Fig. 3-e,f)*, and severe in five, two, and other three eyes, respectively. The vision was restored despite moderate and severe corneal opacity in six of the patients. The CPF technique's success rate was 70% regarding corneal transparency, while corneal integration and healing process revealed a success rate of 90% in a one-step intervention. Moreover, the recurrence rate was 0% in the follow-ups.

In the PRFMG technique, A circumferential (360 degrees) superficial corneal neovascularization in all cases, the platelet-rich fibrin membrane on the ocular surface was found to have appeared much thinner than was observed with the CPF and even merged substantially and was replaced by granulation tissue with marked vascularization *(Fig. 5-f)*. An inflammatory reaction characterized by superficial inflammatory granulomas surrounding the stitches and granulation tissue formation were noted on the membrane graft *(Fig. 7-d)*. A complete integration of the PRFMG characterized by prominent re-epithelialization, granulation tissue formation and corneal neovascularization (50%) were quite prominent at the fourth week *(Fig. 7-e)*, fully covering up the entire ulcer area, which started to have subsided after this period. In the following 5-6 weeks, the granulation tissue formation completely disappeared during the corneal clearing phase *(Fig. 5-g)*. Corneal neovascularization was regressed 20% at six-eight weeks after surgery in all eyes *(Fig.7-f,g)*. The anterior segment had become quite distinguishable at the lesion site at the fourth week. After 1.5-2 months, central corneal opacity diminished 70%. Four eyes of four cases healed without any complications, while four eyes of the other four healed with minimal corneal vascularization and fibrosis *(Fig. 7-h)*. A mild brownish discoloration spot occurred in three eyes of three cases. A new area of necrosis developed in one case at the border of the former necrotic area (7 weeks later) *(Fig. 6-d,e,f)*, and in one other case, necrosis developed at the site of lamellar keratectomy (8 weeks later). The corneal restoration was achieved in all cases, and no visual impairment was monitored *(Fig. 5-h, Fig. 7-ı)*. After 1.5-2 months, mild corneal opacity was observed in three eyes, and full transparency in the other four eyes, while moderate brownish discoloration occurred in one eye, and a recurrence developed in two. The procedure's anatomical outcome was considered favorable regarding a satisfactory cosmetic appearance and successfully achieved visual recovery in a respectively short period. The success

Table 1. *The signalment, etiology of necrosis, summary of surgical and visual outcomes of all the patient's*

No	Breed	Age	Sex	Affected Eye	Etiology	Surgical Technique	Lamellar Keratectomy	Surgical Outcomes	Corneal Clarity
1	Persian	8 years	F	R	E	CPF	I	NR	Moderate opacity
2	Persian	2 years	F	R	U	CPF	C	NR	Severe opacity
3	British S	4 years	M	L	E	NMF	C	NR	Mild opacity
4	Sphynx	3 years	F	R	U	NMF	C	NR	Clear
5	Persian	2 years	F	R	U	NMF	I	R	Mild brownness
6	British S	1.5 years	M	R	C UI	NMF	I	NR	Mild opacity
7	Persian	1.5 years	M	R	E	NMF	C	NR	Clear
8	British S	3.5 years	M	R	T	NMF	I	NR	Clear
9	Persian	3 years	M	L	U	CPF	C	NR	Moderate opacity
10	Persian	3 years	M	R	E	CPF	C	NR	Severe opacity
11	Persian	4 years	F	R	E	CPF	C	NR	Mild corneal opacity
12	Persian	1.5 years	F	R	C	NMF	C	NR	Clear
13	Persian	5 years	F	L	U	NMF	I	R	Mild brownness
14	Persian	4.5 years	M	R	T	NMF	C	NR	Clear
15	Persian	13 years	F	R	E	PRFMG	I	R	Moderate brownness
16	Persian	6 years	F	R	U	PRFMG	C	NR	Mild corneal opacity
17	Persian	1.5 years	M	L	E	PRFMG	C	NR	Clear
18	Persian	3.5 years	F	R	In	PRFMG	C	NR	Clear
19	Persian	1.5 years	M	L	C UI	PRFMG	C	R	Moderate corneal brownness
20	Persian	5 years	M	L	U	CPF	I	NR	Mild corneal opacity
21	Persian	8 years	F	R	E	CPF	I	NR	Severe corneal opacity
22	Persian	1.5 years	F	R	In	CPF	I	NR	Mild corneal opacity
23	Persian	3 years	F	L	U	NMF	I	NR	Mild corneal opacity
24	Domestic S	1 years	F	R	T	CPF	C	NR	Mild corneal opacity
25	Persian	2 years	M	L	U	PRFMG	C	NR	Clear
26	Persian	5 years	M	L	In	PRFMG	I	R	Moderate corneal brownness
27	Persian	2 years	M	R+L	E	PRFMG	C	NR	Mild corneal opacity
28	British S	1.5 years	M	R	E	PRFMG	C	NR	Mild corneal opacity
29	Persian	3 years	M	R	E	CPF	C	NR	Mild corneal opacity
30	British S	2 years	F	L	E	PRFMG	C	NR	Clear

NR: *No recurrence,* ***R:*** *Recurrence,* ***I:*** *Insufficient,* ***C:*** *Complete,* ***E:*** *Entropion,* ***U:*** *Unknown,* ***In:*** *Infection,* ***T:*** *Trauma,* ***C UI:*** *Chronic Ulcer*

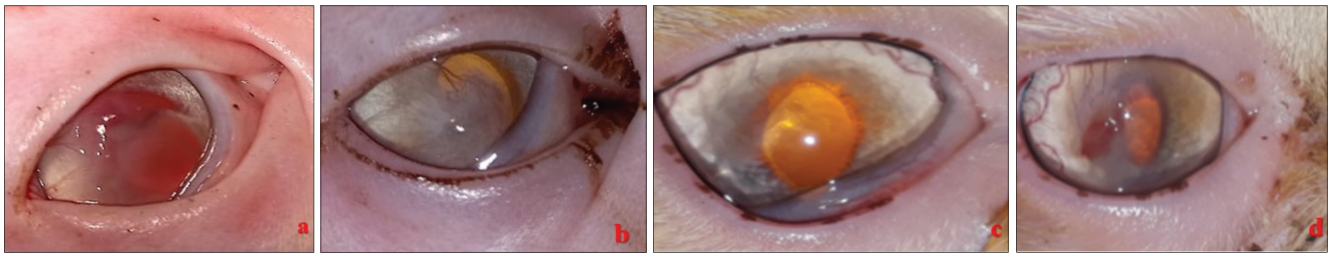


Fig 8. The appearance of the corneas 3 weeks **(a)** and 4 weeks **(b)** after membrana nictitans flap application is in case 4. The appearance of the cornea 30 days **(c)** and 45 days **(d)** after surgery in case 7

rate of the PRFMG technique was 72.7%, 81.8%, and 27.2% in terms of corneal transparency, corneal healing, and recurrence, respectively.

In the NMF technique, in the third week, the fluorescein stain-test was positive in three cases despite the well-filled defect cavity at the central cornea corresponding to the site of keratectomy and vascularization, apart from granulation tissue formation. Corneal epithelialization has not been fully completed. The anterior segment was distinguishable at the lesion site *(Fig. 8-a,b)*. Fluorescein staining was repeated at the fourth week, and corneal integrity and epithelialization were determined to have been achieved and corneal vascularization decreased to 50% at four weeks and to 20% at sixth to eight weeks of clinical examinations. The healing period was rapid, and the duration of medical therapy administered after surgery to reduce or provide full resolution of corneal fibrosis was remarkably shortened *(Fig. 8-c,d)* and even completed in 4-5-weeks in this procedure. After 1.5-2 months, central corneal opacity diminished 100%. In the cases with necrotic tissue's complete resection, no long-term complications

were monitored during 1-2-year follow-ups. Complete corneal transparency was achieved, and corneal fibrosis was indistinct. A mild corneal discoloration was noted only in two cases, which was associated with incomplete keratectomy due to the lesion's extreme depth. On the other hand, even though the necrotic tissue was unable to be excised entirely, neither the lesion recurred nor the remaining necrotic tissue fragment expanded with this technique. The NMF technique offered a less lengthy recovery period even with a deep keratectomy. The success rate of the NMF technique regarding corneal transparency was 80%. A rapid corneal restoration was determined in cats treated with the NMF method.

Discussion

In the presented study, three different techniques were utilized to repair corneal necrosis seen in cats: 1- The CPF technique, which is frequently used in the clinics, 2- The NMF technique, which is not preferable for deep lesions, and 3- The PRFMG technique, which was applied as a new technique for such lesions.

The nictitating membrane flapping is a simple, non-invasive alternative method to treat simple and spontaneous persistent corneal ulcers and corneal lacerations. The technique is also applied for postoperative fixation of corneal grafts and removal of uncomplicated corneal necrosis in cats. Its feasibility is suggested in corneal sequestrum to provide mechanical protection for the corneal surface against external trauma and eyelid friction during the corneal repair. The cornea's contact with the conjunctival mucosa's vascular network provides the release of a serous transudate that supplies the necessary substances for the cornea and also supports high temperature-responsive corneal repair processes [12]. However, unlike conjunctival pedicle flaps, NMF does not provide structural support to the cornea, including fibrovascular tissue, blood vessels, immune components, and natural anti-collegenasese. Some disadvantages arise, such as the inability to monitor the cornea during the healing process, prevention of drug penetration, the effect of pH, retention of inflammatory exudates that are in contact with the cornea, and potential surface friction. Ragozzino et al.[12] reported that the nictitating membrane flap supported the cornea, and satisfactory corneal transparency was achieved at the therapy's cessation. Neither complications nor recurrence was monitored during 1-6-year follow-ups. It was considered a successful method due to the shortened recovery time, favorable patient outcomes, and lack of recurrence [12]. However, some authors pointed out the insufficiency of the technique in deep stromal corneal defects due to the inability to perform total keratectomy in these cats, which leads to recurrence, despite the success of NMF in superficial lesions [11]. Therefore, the NMF technique was replaced by conjunctival grafting and keratoplasty targeting structural stabilization of specific lesions in the management of corneal disorders. Despite the reported disadvantages, the patients' outcomes were quite favorable with this technique. The necrosis repetition frequency in the NMF application after lamellar keratectomy was reported to have varied from 12% to 38% [12]. In the presented study, deep corneal sequestrum treated with the NMF application after keratectomy healed successfully with minimal or no scarring despite the severe stromal tissue damage even in centrally located lesions, and no vision impairment was noted. Three-year follow-ups revealed recurrence in two cats after 3-6 months. Conjunctival hyperemia and occasionally occurring blepharospasm were monitored in the other two cats, yet no recurrence was noted. The necrosis recurrence rate of the NMF application after lamellar keratectomy was estimated as 20%. Recurrence developed in two of five cases (40%) with incomplete removal. The findings were considered compatible with the previous reports, which were associated with the incomplete resection of the defected stromal tissues rather than the technique's insufficiency.

The conjunctival pedicle flapping is a simple, feasible, and cost-effective treatment method. It has long been used to treat corneal ulcers; however, its popularity has declined since new treatment options emerged. The technique aids in minimizing corneal vascularization by delivering nutrients and cellular components, and thus providing metabolic and mechanical support to the defected area, and retrieving ocular surface integrity, which -as a result- promotes wound healing and offers comfort to the patient [5,13]. It facilitates convenient monitoring of the disorder's post-operative status since it covers solely the defected area [3]. It can be easily performed in autotransplantation and donor tissue grafting with large dimensions; however, it causes the development of leucoma or scarring at the transplantation site [14]. On the other hand, the CPF technique avoids potential recurrence followed by partial excision of the necrotic stromal layer when the necrosis has invaded the deeper corneal layers. Also, it has been supported the conjunctival pedicle grafting due to its advantage as a one-step application not requiring further surgical interventions in feline corneal necrosis with persistency and potential recurrence [3,15]. In contrast, some authors suggested that it was not significantly efficient in preventing recurrence, attributing to the occurrence of persistent corneal opacity [16]. It was also indicated that the graft's overlength and flaccidity might have caused displacement due to its horizontal shifting generated by eye and eyelid movements [3]. Furthermore, insufficient debridement while preparing the corneal graft bed, the distance between the graft and the defected area, positioning the graft with an angle more than 45 degrees, the graft's inadequate size or over-thickness, excessive pressure applied on the graft, softening of the cornea, and use of improper sutures were also reported to have resulted in the conjunctival grafts' dislocation [15]. In the presented study, the pedicle flap's loosening in one patient

was associated with the ocular movements and the flap's overstretching due to its inadequacy in size. When the lesion is localized in the central cornea affecting the visual axis clarity, it is considered irrational to create transparency at the transplantation site if an optical surface is desired. It takes three weeks for conjunctivalization, and corneal transparency enters restoration at least 2.5-3 months after the operation [13,14]. Saroğlu et al.[16] reported that no vision loss was monitored with conjunctival pedicle flap, which ended up with inconspicuous, mild, moderate scarring in two, three, and two cases, respectively. In the study, in the CPF-applied patients, the lesions were centrally located in 8 cases and were paracentral in two. Five, two, and the other three patients ended up with mild, moderate, and severe scarring, respectively. Therefore, the vision was impaired to a certain degree. The recurrence rate was similar to those reported by the other researchers who performed the CPF after keratectomy. Even if there were cases where the total necrotic area was unable to be removed (n = 3), no recurrence was observed. Although it does not provide as excellent corneal transparency as other techniques and a more extended period is required to achieve corneal transparency, an improvement and vision restoration was achieved in all patients by the aid of concurrent medical therapy, which is considered a satisfactory result with an indication of the technique's feasibility.

The platelet-rich fibrin membrane is a new second-generation fibrin concentrate rich in autologous platelets developed by Choukroun et al.[17] for maxillofacial surgery. It is used in periodontal and maxillofacial plastic and reconstructive surgery and orthopedics to repair bone defects in humans [10], while it was reported to have been applied in veterinary medicine for esophageal [18] and tendon repair surgery of dogs [19,20].

The PRF membrane grafting is a successful procedure with a favorable outcome in human corneal reconstruction due to the membrane's anti-inflammatory properties and the healing process with minimal scarring. It is considered a simple, practical, and laborless technique that provides a cost-effective treatment and excellent corneal integrity [10,21]. Its accomplished application in humans inspired us to use this technique in the surgical management of feline corneal necrosis. The presented study is about the use of PRFMG material as a new method in the treatment of these lesions in cats. The fibrin matrix was transformed into a membrane graft as previously proposed by Alio et al.[10]. The membrane graft was sutured onto the intact corneal tissues while covering up the entire necrotic area. The PRFMG proved to be a successful technique applied after keratectomy to treat feline corneal sequestrum in the presented study. It was found superior to the other techniques like conjunctival pedicle flapping with minimal corneal scarring. The membrane properly integrated with the cornea, and no repositioning or correction like trimming was required. The cosmetic appearance was also quite satisfactory. Corneal transparency improved in time, and the fibrin membrane graft was replaced by corneal epithelialization. The membrane grafted provided a structural framework for full corneal recovery, and thus it is indicated in the treatment of ulcerative keratitis for supporting the corneal repair with indistinct scarring. The graft material merged with the cornea three weeks after the application, and the healing was even more satisfactory at the fourth week. Corneal thickness was within normal limits with a subtle opacity. However, it did not prevent a recurrence, particularly in cases with incomplete excision of the degenerated collagen. Recurrence developed in three of eleven eyes treated with the PRFMG technique 6-8 months after surgery, whereas no recurrence had been noted with the conjunctival pedicle flap grafting. Recurrent corneal sequestrum detected in the early postoperative period (4 weeks after surgery) occurred in two cats with incomplete excision, and vascularization during the healing process hampered distinguishing the discoloration in the defected area. Corneal discoloration recurred in one cat at the margins of the excision site despite the complete excision. It was only possible to perform an incomplete keratectomy in ten cases (3 from CPF, 3 from PRFMG, and 4 from NMF) due to the discoloration expanding into the deepest layer to avoid potential perforation. The recurrence rate was 40% (2 from PRF and 2 from NMF) in the eyes that underwent incomplete keratectomy, which was considered to be associated with the fact that residual pigmentation might have served as a potential risk factor for recurrence. The recurrence rate of the eyes that underwent incomplete keratectomy with concurrent PRFMG application was 27.2%. Nevertheless, it can be deduced that minimal scarring and rapid recovery of corneal transparency were achieved with the PRFMG technique compared with conjunctival pedicle flapping.

The study introduced some impediments to be considered. The study's retrospective design, relatively low cat population, enrolled and breed, age, and gender-wise variability were the main issues. The limited number of patients that developed recurrence hindered evaluating risk factors for recurrent corneal sequestration. Nevertheless, the study offered favorable functional and structural outcomes, with an adequate follow-up period. The conjunctival grafting followed by keratectomy reduced the potential recurrence of corneal necrosis and provided tectonic support due to a fortiori corneal scarring of varying degrees. The patients that underwent the NMF technique followed by keratectomy allowed successfully treating deep corneal necrosis, including full-thickness defects, and proved eligible for deep lesions, unlike previously described techniques. Our results revealed that the autologous PRFMG is a safe and efficient procedure in the surgical management of deep corneal necrosis and rendered merely minimal scarring compared to the CPF technique. Apart from a scaffold provided by an autologous 3-D fibrin network, the long-term release of growth

factors crucial for tissue repair, inducing the synthesis of novel collagen lamellae, enabling the emerging of the defected area smoothly into the adjacent healthy corneal tissue, is the main advantage of the technique. Briefly, the PRFMG, allowing a fifteen-minute lengthy application, was considered a well-deserved alternative to either conjunctival flapping providing tectonic support yet triggering the likelihood of axial visual impairment or other costly and more complicated methods. Even though the study was designed to investigate the efficacy of a novel technique in the surgical treatment of deep corneal necrosis, further randomized clinical trials comprising a larger patient population are required to verify the procedure's success and reveal the autologous nature of tissue repair to be offered by the technique. Consequently, the PRF membrane grafting was considered a promising treatment choice regarding the surgical approach to the patients with central and paracentral deep corneal necrosis despite the relatively low number of patients included in the presented study, which is hampering an overall inference.

Availability of Data and Materials

The authors declare that the data supporting the study findings were obtained from the corresponding author (A. Demir).

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Competing Interests

The authors declare no conflict of interest regarding this report.

Author Contributions

AD made the design of the study, the writing of the article, the treatment and controls, and the revision of the manuscript. YA provided data collection, article writing, revision of the article draft, submission of the article and entry of subsequent revisions into the system.

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RESEARCH ARTICLE

The Development of a SYBR Green I Multiple Real-time Fluorescence PCR Assay for Detection of *Actinobacillus pleuropneumoniae, Haemophilus parasuis* and *Pasteurella multocida*

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Abstract

Actinobacillus pleuropneumoniae, Haemophilus parasuis, and *Pasteurelle multocida* are common pathogens of respiratory diseases in the pig industry, and they may cause secondary infections and serious economic losses to the pig industry. The clinical symptoms caused by these three pathogens are difficult to distinguish with the naked eye, and mix infections bring difficulties to the diagnosis of diseases. In this study, specific primers were designed on the basis of *A. pleuropneumoniae Apx IV, H. parasuis Omp P2* and *P. multocida PlpE* gene. The expected amplified products of *A. pleuropneumoniae, H. parasuis,* and *P. multocida* were 157, 120 and 305 bp, respectively. After the amplified fragment was cloned into a vector, a standard plasmid was constructed. By using the standard plasmid as template, a fluorescence quantitative PCR method for simultaneous detection of *A. pleuropneumoniae, H. parasuis,* and *P. multocida* multiple SYBR Green I was established. Combined with melting curve analysis, the sensitivity, specificity, and repeatability were also evaluated. The results showed that the sensitivity of the method for detecting the three pathogens were 147, 145, and 61 copies/μL. On the same melting curve that produced three specific Tm peaks, no cross reaction with other bacteria was observed, and the method demonstrated good specificity and repeatability. This method could be used for the simultaneous detection of the three pathogens, thus providing an effective detection tool for disease prevention and treatment.

Keywords: *Actinobacillus pleuropneumoniae, Haemophilus parasuis, Pasteurelle multocida, SYBR Green I, Multiplex PCR*

Actinobacillus pleuropneumoniae, Haemophilus parasuis ve *Pasteurella multocida*'nın Saptanmasında SYBR Green I Multiple Gerçek Zamanlı Floresan PCR Yönteminin Geliştirilmesi

Öz

Actinobacillus pleuropneumoniae, Haemophilus parasuis ve *Pasteurelle multocida,* domuz yetiştiriciliğinde yaygın solunum sistemi hastalıkları patojenleridir ve domuz endüstrisinde sekonder enfeksiyonlara ve ciddi ekonomik kayıplara neden olabilirler. Bu üç patojenin neden olduğu klinik semptomların çıplak gözle ayırt edilmesi güçtür ve miks enfeksiyonlar hastalıkların tanısını zorlaştırır. Bu çalışmada, *A. pleuropneumoniae Apx IV, H. parasuis Omp P2* ve *P. multocida PlpE* gen bazında spesifik primerler tasarlandı. *A. pleuropneumoniae, H. parasuis* ve *P. multocida* için sırasıyla 157, 120 ve 305 bp amplifiye ürünler beklendi. Amplifiye fragment bir vektöre klonlanarak standart bir plazmit oluşturuldu. Kalıp olarak standart plazmit kullanılarak, *A. pleuropneumoniae, H. parasuis* ve *P. multocida* multipl SYBR Green I'in eşzamanlı tespiti için kantitatif bir floresan PCR yöntemi geliştirildi. Erime eğrisi analizi ile birlikte duyarlılık, özgüllük ve tekrarlanabilirlik de değerlendirildi. Sonuçlar, bu yöntemin, bu üç patojeni saptamada duyarlılığının 147, 145 ve 61 bakteri/μL olduğunu gösterdi. Üç spesifik Tm piki veren aynı erime eğrisinde diğer bakterilerle çapraz reaksiyon gözlenmedi ve yöntem iyi bir özgüllük ve tekrarlanabilirlik sergiledi. Bu yöntem, üç patojenin eşzamanlı tespiti için kullanılabilir ve böylelikle hastalıkların önlemesi ve tedavisi için etkili bir teşhis aracı niteliğindedir.

Anahtar sözcükler: *Actinobacillus pleuropneumoniae, Haemophilus parasuis, Pasteurella multocida, SYBR Green I, Multipleks PCR*

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Introduction

Actinobacillus pleuropneumoniae is one of the pathogens causing porcine contagious pleuropneumonia, which is distributed all over the world [1]. This pathogen has many different serotypes, no cross-reaction exists between different serotypes; the serotypes prevalent in different countries and regions are not exactly the same, making it difficult to accurately diagnose *A. pleuropneumoniae* infection [2,3]. When pigs are infected with *A. pleuropneumoniae*, they are mainly characterized by acute hemorrhage and chronic fibrinous necrotizing pleuropneumonia. They have a high mortality rate and cause huge losses in the pig industry.

Haemophilus parasuis is a pathogenic bacterium that can cause infections in pigs. *H. parasuis* is a Gram-negative bacterium belonging to the *Pasteurellaceae* family; it exists in the upper respiratory tract of healthy pigs [4]. When tolerance to the environment is weakened, it can invade various organs of the body and cause disease. In pigs, the clinical symptoms are fibrinous serositis, meningitis, and arthritis, and the disease is also known as pig Glasser's disease [5]. The disease is prevalent worldwide, and it mainly harms nursery pigs and weaned piglets, with a high incidence and fatality rate. It is one of the major swine diseases affecting the development of the pig industry [6,7].

Pasteurella multocida is an important Gram-negative pathogen that usually exists in the nasal cavity, peach body, lung, and other parts of pigs; it can cause diseases, such as swine pneumonitis and swine infectious atrophic rhinitis [8]. Clinically, the most acute type is mainly manifested as sepsis. The acute type is the most common, with inflammation and swelling of the throat, unsmooth breathing, late weakness, and death from suffocation. The chronic type is rare, and the symptoms are not obvious. The disease is generally not affected by the season, it is endemic, and pigs of any age could be infected with it [9]. It also has caused huge economic losses to the pig industry [10].

Clinically, the symptoms of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* are difficult to distinguish as they are all common and important pathogenic bacteria in the respiratory tract of pigs, and they may cause mixed infections, which are also difficult to distinguish [11]. How to quickly and accurately distinguish and identify these three kinds of bacteria has attracted extensive attention. At present, the traditional methods of pathogen isolation and identification and serological diagnosis are still widely used in veterinary clinical diagnosis of these three diseases, but the methods of pathogen isolation and serological diagnosis are time-consuming, with low sensitivity and specificity, thus not meeting the needs of rapid clinical diagnosis. Although conventional PCR detection methods could be used to detect the three pathogens, they need to be tested separately, thus time-consuming, laborious, and cumbersome to operate, resulting in great inconvenience. Therefore, establishing a rapid, efficient, and accurate detection method is considerably important [12].

Multiplex PCR technology is a method to simultaneously amplify nucleic acid fragments of multiple purposes by adding two or more pairs of primers into the same PCR amplification system, and simultaneously identify and detect various pathogens [13,14]. It has the advantages of strong specificity, high sensitivity and fast diagnosis [17]. Real-time fluorescence quantitative PCR is a new technique developed in the 1990s to detect nucleic acid molecules. Fluorescent dyes are added in the PCR system, and through the change in fluorescence intensity, this system could be used for real-time PCR process inspection; finally, through the standard curve of nucleic acids, it could be used in the accurate quantitative analysis of unknown samples and qualitative analysis through the dissolution curve of unknown samples; it has high sensitivity, short reaction time, observational results, and many other advantages [15]. Multiplex real-time fluorescence quantitative PCR is a detection method that uses multiple specific primers to simultaneously amplify multiple target nucleic acid fragments in the same PCR reaction system. In accordance with different Tm values, multiple specific Tm peaks could be generated on the same melting curve to achieve the purpose of simultaneous detection.

In this study, a multiple SYBR Green I fluorescence quantitative PCR method for simultaneous detection of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* was established. Through the melting curve, this method could simultaneously detect one, two, or three kinds of pathogens, thus providing technical support for the rapid and accurate detection of diseases in clinical practice.

Material and Methods

Strains

A. pleuropneumoniae, *H. parasuis*, *P. multocida*, *Staphylococcus aureus*, *Staphylococcus suis* and *Escherichia coli* are all preserved in the laboratory of Henan Institute of Science and Technology.

Primers

According to the conserved sequences of *A. pleuropneumoniae Apx IV*, *H. parasuis Omp P2,* and *P. multocida PlpE* by GenBank, three pairs of specific primers were designed by using the software Premier 5, and the modified primers were synthesized by Shanghai Shenggong Bioengineering Co., Ltd. The sequence of the three pairs of primers is shown in *Table 1*.

Standard Recombinant Plasmid Construction

Bacterial genomic DNA was extracted in accordance with the operating instructions of the TAKARA DNA extraction kit. It was stored at -20ºC for later use. The extracted *A.*

Table 1. *Primers used in the FQ-PCR of A. pleuropneumoniae, H. parasuis and P. multocida gene*

Gene	Primer	Sequence (5′-3′)	Tm/°C	Length/bp
A. pleuropneumoniae	Forward	GCAGCTACGGTGCGGACA	61.8	157
	Reverse	TCATTATCTACTCGTCGGAATTTCACT	54.8	
H. parasuis	Forward	AAAAGATACCAAAGGCAAGG	49.9	120
	Reverse	ACCACAGTAATAGTTTCACCGA	52.9	
P. multocida	Forward	TGGCTACCTTGTTACGACTTC	54.3	305
	Reverse	CATGAGGGCAGGAGAGGAG	54.8	

pleuropneumoniae, *H. parasuis*, and *P. multocida* were used as templates to amplify the target gene. DNA STAR enzyme, primers, and template were added to 20 μL reaction system in sequence, finally making up to 20 μL with double-distilled water. The EP tube was placed in the PCR machine, and amplification was performed according to the following procedure: after pre-denaturation at 95°C for 5 min, cycle at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; after 30 cycles, 72°C for 10 min; and save at 4°C. Then, the amplified product was electrophorized on agarose gel, and the target fragment was recovered according to the TAKARA gel recovery kit. The recovered product was linked with pMD-19T vector, transformed into DH-5a competent cells, and spread on LB plate containing ampicin antibiotics. White colonies were selected and identified by PCR. Plasmids were extracted using the TAKARA plasmid extraction kit and sent to Beijing Genomics Institution in Beijing for sequencing. The correct plasmid verified by sequencing was used as the standard substance to establish the standard curve.

Simplex SYBR Green I Fluorescence Quantitative PCR Assay

Single SYBR Green I fluorescence quantitative PCR was established for *A. pleuropneumoniae*, *H. parasuis* and *P. multocida*, respectively. OD26 was measured by the recombinant plasmid of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida*. Its copies/uL was calculated by substituting the value into the following formula (copies/μL = (6.02 x 10^{23}) x (ng/μL x 10^{-9})/(DNA length x 660). Then, a gradient dilution was established, with dilution of concentrations of 10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4}, 10^{3}, 10^{2}, 10^{1}, 10^{0} copies/μL, making the standard curve as a reference standard. The total reaction system of SYBR Green I was 10 μL, 5 μL SYBR PreMix enzyme with 0.5 μL each for F and R; 1 μL template; and 3 μL double-distilled water were added. Fluorescent quantitative PCR instrument was used for reaction amplification. The program was set as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, with a total of 40 cycles.

Duplex SYBR Green I Fluorescence Real-time PCR Assay

On the basis of the constructed simplex fluorescence real-time PCR method, duplex SYBR Green I fluorescence quantitative PCR was established for *A. pleuropneumoniae* and *H. parasuis*, *A. pleuropneumoniae* and *P. multocida*, and *H. parasuis* and *P. multocida*. The SYBR Green I real-time fluorescent PCR 20 μL reaction system was as follows: SYBR Premix enzyme 10 μL, with 0.5 μL for the F and R of each two pathogenic bacterial primers, 1 μL each of the two pathogenic bacteria templates; and 6 μL double-distilled water. After instantaneous centrifugation, the EP tube was placed on a fluorescence quantitative PCR instrument. After pre-denaturation at 95°C for 5 min, then at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, 40 cycles were performed for PCR reaction, and a negative control without template was set up.

Multiple SYBR Green I Fluorescence Real-time PCR Assay

Based on the constructed single and double fluorescence real-time PCR methods, multiple SYBR Green I fluorescence quantitative PCR was established. The 20 μL system of SYBR Green I real-time fluorescent PCR reaction was as follows: SYBR Pre Mix enzyme 10 μL, *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* primer F, R each 0.5 μL, template 1 μL each, and 4 μL double-distilled water. After instantaneous centrifugation, the EP tube was placed on the fluorescence quantitative PCR instrument for pre-denaturation at 95°C for 5 min, then for 30 s at 95°C; 55°C for 30 s; 72°C for 1 min, 40 cycles were performed for PCR reaction, and a negative control without template was set up.

Sensitivity, Specificity and Repeatability Analysis

By using 1 μL DNA of *A. pleuropneumoniae*, *H. parasuis*, *P. multocida*, *S. aureus*, *S. suis,* and *E. coli* as templates and double-distilled water as negative control, fluorescence quantitative PCR was performed to verify the specificity.

Plasmid standard samples with the same concentration of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were selected for multiple SYBR Green I real-time PCR repeatability test, and the reaction was repeated three times. The stability of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple SYBR Green I real-time PCR method was verified by analyzing the Tm values and melting curves of each bacterium.

Plasmid standard samples with the same concentration

of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* were selected for multiple SYBR Green I real-time fluorescent PCR reaction repeatability test, and the reaction was repeated three times. The stability of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* multiple SYBR Green I real-time PCR method was verified by analyzing the Tm values and melting curves of each bacterium.

The OD260 value of recombinant plasmids *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* was measured, and the copy number per microliter was calculated. Then, the 10 times gradient dilution was performed. The *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* standard plasmids after gradient dilution were used as templates for PCR amplification by using the proposed method, and the sensitivity of fluorescence quantitative PCR reaction for the detection of bacteria was determined.

Results

Construction and Identification of Standard Recombinant Plasmid

Specific primers were used to amplify *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida*, and the results showed that the sizes of their target bands were 157, 120, and 305 bp, respectively *(Fig. 1)*. After enzymatic digestion was identified, the product was found to be consistent with the expected band.

Establishment of Standard Curve of Simplex Fluorescence Quantitative PCR

The concentration of the extracted positive plasmid was determined using a protein nucleic acid quantifier. The concentrations of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were 254.6, 191.1, and 205.7 ng/μL respectively. By using the above formula, the actual concentration of copies could be calculated. The *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* plasmid template copy number were 1.47 x 10^{12}, 1.45 x 10^{12}, and 6.05 x 10^{11} copies/μL respectively. According to the results obtained, the plasmid was diluted 10 times to the gradient concentration and used as a standard sample to make a standard curve. From *Fig. 2-A,B, Fig. 3-A,B,* and *Fig. 4-A,B,* the amplification curves were smooth and evenly spaced; the Ct values of the repeated samples of each dilution gradient were the same; and the dissolution temperatures of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* standard at each dilution degree were approximately 76.9°C, 80.0°C and 83.8°C, respectively. The height of the peak was positively correlated with the concentration of DNA, and the CT value gradually increased with the increase in the dilution degree of the standard substance. *A. pleuropneumoniae* standard curve: Y = -3.449X + 39.682, E = 95.0%, R^2 = 0.9968; *H. parasuis* standard curve: Y = -3.5805X + 41.26, E = 90.2%, R^2 = 0.996;

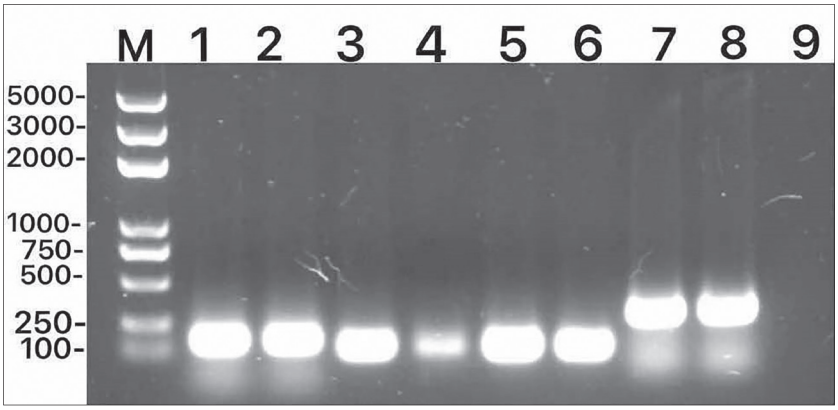

Fig 1. PCR amplification of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* plasmid. M:DL 5000 Marker; 1,2: amplification product of *A. pleuropneumoniae* plasmid; 3-6: amplification product of *H. parasuis* plasmid; 7,8: amplification product of *P. multocida* plasmid; 9: Blank control

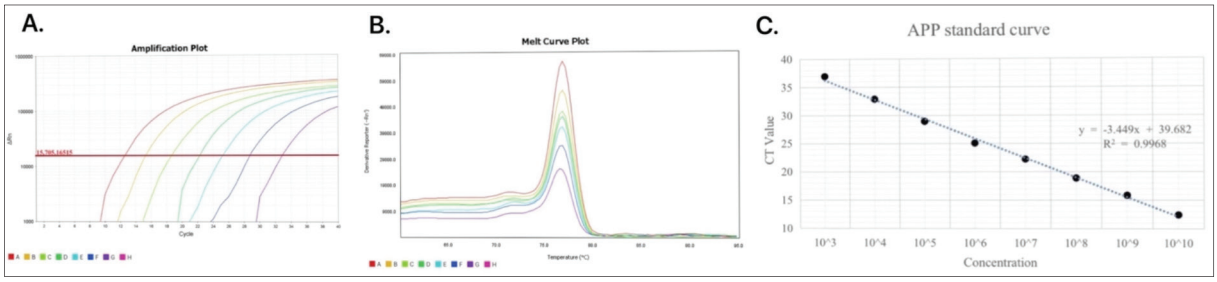

Fig 2. Simplex real-time PCR assay of *A. pleuropneumoniae*. **(A)** The amplification curves of different plasmid concentrations of *A. pleuropneumoniae*; **(B)** The melting curves of different plasmid concentrations of *A. pleuropneumoniae*; **(C)** The standard curve of *A. pleuropneumoniae*

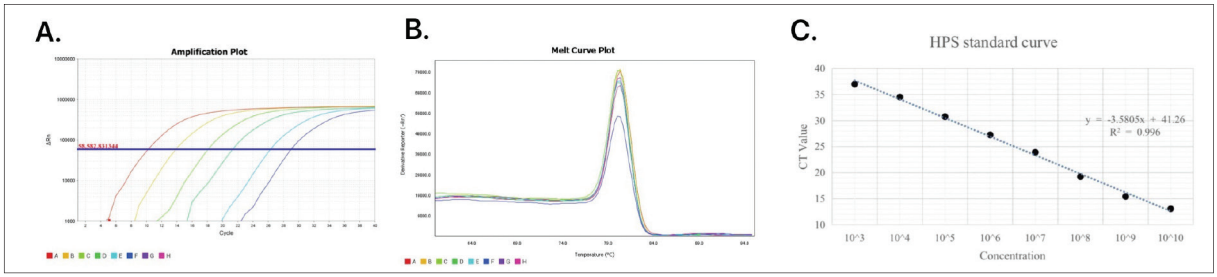

Fig 3. Simplex real-time PCR assay of *H. parasuis.* **(A)** The amplification curves of different plasmid concentrations of *H. parasuis*; **(B)** The melting curves of different plasmid concentrations of *H. parasuis*; **(C)** The standard curve of *H. parasuis*

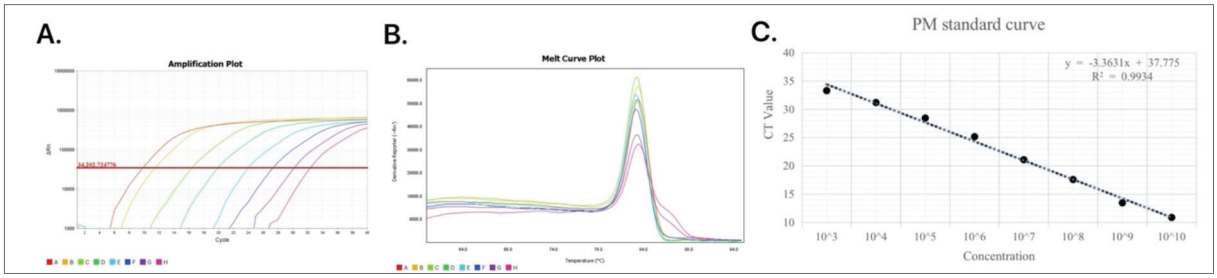

Fig 4. Simplex real-time PCR assay of *P. multocida.* **(A)** The amplification curves of different plasmid concentrations of *P. multocida*; **(B)** The melting curves of different plasmid concentrations of *P. multocida*; **(C)** The standard curve of *P. multocida*

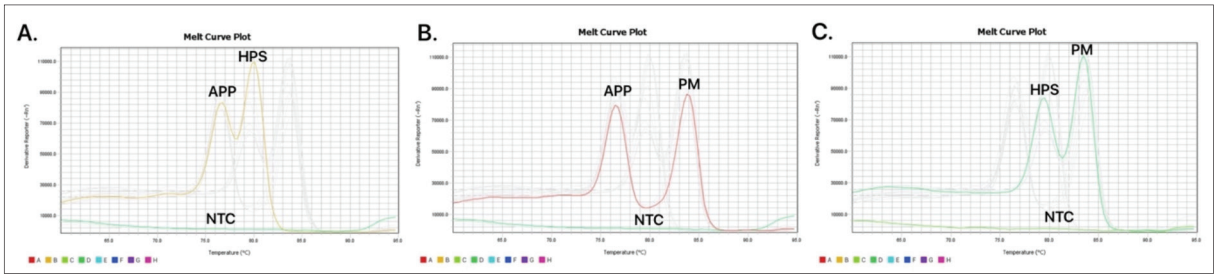

Fig 5. Double real-time PCR results. **(A)** *A. pleuropneumoniae* and *H. parasuis* double SYBR Green I real-time fluorescent quantitative PCR results; Targets are indicated above each peak along with non-target negative controls (NTC). **(B)** *A. pleuropneumoniae* and *P. multocida* double SYBR Green I real-time fluorescent quantitative PCR results; Targets are indicated above each peak along with non-target negative controls (NTC). **(C)** *H. parasuis* and *P. multocida* double SYBR Green I real-time fluorescent quantitative PCR results: Targets are indicated above each peak along with non-target negative controls (NTC)

and *P. multocida* standard curve: Y = -3.3631X + 37.775, E = 98.3%, R^2 = 0.9934. The results showed a good linear relationship among the three plasmids.

According to the fluorescence quantitative PCR test, the melting curve showed that the temperature corresponding to the two specific peaks is the Tm value of the pathogen. The Tm values of *A. pleuropneumoniae* and *H. parasuis* in *Fig. 5-A,* Tm values of *A. pleuropneumoniae* and *P. multocida* in *Fig. 5-B,* Tm values of *H. parasuis* and *P. multocida* in *Fig. 5-C,* that displayed a same degree in the single fluorescence quantitative PCR test. No specific peak was found in the negative control.

Establishment of Multiplex Fluorescence Real-Time PCR Assay

The recombinant plasmid of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* was used as the template for PCR reaction, and the dissolution curve was obtained using the fluorescence quantitative PCR instrument. From *Fig. 6-A,* shows three specific Tm peaks on the same melting curve, and these peaks were the Tm values of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida*. The Tm values of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were 76.47-76.93°C, 79.77-80.10°C, and 83.67-83.81°C, respectively, whereas the negative control had no peak value.

Specificity, Repeatability, and Sensitivity Analysis

The results of multiple SYBR Green I real-time fluorescence PCR specificity test on *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* in *Fig. 6-B* showed that in the control group, *S. aureus*, *S. suis, E. coli* and the negative control had no specific peaks. Only the *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple real-time PCR in the test group produced three specific peak values, and no cross-

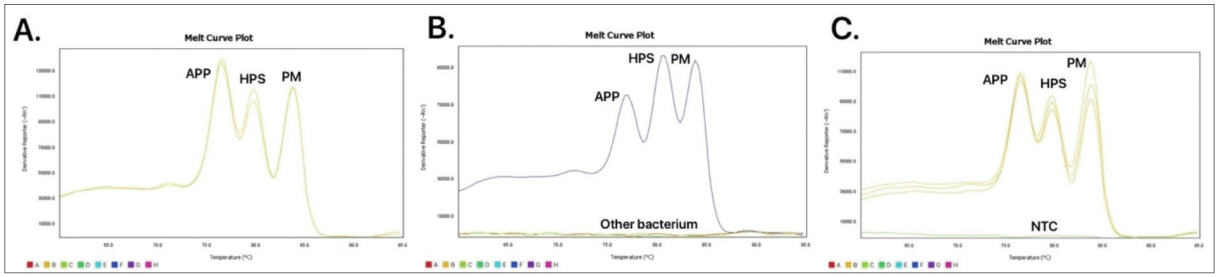

Fig 6. *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* multiple SYBR Green I real-time fluorescent PCR results. **(A)** The determination melting curve; Targets are indicated above each peak along with non-target negative controls (NTC). **(B)** The specific test results; N = 3 **(C)** The repeatability detection results of different concentration gradients; Targets are indicated above each peak along with non-target negative controls (NTC)

Table 2. *The Tm analysis of repeatability for the multiplex SYRR Green I real-time PCR intra-assay*

Pathogenic Species	The Tm/°C Values of 3 Tests			Means	S	CV(%)
	1st	2nd	3rd			
A. pleuropneumoniae	76.47	76.47	76.47	76.63	0	0
H. parasuis	79.77	79.77	79.77	79.77	0	0
P. multocida	83.66	83.81	83.81	83.76	0.07	0.0836

reaction occurred in the other test groups. The Tm values of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were 76.9°C, 79.8°C, and 83.7°C respectively. These results showed that the method had strong specificity.

In the three repeated experiments, the Tm value of each pathogen was relatively stable. The results are shown in *Fig. 6-C* and *Table 2*. The melting curves showed a high degree of overlap and the corresponding Tm values were relatively stable. The standard deviations were all less than 0.1, and the coefficients of variation were less than 0.1%, indicating that the real-time fluorescence PCR reaction of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple SYBR Green I under the same concentration had good stability and repeatability.

The established *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple fluorescent quantitative PCR method was used to detect the standard plasmids diluted in multiple ratios. The sensitivity of the *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* recombinant plasmids could reach 147, 145, and 61 copies/μL, respectively.

Discussion

Respiratory infectious diseases are one of the three syndromes that harm the pig industry. Especially with the development of large-scale pig industry in recent years, the respiratory diseases show an increasing trend. *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* are important common pathogenic bacteria in pig respiratory tract, and distinguishing the clinical symptoms caused by them is difficult [16]. They separately cause pig disease and often infect each other or combine with other pathogen infections [11]. These three bacteria are all Gram-negative bacilli of the Pasteurella family. They also require two-stage concentrated staining, and they are pleomorphic, thus difficult to complete in clinical isolation and identification [17]. Therefore, obtaining a convenient and effective detection method is particularly important.

SYBR Green I is a non-sequence-specific fluorescent dye that can bind to double-stranded DNA heterotectically [18]. At present, it is widely used in quantitative PCR. SYBR Green I real-time PCR not only can accurately quantify the nucleic acid of the target but also carry out multiple PCR reactions in the same PCR tube by analyzing the dissolution curve and using different Tm values of the target fragment, thus providing a new method for simultaneous diagnosis of multiple diseases.

A real-time fluorescent quantitative PCR method based on SYBR Green I was developed for rapid identification of multiple bacteria or viruses. The experiment was highly specific, and it did not cross-react with other common bacteria. This method has been used in many articles, and the detection efficacy is very good [19-21].

In this study, specific primers for *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were designed, and multiple SYBR Green I real-time fluorescence PCR technology was used to amplify the three pathogens. A multiple SYBR Green I real-time quantitative PCR method that could simultaneously detect the three bacteria was also established. In the experiment, different Tm values were used to distinguish nucleic acid fragments. The Tm value of nucleic acid fragments is mainly related to sequence length and sequence structure. Three specific Tm peaks were produced on the same melting curve. Therefore, the purpose of differential diagnosis could be achieved by

monitoring the location of the peak Tm of the fusion curve of the target product.

A. pleuropneumoniae, *H. parasuis*, and *P. multocida* single SYBR Green I real-time quantitative PCR assays; *A. pleuropneumoniae* and *H. parasuis*, *A. pleuropneumoniae* and *P. multocida*, and *H. parasuis* and *P. multocida* double SYBR Green I real-time quantitative PCR assays; and *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple SYBR Green I real-time quantitative PCR assays were successfully established. The specificity test results of this study were good, with only three specific peaks appearing at Tm values of 76.9°C, 79.8°C and 83.7°C respectively. The repeatability test had good stability. The sensitivity test showed that the minimum detection limits of multiple quantitative PCR for *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* plasmid standard were 147, 145, and 61 copies/μL, respectively. The greatest advantage of this study is that *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* could be detected simultaneously, which is conducive to the identification and diagnosis of pig respiratory diseases.

Availability of Data and Materials

The data sets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Competing Interest

The authors declare no conflict of interest.

Authors' Contribution

YZ performed experiments and wrote the manuscript. YZ, YD, YX, ZW, NY performed experiments, HL and LW wrote the article and conceived the experiments.

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RESEARCH ARTICLE

Growth, Hematological and Histopathological Responses to Guar *(Cyamopsis tetragonoloba)* and Salinomycin Sodium for Ameliorating Deleterious Effects of Coccidiosis in Broiler Chicken

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Abstract

Coccidiosis is a preeminent threat to productivity in broiler industry. This prospective study was undertaken to evaluate the anti-coccidial efficacy of guar *(Cyamopsis tetragonoloba)* and salinomycin sodium in broiler Chicken. One hundred and twenty broiler chicks were divided into four groups with each having 30 birds. Group A had salinomycin sodium supplementation, group B received Guar *(Cyamopsis tetragonoloba)* whereas groups C and D were positive and negative controls respectively. Salinomycin and guar *(Cyamopsis tetragonoloba)* were administered following oral inoculation of 50.000 oocysts of Eimeria specie in all birds except the ones inducted into negative control group. Weight gain, feed conversion ratio, Fecal oocysts and blood profile of these birds were determined weekly. When histopathology was performed only a couple of coccidian parasites were identified in the intestinal caeca of birds treated with guar, whereas none were seen in birds treated with salinomycin sodium. It was concluded that results in terms of weight gain and feed conversion ratio were more favorable in case of salinomycin supplementation. However, guar exhibited greater efficacy as an antiprotozoal agent with significant reduction in fecal shedding of oocysts. In contrast to groups receiving salinomycin, severity of bloody diarrhea and mortality rates amongst infected birds subjected to guar were appreciably lower as well.

Keywords: *Age, Blood profile, Hemoglobin, Infection, Oocysts, Broiler chicken, Guar*

Etlik Piliçlerde Koksidiyozun Zararlı Etkilerinin İyileştirilmesi İçin Kullanılan Guar *(Cyamopsis tetragonoloba)* ve Salinomisin Sodyuma Karşı Büyüme, Hematolojik ve Histopatolojik Tepkiler

Öz

Koksidiyoz, broiler endüstrisinde üretkenlik için önde gelen tehditlerden birisidir. Bu prospektif çalışmada, broiler tavuklarda guar *(Cyamopsis tetragonoloba)* ve salinomisin sodyumun antikoksidiyal etkinliği değerlendirildi. Yüzyirmi broier civciv, her birinde 30 hayvan olacak şekilde dört gruba ayrıldı. A grubuna salinomisin sodyum ve B grubuna guar *(Cyamopsis tetragonoloba)* verilirken, C ve D grupları sırasıyla pozitif ve negatif kontrolleri oluşturdu. Negatif kontrol grubu hariç diğer gruplardaki tüm hayvanlara Eimeria türüne ait 50.000 ookist ağız yoluyla verildikten sonra, salinomisin ve guar *(Cyamopsis tetragonoloba)* uygulandı. Civcivlerin, haftalık ağırlık artışı, yemden yararlanma oranı, dışkı ookistleri ve kan profilleri belirlendi. Histopatolojik analizde, guar uygulanmış hayvanların sekumunda sadece birkaç koksidial etken tespit edilirken, salinomisin sodyum uygulanan hayvanların hiçbirinde etken saptanmadı. Salinomisin ilavesinin, hayvanlarda ağırlık artışı ve yemden yararlanma oranı açısından daha olumlu sonuçlar verdiği saptandı. Bununla birlikte, guar, ookistlerin dışkı ile saçılımlarında önemli bir azalma ile antiprotozoal bir ajan olarak daha fazla etkinlik gösterdi. Salinomisin uygulanan grubun aksine, kanlı ishalin şiddeti ve enfekte kuşlar arasındaki ölüm oranları guar uygulanan grupta kayda değer ölçüde daha düşüktü.

Anahtar sözcükler: *Yaş, Kan profili, Hemoglobin, Infeksiyon, Ookist, Broiler tavuklar, Guar*

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Introduction

Productivity in Pakistani broiler sector has become stagnant in recent years due to a myriad of bacterial, viral, fungal and parasitic diseases causing devastating economic losses [1,2]. *Eimeria* species (phylum Apicomplexa) being a protozoan parasite, is believed to be the root cause of Coccidiosis in broiler [3], infecting upto 70% commercialized broiler production systems [4], in developing countries [5]. Seven subspecies of *Eimeria* have been isolated from chicken i.e. *E. acervulina, E. maxima, E. tenella, E. brunette, E. necatrix, E. mitis* and *E. praecox* [4,6,7]. However, in Pakistan *E. tenella* has been observed as the most prevalent pathogenic species [8]. Coccidiostats have been employed all over the world for efficient control of avian coccidiosis but their repeated and inefficient usage has produced several drug resistant strains [4]. This dilemma has promoted a search for plant based anti-microbial nutraceutical agents [9].

Guar legume *(Cyamopsis tetragonoloba),* grown for gum, produces guar hulls as a by-product containing high level of antiprotozoal Saponins [10] whereas Salinomycin is a tri-cyclic spiroketal hexagonal ring system possessing ionophoric properties [11]. Hematology values indicate the physiological profile of birds [11] and are used as an important diagnostic tool in many anemic disorders [12]. It has been reported that erythrocytes (RBC), leukocytes (WBC), hematocrit (hct) and hemoglobin (Hb) levels increase with age and their values change significantly in diseased birds [13,14]. As coccidial oocysts are ingested and intestinal mucosae of birds are colonized by *Eimeria* species, impaired food conversion ratio (FCR) leads to reduced productivity [9,15]. Furthermore, coccidiosis also destroys natural intestinal microflora namely Enterobacteriaceae, that assist in feed digestion. This creates favorable condition for the progenation of microbes such as *Lactobacillales* spp. thereby further deteriorating overall gut health. Poor feed conversion causes depressed immune system which eventually enhances the probability of secondary bacterial infections [16,17].

So the current study was designed to examine the anti-coccidial efficacy of guar and salinomycin sodium in broilers chicken. Efficiency of these drugs in diseased broiler chickens was to be evaluated based on production, hematological profile and histopathological changes.

Material and Methods

Ethical Considerations

Ethical approval of this study was obtained from University of Veterinary and Animal Sciences, Lahore, Ethical Committee. The animals were treated in compliance with ethical standards.

Grouping of Birds

The study was conducted on 120 birds which were deemed free from any coccidial infection by fecal examination. Day old chicks were sourced from local market. They were weighed and randomly assigned into four (4) groups namely A, B, C and D. Each group had 30 birds. Group A (GA) and Group B (GB) were treatment groups whereas group C was positive control (PC) and group D was negative control (NC). The experimental birds were vaccinated against Newcastle and Infectious Bursal Disease. All birds inducted into the study were offered an experimental feed. Nutritional composition of the aforementioned feed has been presented in *Table 1*.

Group A had salinomycin sodium supplementation; group B had Guar *(Cyamopsis tetragonoloba)* supplementation whereas groups C and D were positive and negative control respectively.

Infection and Medication

Broiler chicken suspected with coccidiosis were collected from different broiler farms. Postmortem examinations were performed and samples were collected from suspected *Eimeria tenella* infections. Collected specimens were evaluated with PCR analysis to confirm presence of aforementioned coccidial specie. A suspension containing *E. tenella* oocysts was prepared by using Clorox digestion procedure [21]. Coccidial eggs were isolated and 1 mL inoculum containing 50.000 sporulated oocysts was introduced directly into the crop of each bird of groups A, B and C on day 21. Medication was started when clinical signs of coccidiosis appeared after 7 days of infection [18]. All birds were fed the same experimental diet details of which are given in *Table 1*. In addition to that GA was offered salinomycin sodium 5 gms/10 kg of feed [19] and GB was offered guar at 5% of feed [20].

Drug Efficacy and Blood Profile

Mortality rates and weight gain were determined on weekly basis in the birds of all groups. The feed conversion ratio (FCR) was determined as described by Tanweer et al.[6] and oocysts per gram (OPG) were counted as described by Velkers et al.[21] to determine the efficacy of the drug. Complete blood count (CBC) included, Total Red blood cell count, Total leucocyte count, Blood hemoglobin, Mean corpuscular volume, Packed cell volume and Mean

Table 1. *Nutritional composition of experimental feed offered to broilers*

Composition	%, Unless Indicated
Moisture	9.8
Dry matter	90.2
Crude protein	18.4
Crude fiber	4.8
Ether extract	8.9
Crude ash	2.5
Nitrogen-free extract	55.6
Metabolizable energy (K Cal/kg)	2896

corpuscular hemoglobin were measured by using an automatic hematology analyzer (Hematology Analyzer, Abacus Junior Vet, Austria) as described by Odunitan-Wayas et al.[22] to determine the effects of guar and salinomycin sodium on the blood profile of the birds every week.

Histopathological Examination

Birds were slaughtered by Halal method as described by Abdullah et al.[23] and 3 cm intestinal segments at midpoints were collected from all the groups preserving them in 4% paraformaldehyde solution. Samples were dehydrated and histological sectioning was performed after embedding in paraffin. Slides were stained with haematoxylin and eosin (HE) [9].

Statistical Analysis

Statistical analysis was done using Statistical Package for Social Science (SPSS for windows version 17.0, SPSS Inc, Chicago, IL). Normal distribution of the data was tested with Shapiro-Wilk test [24]. Analysis of data was done by multiple analyses of variance (MANOVA). Significant differences among the groups were analyzed by Duncan's Multiple Range test (Duncan 1955), ($P<0.05$).

Results

No dead birds were observed in GA, GB and NC till 28th day post infection, but several birds of PC group died by 28th day. No mortality was observed in NC throughout the duration of trial. However, all birds inducted into Positive Control group died by 42nd day *(Fig. 1)*.

Higher mortality was observed in GA as compared to GB showing that guar reduces mortality in coccidiosis more efficiently as compared to salinomycin sodium *(Fig. 2)*.

Significant results were observed for weight gain, FCR and OPG between weeks. Weight gain and FCR increased with increasing age and was found to be most in 6th week. However, oocyst per gram (OPG) decreased gradually with time in GA and GB but increased in PC *(Table 2)*.

Erythrocyte count amplified as birds aged in NC but plummeted in groups infected with coccidiosis. Similarly, TLC increased in NC with age whereas decreased in the rest of experimental groups as infection became severe. A significant difference ($P<0.05$) was observed for PCV, hemoglobin, MCV and MCH throughout the duration of study *(Table 3)*.

NC had the highest values for TEC and TLC followed by GB and GA whereas as PC had the least values for TEC and TLC. NC had the most values for hemoglobin and PCV followed by GB and GA, as PC had least values for PCV and hemoglobin. GA and GB showed no significance for MCH and MCV during 5th week while NC had lowest values for MCV and MCH. However, during the 4th week group B had the lowest value for MCH *(Table 3)*. Postmortem lesions

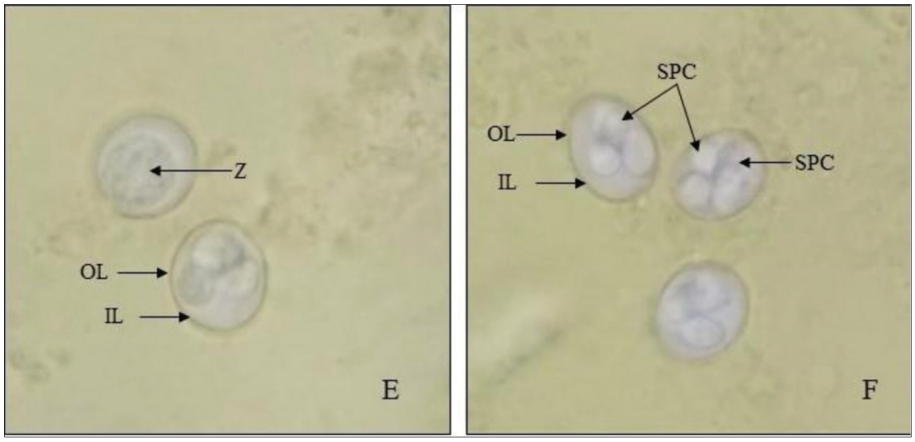

Fig 1. Oocysts morphological features: (E) Photomicrograph of freshly shed un-sporulated oocyst (Z-zygote); Two layers of the oocyst wall (OL-outward, IL-inward) (400×). (F) Photomicrograph of the sporulated spherical oocyst with four sporocysts (SPC) each containing two sporozoites (SPO) (400×)

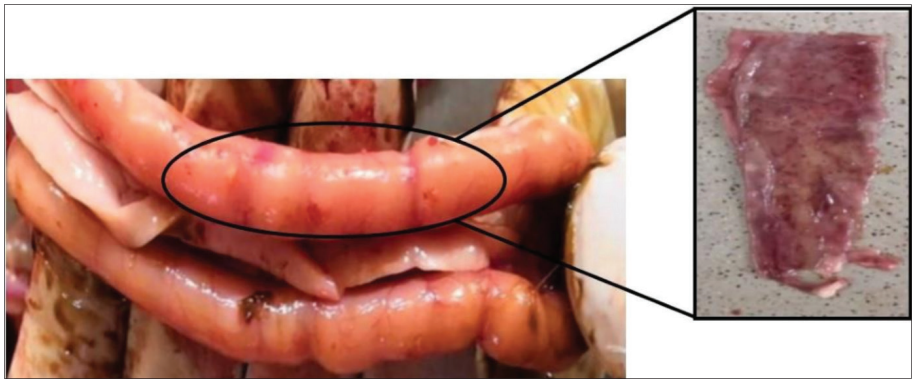

Fig 2. Ballooned intestines and hemorrhagic spots on caecal wall with blood contents

Table 2. *Effects of guar and salinomycin sodium supplementation on production of broilers post infection*

Variables	Weight Gain (gms)			FCR[+]			OPG[Ψ]		
	Weeks			Weeks			Weeks		
	4th	5th	6th	4th	5th	6th	4th	5th	6th
Salinomycin Sodium	1148.0±11.56 ^{A,c}	1750.23±11.77 ^{B,c}	2351.94±10.59 ^{C,b}	1.47±0.01 ^{A,a}	1.65±0.01 ^{B,b}	1.67±0.01 ^{C,b}	7153.76±35.29 ^{C,b}	4168.61±3.86 ^{B,b}	2146.27±18.01 ^{A,b}
Guar *(Cyamopsis tetragonoloba)*	1079.66±14.19 ^{A,b}	1641.25±13.04 ^{B,b}	2068.47±11.02 ^{C,a}	1.56±0.02 ^{A,b}	1.78±0.01 ^{B,c}	1.88±0.01 ^{C,c}	6269.23±25.84 ^{C,a}	3297.29±127.23 ^{B,a}	1147.65±16.98 ^{A,a}
Positive Control	881.60±18.52 ^{A,a}	1000.94±13.37 ^{B,a}	ABD[∞]	1.92±0.04 ^{A,c}	2.92±0.03 ^{B,d}	ABD[∞]	7766.44±17.82 ^{A,c}	8950.17±349.97 ^{B,c}	ABD[∞]
Negative Control	1147.50±9.97 ^{A,c}	1808.83±14.42 ^{B,d}	2539.50±13.15 ^{C,c}	1.47±0.01 ^{A,a}	1.52±0.01 ^{B,a}	1.53±0.01 ^{C,a}	N.O[♭]	N.O[♭]	N.O[♭]

[+] FCR = feed conversion ratio, [Ψ] OPG = oocysts per gram, [∞] ABD = all birds died, [♭] N.O = no oocysts. Results are written as Mean ± Standard Deviation. Superscripts [A-C] within a row indicates that values are statistically significant (P<0.05). Superscripts [a-d] within a column indicates that values are statistically significant (P<0.05)

Table 3. *Effects of guar and salinomycin sodium supplementation on blood profile of broilers post infection*

Variables	TEC[χ] X 10^6/µL			TLC[ω] X 10^3/µL			Hb[ε] (gm %)			PCV[Ψ] (%)			MCV[δ] (fl)			MCH[γ] (pg)		
	Weeks			Weeks			Weeks			Weeks			Weeks			Weeks		
	4th	5th	6th	4th	5th	6th	4th	5th	6th	4th	5th	6th	4th	5th	6th	4th	5th	6th
Salinomycin Sodium	3.17±0.01 ^{C,b}	3.07±0.01 ^{B,b}	3.02±0.01 ^{A,a}	8.54±0.02 ^{C,b}	8.47±0.01 ^{B,b}	8.42±0.01 ^{A,a}	8.67±0.01 ^{C,b}	8.62±0.01 ^{B,b}	8.56±0.01 ^{A,a}	25.51±0.03 ^{C,b}	25.36±0.03 ^{B,b}	25.20±0.04 ^{A,a}	80.44±0.35 ^{A,c}	82.56±0.40 ^{B,c}	83.18±0.37 ^{C,c}	27.35±0.12 ^{A,c}	28.07±0.13 ^{B,c}	28.28±0.12 ^{C,c}
Guar *(Cyamopsis tetragonoloba)*	3.25±0.03 ^{C,c}	3.16±0.02 ^{B,c}	3.13±0.01 ^{A,b}	8.93±0.03 ^{C,c}	8.85±0.03 ^{B,c}	8.67±0.02 ^{A,b}	8.85±0.02 ^{C,c}	8.80±0.02 ^{B,c}	8.75±0.02 ^{A,b}	26.05±0.06 ^{C,c}	25.89±0.07 ^{B,c}	25.75±0.07 ^{A,b}	79.10±0.82 ^{A,b}	82.20±0.73 ^{B,c}	82.72±0.29 ^{C,b}	26.89±0.28 ^{A,a}	27.95±0.25 ^{B,c}	28.12±0.10 ^{C,b}
Positive Control	3.09±0.01 ^{B,a}	3.01±0.01 ^{A,a}	ABD[∞]	8.44±0.02 ^{B,a}	8.35±0.01 ^{A,a}	ABD[∞]	8.58±0.02 ^{B,a}	8.22±0.01 ^{A,a}	ABD[∞]	25.25±0.07 ^{B,a}	24.19±0.07 ^{A,a}	ABD[∞]	80.19±0.66 ^{A,d}	81.62±0.45 ^{B,b}	ABD[∞]	27.26±0.22 ^{A,d}	27.75±0.15 ^{B,b}	ABD[∞]
Negative Control	3.41±0.04 ^{A,d}	3.55±0.02 ^{B,d}	3.65±0.03 ^{C,c}	8.90±0.05 ^{A,d}	9.04±0.04 ^{B,d}	9.33±0.08 ^{C,c}	8.88±0.04 ^{A,d}	9.07±0.03 ^{B,d}	9.11±0.03 ^{C,c}	26.14±0.13 ^{A,d}	26.68±0.08 ^{B,d}	26.79±0.09 ^{C,c}	76.46±0.84 ^{C,a}	74.98±0.70 ^{B,a}	73.32±0.79 ^{A,a}	26.96±0.69 ^{C,b}	27.05±1.14 ^{B,a}	26.81±1.63 ^{A,a}

[χ] TEC = total erythrocyte count, [ω] TLC = total leucocyte count, [ε] Hb = hemoglobin, [Ψ] PCV = packed cell volume, [δ] MCV = mean corpuscular volume, [γ] MCH = mean corpuscular volume, [∞] ABD = all birds died. Results are written as Mean ± Standard Deviation. Superscripts [A-C] within a row indicates that values are statistically significant (P<0.05). Superscripts [a-d] within a column indicates that values are statistically significant (P<0.05)

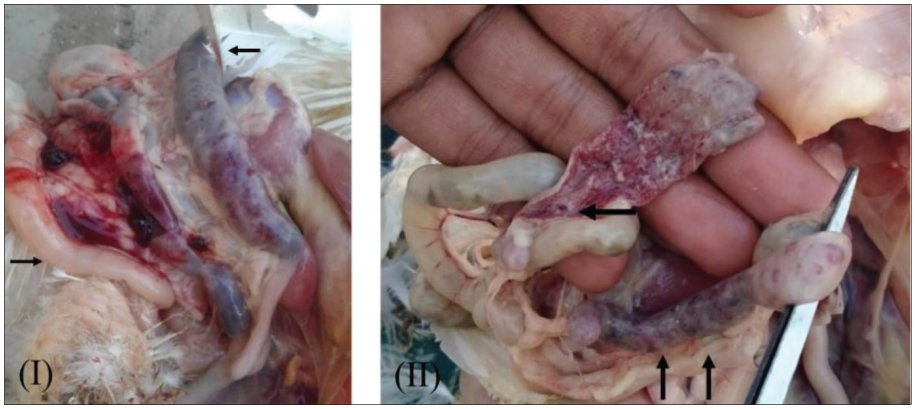

Fig 3. **(I)** *Eimeria tenella* infected broiler chicken caeca, **(II)** Opened caecum is filled with blood

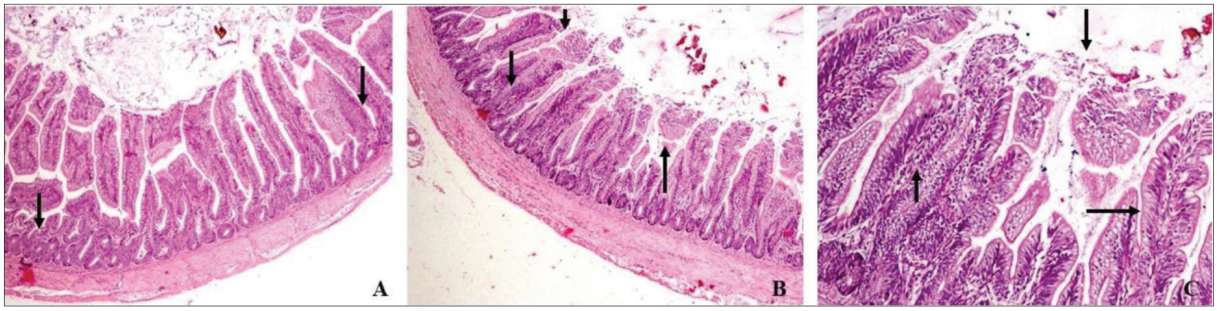

Fig 4. Progressive cytopathic changes observed in intestinal histopathological slides as a consequence of coccidiosis in group C. **(A)** Intestinal villi preserved with few trophozoites whereby arrows mark the inflammatory infiltration by heterophils and mononuclear cells (H.E. staining, 200×), **(B)** Desquamation of enterocytes and fibrosis in muscularis marked by arrows indicate proliferation of meronts (H.E. staining, 200×), **(C)** Degradation of sub-mucosal glandular epithelium, edema and fibrosis along with shedding of oocysts in the glandular lumen (arrow) (H.E. staining, 400×)

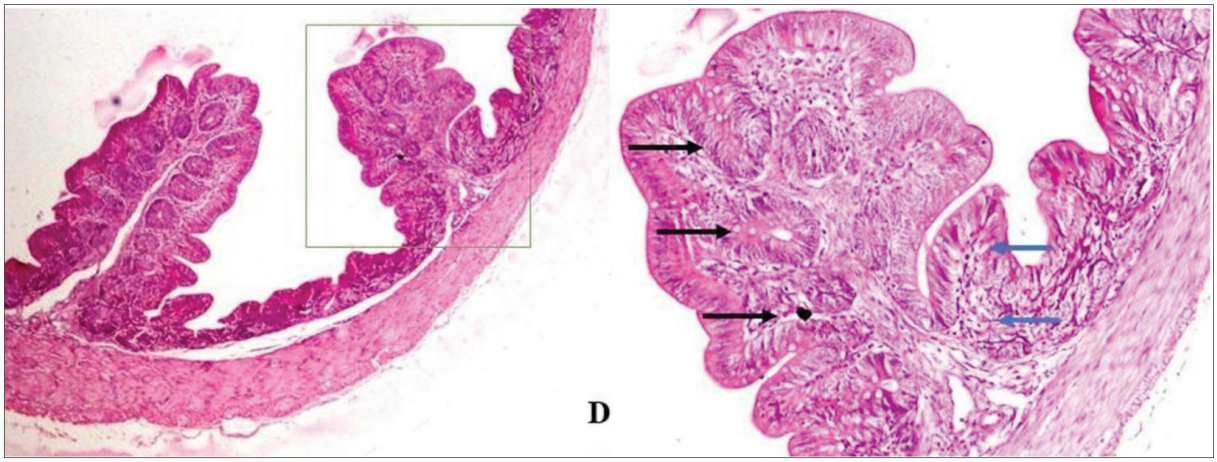

Fig 5. Photomicrographs of intestinal mucosa of birds having guar supplementation **(D)** Mitigation of inflammatory process between crypts. Intestinal villi exhibit recovery after experiencing hypoplasia and sloughing (indicated by presence of fibrinous proliferation in lamina propria) (H.E. staining, 400×)

pathognomonic to *Eimeria* infection were evident in clinical cases *(Fig. 3)*.

Histopathology of caecal coccidiosis showed epithelial tissue destruction and vascular congestion along with presence of hyperplastic lymphoid cells in the lumen. Caecum and intestine of birds were full of *Eimeria* oocyst. Progressive cytopathic changes were observed in intestinal histopathological slides as a consequence of coccidiosis in group C *(Fig. 4)*.

Whereas birds supplemented with guar supplementation exhibited prompt recovery of Intestinal villi after experiencing mucosal sloughing *(Fig. 5)*.

Discussion

Birds of Group A treated with salinomycine sodium exhibited greater weight gains as compared to other groups. Similar findings were reported by Rychen et al.[25] and Hassan [26], whereby administration of synthetic ionophores reduced coccidiosis-related mortalities while concurrently acted as growth promoters. These observations were attributed to the innate ability of salinomycine sodium to neutralize certain Gram-positive bacteria responsible for intestinal maladies [27]. In several studies salinomycine sodium (SAL) has been hailed as an immensely efficacious anticoccidial agent. However, Demirulus et al.[28] thoroughly contradicted with prior suggestions and reported negative effects of SAL on weight gain in broiler birds. Concurrently certain studies indicated favorable outcomes when guar was administered to mitigate coccidiosis in broiler birds. Howbeit, poor weight gains were observed again which were rationalized by the presence of anti-nutritional factors such as saponins in guar based feeds [25,29]1983. Reyer et al.[30] justified improved FCR in guar fed broiler birds by hypothesizing the emulsifying effects of saponins thereby improving membrane permeability and nutrient absorption of diet. Furthermore, a recent study of guar in broiler birds has indicated elevated digestibility of both essential and non-essential amino acids [31]. But present study could not corroborate these findings as birds which received SAL supplementation observably gained greater weight than the ones that fed with guar. Yet in certain cases, supplementation of feed with phytogenic additives has been judiciously warranted by experimental trials [32-34].

Oral-fecal route has been a primary device for the spread of coccidiosis throughout modern broiler farming systems [35,36]. Authors have reported an increase in OPG with age of PC after inoculation. This has been previously reported by Rambozzi et al.[37] who suggested that oocysts increase with age in coccidiosis provided that birds received no therapeutic treatment. We observed marked reduction in OPG in all treatment groups whereby Guar showed better anticoccidial activity when compared with salinomycin sodium. Efficacy of guar supplementation has been attributed to the presence of saponins which elicited anticoccidial activity by binding with the sterol molecules that are present on the protozoan cell membranes [20,38] because they do not results to tissue residue and drug resistance. In order to evaluate the effects of herbal extracts to control avian coccidiosis, 180 one-day-old broiler chickens were randomly divided into nine equal groups, as follows: (1. Consistent with previous reports, the number of fecal oocysts in birds challenged with coccidia were effectively reduced in response to the phytogenic products having saponins [6,35,39,40].

Similar to prior findings [14], it was observed that TEC, TLC, hemoglobin and percentage of PCV increased as birds continued to age whereas MCV and MCH decreased in ageing birds that were infected with *Eimeria* spp. *(Table 2)* [14]. Patra et al.[41] has also reported overall reduction in TEC and hemoglobin of infected birds due to hemorrhagic cecal lesions. GA had the lowest TLC values than NC and GB as salinomycin caused fragility of red blood cells which ultimately led to their lysis [42]. TLC was reportedly lowest in GA as compared to NC and GB validating prior findings [43], which suggested that salinomycin significantly decreased size of bursa leading to a reduction of white blood cell count [43]. In our study, decrease in PCV and relative increase of MCV and MCH values were comparable to that of published data [44]. Lower TEC for GA relative to GB indicated that PCV and hemoglobin levels would be depressed as well *(Table 2)*. Likewise, MCV and MCH decreased more so in GB than in GA due to comparative difference in TEC as it affected MCV and MCH [45].

Histological sections of necrosed cecal epithelia in birds infected with *Eimeria tenella* oocyst was illustrated by Wasae [46] and other researchers [47-50] to exhibit deterioration of intestinal epithelium and caeca in broiler birds. Fewer cecal coccidian sporozoites were observed birds treated with guar, while multiple developmental stages were seen in the infected and untreated groups [38]. Salinomycin sodium was effective in eliminating parasitic infestation but histopathological evidence of cecal destruction was still evident [38].

In context of present study, we concluded that although both salinomycin sodium and guar *(Cyamopsis tetragonoloba)* exhibited adequatevanticoccidial properties but guar was considerably more efficacious in controlling manifestation of clinical signs and oocyte shedding through feces. Whereas, Salinomycin sodium supplementation resulted in better body weight and FCR gains.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author.

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Declaration of Conflict of Interest

There was no conflict of interest in regards to authors reporting their findings.

Author Contributions

Experimental Design was conceived by ON, JAK and MS. Data was collected by AHR, ASA, YRK, AA and KH. Statistical

analysis was conducted by JN, MLS and US. Original draft was written by MB and MW. All authors have contributed to the revision and final proof-reading of the manuscript.

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RESEARCH ARTICLE

Encapsulation of Progesterone-Like Compounds in 10% Liposome Increases Their Concentration in Rats Administered an Injectable Dosage Form of These Compounds

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Abstract

The use of herbal medicine to fill the void in synthetic medicine is very necessary for health lifestyle. Progesterone-like compounds (PLCs) from the extract of the leaves of *Dendrophthoe pentandra* L. *Miq* from the subspecies, *Benalu Duku* (BD), are known to contain beneficial compounds that contain anti-cancer and androgenic substances. The slow release of active compounds can be achieved using slow release vehicles. Liposomes that are small unilamellar vesicles (SUVs) are one of the excipient substances that can be reliably used as a vehicle to achieve timely release of bioactive substances. This study was conducted to demonstrate that encapsulating PLCs in 10% liposomal SUV enables the gradual release of bioactive compounds. Three single doses of 3; 5; 7 mg PLCs-Liposome SUV/100 g per body weight of rats were injected into rats in the trial groups (15 of rats). Thereafter, the plasma concentrations of PLCs were assessed using liquid chromatography electrospray ionization mass spectrometry (LC-ESI MS). The concentrations of PLCs in the trial groups were found to be 1.20 to 2.40 fold higher than those in the control group. Such findings indicate that encapsulating drugs in 10% liposomes can result in a higher drug level in blood than that obtained without drug encapsulation (P<0.05).

Keywords: *Androgenic, Health Lifestyle, Mistletoe plant, Progesterone-like compounds, Small unilamellar vesicle*

Progesteron-Benzeri Bileşiklerin %10 Lipozom İçerisinde Kapsüllenmesi ve Bu Bileşiklerin Enjektabl Dozaj Formunun Uygulandığı Sıçanlardaki Konsantrasyonlarını Artırır

Öz

Sentetik tıptaki boşluğu doldurmak için bitkisel ilaçların kullanılması sağlıklı yaşam tarzları için oldukça gereklidir. *Benalu Duku* (BD) alt türü olan *Dendrophthoe pentandra* L. *Miq*'in yaprak ekstraklarından elde edilen progesteron benzeri bileşiklerin (PLC), anti-kanserojenik ve androjenik maddelerden oluşan faydalı bileşikler içerdiği bilinmektedir. Aktif bileşiklerin yavaş salınımı, yavaş salınan araçlar kullanılarak elde edilebilir. Küçük tek katmanlı veziküller (SUV) olan lipozomlar, biyoaktif maddelerin zamanında salınımını sağlamak için güvenilir bir şekilde kullanılabilen eksipiyan maddelerden biridir. Bu çalışma, PLC'lerin %10 lipozomal SUV içinde kapsüllenmesinin biyoaktif bileşiklerin kademeli olarak salınmasını sağladığını göstermek için yapılmıştır. 100 g vücut ağırlığı başına üç farklı doz (3; 5; 7 mg) PLC-Lipozom SUV deneme gruplarındaki ratlara (15 rat) enjekte edildi. Daha sonra PLC'lerin plazma konsantrasyonları, sıvı kromatografi-elektrosprey iyonizasyon kütle spektrometrisi (LC-ESI MS) kullanılarak değerlendirildi. Deneme gruplarındaki PLC konsantrasyonlarının, kontrol grubundakilerden 1.20 ile 2.40 kat daha yüksek olduğu saptandı. Bu bulgular, %10'luk lipozomlarda kapsüllenen ilaçların, ilaç kapsüllenmesi olmadan elde edilene göre kanda daha yüksek bir ilaç seviyesi ile sonuçlanabileceğini göstermektedir (P<0.05).

Anahtar sözcükler: *Androjenik, Sağlıklı yaşam tarzı, Ökseotu bitkisi, Progesteron benzeri bileşikler, Küçük tek lamelli kesecik*

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Introduction

Many bioactive compounds, such as amino acids, phytohormones, plant enzymes, alkaloids, flavonoids, and other substances, can be found in mistletoe plants, especially their leaves [1]. According to current reports, more than 60% of the bioactive compounds in mistletoe plants have been used in pharmaceutical products. As a result, these compounds have had a positive impact on the healthcare system owing to their use as treatments for diseases associated with hormone deficiency disorder [2]. The subspecies of *Dendrophthoe pentandra L. Miq, Benalu Duku* (BD) produces a small number of diverse bioactive compounds, namely progesterone-like compounds (PLCs), that can be used to treat cancers and androgenic hormone disorders so make a better performance to health lifestyle [3,4]. High concentrations of progesterone, which may protect against libido disorders, accumulate in the crude methanol extract of BD leaves [5-7].

Plants containing beneficial PLCs that are used as herbal medicines may supplement the needs of the human body through mechanisms involving normal physiological hormones [8]. Generally, treatment using hormones as bioactive compounds requires the administration of a high dose. An alternative option to reduce the high dose required is to produce the drug as an injectable dosage form. Nanoparticle technology or ultra-fine technology can help to accelerate the pharmacodynamic action of injectable dosage forms for applications to non-vascular administration [9]. The effect of the drugs will be better if the concentrations of the bioactive compounds can be maintained for a long period. These concepts can be modified to produce new concepts that vehicle as an excipient to control the release time of the active substance. Small unilamellar vesicle (SUV) is a phospholipid vesicle consisting of one or more concentric lipid bilayers enclosing discrete aqueous-oil spaces [10,11]. The unique ability of liposomal systems to entrap both lipophilic and hydrophilic bioactive compounds enable a diverse range of drugs to be encapsulated by these vesicles [12,13]. This ability causes the pharmacodynamic action of the bioactive molecules to be increased through a controlled dispensing system and enables the time interval of drug administration to be reduced [14]. The gradual release concentrations of PLCs encapsulated of SUV can be observed after comparing to concentrations of Certified Reference Material (CRM) progesterone [15]. Thus, the availability of drugs in the test group is expected to be longer than control group. The explored model, which enabled the gradual release of drugs from liposome employed as a vehicle, was identified as a suitable test to prove the pattern of drug dispensing to produce long-acting effects. Such test will be significant if the drug will be dispensed via non-vascular parenteral routes (intramuscular, sub-cutaneous, intra-peritoneal etc.). It is known that injections do not exhibit the first-pass phenomena, and small particles will cause the surface area of the particles to become larger, facilitating the absorption and adsorption processes. This strategy will be easily realized if the drug is designed in the form of injection and uses a slow-release vehicle [16]. Some substances that can be used as vehicles include fats or oils, one of which is liposomes. However, this strategy has yet to be investigated.

Based on the above information, this research will be carried out to determine the benefits of encapsulating PLCs in liposomes for administration as an injectable dosage form. The hypothesis of this research is that encapsulated liposomes of active compounds, namely, PLCs will increase the concentration of progesterone in blood plasma. The present study was designed to estimate the concentrations of bioactive PLCs in rat plasma after encapsulation in SUV.

Material and Methods

Chemical

The reagent and chemicals used to extract and analyze PLCs by HPLC or LC-ESI MS and to produce the injectable dosage form were of CRM grade or pro analysis (PA) grade. SUV was obtained from Merck Corp. (Germany) [17]. The liposome component as small unilamellar vehicles on injection dosage form at levels sterile, pyrogen free, isotonic, free from ion compounds was used at compositions as follows; cholesterol 9 µmol, L-α-phosphatidylcholine 63 µmol, and stearylamine 18 µmol. Samples of BD were collected from the Muara Enim District of Palembang, South Sumatera, Indonesia.

Extraction and Isolation of BD

Leaves of BD were manually separated, washed, shade-dried, and ground to fine powders. To remove the impurities, hydrolysis was performed. Briefly, 20 g of each powdered plant sample was mixed with 100 mL of 0.5 M acidified (HCl) methanol (w/v) and agitated at room temperature for 24 h [18]. The total hydrolyzed extract was separated from other impurities that were not dissolved in methanol:water (70:30) by SPE with a C_{18} cartridge. First, reverse phase activation of the C_{18} SPE cartridge was carried out by adding 5 mL methanol to the cartridge. Thereafter, the hydrolyzed plant extract dissolved in 5 mL methanol was added to the cartridge. Clean-up was carried out with the addition of 5 mL of water to SPE followed by drying for 10-15 min. The pure BD extract containing PLCs was eluted using fractions of methanol:water (70:30) 5 mL and all elution results [19].

Purifying PLCs From the Hydrolyzed Powders of The BD Leaf Extract

HPLC was performed using a Shimadzu LC-6AD pump, DGU-20A5 degasser, type 20A communication bus module (CBM), UV-visible type SPD-M20A photodiode array detector, and FRC-10A fraction collector. A C_{18} RP LiChrospher

100 column was employed. For HPLC, isocratic elution was carried out at wavelength, 254 nm; flow rate, 0.5 mL/min; column temperature, 20°C; mobile phase, methanol:water (70:30); and stop-time, 11.00 min [18].

Encapsulation of Liposomes

A total of 500 mg of dried liposomes was weighed for combination with 5 g of PLCs, melted at 70°C, and added to hot water until a volume of 5 mL was obtained. The mixture was shaken well for 30 min and sonicated for 30 min. Analysis of particle PLCs and particle encapsulate of liposome were using by Scanning Electron Microscope (SEM) of Zeiss MA 10 installed at year 2010 producing from One North Broadway, White Plans, NY 10601 US. The SEM were adjusted as follows; Electron High Tension (HET) at about 15.000 to 30.000 kv, Working Distance (WD) approximately 5 to 10 mn, Signal A at SE 1 as secondary detector, Magnified (Mag) 700 to 10.000 times and scale of object at about 1 to 50 µm. Operating of SEM was used for object particle dried and stable at range of HET workflow.

Injectable Dosage Form

Substances containing PLCs and 10% liposomes were prepared in clean bench room as follows; filtered with a porous membrane filter (20 µm), then adjusted to a pH value between 6.80 and 7.00 using sodium hydroxide.

Animal Experiment

Rats *(Rattus norvegicus*; healthy, adult, male (150-200 g) were employed for the animal experiments. Rats were obtained from the Veterinary Pharma Research Centre, *(http://pusvetma.ditjenpkh.pertanian.go.id/layananpenunjang)*. Before treatment, an ethics test was first carried out using rats at the Faculty of Veterinary Medicine, Universitas Airlangga (Certificate No: 2.KE.102.11.2020). Experimental animals were cared for and maintained in accordance with the animal welfare guidelines, and housed in a comfortable place.

Experimental Design

Research designs were used as an *in vivo* model by post test only control group design in groups as follows; research groups, negative control groups and positive control groups. The sample size for this experiment was calculated using the equation below [20].

$$N = \frac{\left[\left(Z_1 - \frac{\alpha}{2}\right) + Z_\beta\right]^2}{\frac{(d)^2}{(S_a)^2 + (S_b)^2}}$$

Where (Z1- α/2) = 1.96, with a significance of 0.05; Z_β = 1.645 with an error limit of 5%; d = 3.62, Sa = 1.7; and Sb = 1.4. As the N value was rounded to 5, the positive control group should consist of 15 rats, the negative control group should contain 15 rats, and the test groups should also contain 15 rats. The 15 rats allocated to the test groups were further divided into three sub-groups (for each dose) consisting of five individuals.

Each sub-group of test rats was injected intraperitoneally with PLCs encapsulated in 10% liposome; the sub-groups received 3 mg/100 g BW, 5 mg/100 g BW, and 7 mg/100 g BW. The rationale for assigning three doses was based on research reports that the three doses provided minimal pharmacological response of BD as androgenic effect [18,20]. Rats in the positive control group were injected with a dose of pure progesterone. At 30 min after the injection, 1 mL of blood was withdrawn from the heart. After 60 min, more blood was withdrawn. A total of 1 mL of blood was centrifuged for 30 min and plasma (0.5 mL) was obtained. Blood sampling was done by first giving ketamine 1 mg/kg BW intramuscularly.

LC-ESI MS

Acetonitrile was added to the plasma and the mixture was shaken well for 10 min. The supernatant was collected, centrifuged, and separated using a C_{18} SPE column, which was first activated by the addition of methanol. The SPE elution material was then dried and added to the mobile phase for injection into the LC-ESI MS system. PLCs encapsulated in 10% liposomes were subjected to liquid chromatography coupled with mass spectrometry-mass spectrometry (LC/MS-MS) using an Accela TSQ Quantum Access apparatus (Thermo Fisher Scientific, San Jose, CA, USA). To achieve chromatographic separation, vial samples were injected through an auto sampler (Surveyor auto-sampler plus) into the rheodyne system (Surveyor) equipped with a Hypersil GOLD (0.2 µm particle size, length 10 cm) for gradient elution. The elution was carried out at a flow rate of 0.5 mL/min. Solvent A consisted of a mixture of water:acetonitrile:formic acid 90:10:0.1% (v/v), while solvent B was water:acetonitrile:formic acid 10:90:0.1% (v/v). Elution was performed using the following gradient: Solution B increased from 35% to 70% at 20 min. The identification of PLCs was conducted in full scan mode in the range of 100–600 m/z.

Statistical Analysis

The analyzed data between the trial groups compared to the control groups were assessed using the Statistical Package for Social Sciences (SPSS) 24.0, at 5% significance by independent t-test.

Results

The chromatogram peaks of PLCs separated from the crude extract of BD leaves by HPLC appeared at retention times between 6.50 to 8.00 min. In contrast, the pure substance of progesterone was observed at a retention time of 7.119

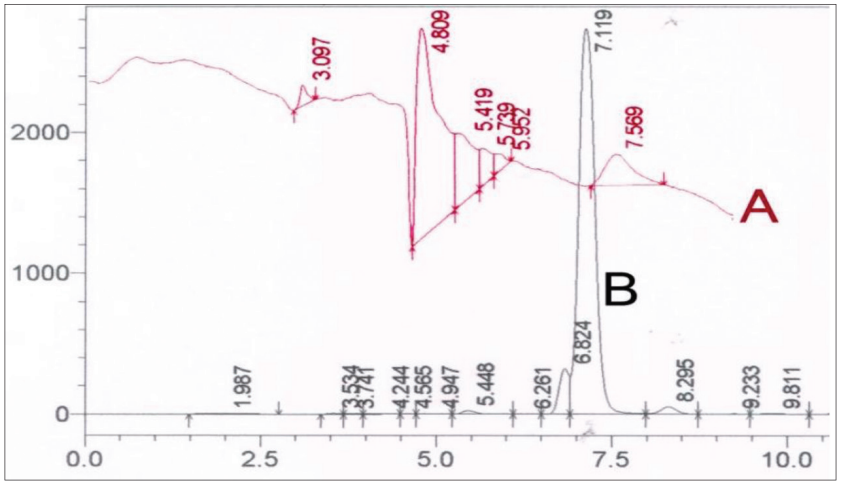

Fig 1. Red line represents the methanol extract of *Benalu Duku* leaves, with peak for the PLCs appearing at a retention time of 7.569 min **(A)**. Black line represents pure progesterone in the mobile phase, with its peak appearing at a retention time of 7.119 min **(B)**

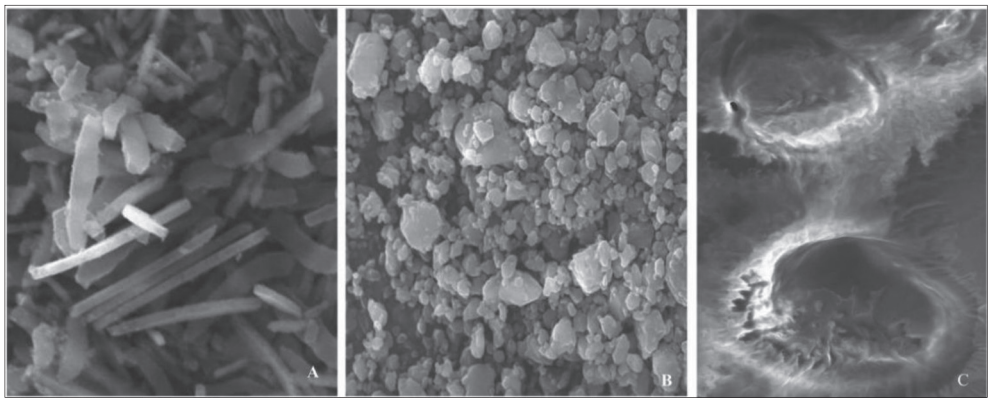

Fig 2. Particle for progesterone-like compounds at working distance 8.0 mm, magnification 10.000x, scale of object 1 µm **(A),** particle for progesterone as a certified reference material at working distance 7.5 mm, magnification 3000x, scale of object 3 µm **(B),** and particle for progesterone-like compounds after encapsulation in 10% liposome for administration as an injectable dosage form at working distance 7.5 mm, magnification 700x, scale of object 10 µm **(C)**

min. In *Fig. 1*, PLCs (red line, A) appeared at a retention time of 7.569 min while the CRM of pure progesterone (black line, B) appeared at a retention time of 7.119 min. The PLCs were collected at a retention time of 7.569 min and obtained at a weight of ±1600 mg. The particles of PLCs before and after encapsulation in 10% liposome (w/w) are shown in *Fig. 2-A,B,C* at 500x to 10.000x magnification.

The optimizations carried out to analyze the progesterone standard after encapsulation in 10% liposome are presented in *Fig. 3* and *Fig. 4*. A gradient elution was carried out on the LC-ESI MS system using acetonitrile: water (30:70) with 0.1% formic acid. *Fig. 4* shows that PLCs encapsulated in 10% liposomes could be identified by MS/MS, with a ratio of ionized mass molecule (m/z) of 326.50-327.50. LC-ESI MS targeted a special molecule mass of progesterone. The peaks for impurities in the biological matrix were found to disappear at the retention time and molecular mass of the analyte. After encapsulation in 10% liposome, the PLCs were identified at a retention time of 4.35 min, and the ESI of the ionic molecular mass spectrum was 326.50. The drifts of retention time and m/z between 0.5 to 1 min or m/z at 0.1 to 1 were indicated precision.

The linearity between concentrations and the area abundance ion between concentrations remained stable. Employing LC-ESI MS analysis also proved satisfactory as the MS detector was very sensitive. Based on the fact presented in the serial concentrations of progesterone as follows; 0.05 µg/mL with 139911 AA, 0.10 µg/mL with 279831 AA, 0.50 µg/mL with 1399155 AA, 0.70 µg/mL with 1958811 AA and 0.90 µg/mL with 2518480 AA, the system suitable test (SST) of the MS detector at square of the correlation coefficient (R^2) 0.961 by equation of Y = 64361 X - 67159.

The coefficient of variation (CV) values for recovery and accuracy are presented as follows; concentrations

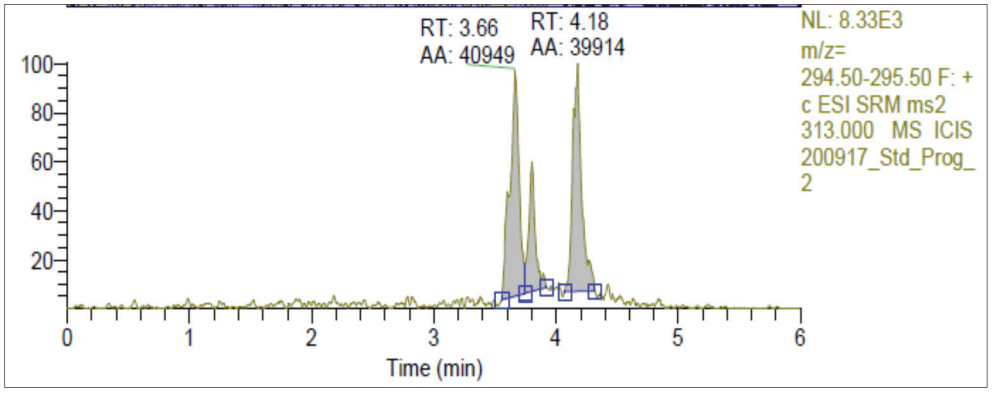

Fig 3. Mass spectrum of the progesterone standard (0.100 ppm) at a retention time of 4.18 min, m/z = 294.50 - 295.50, and MS-MS at 313.000 by LC-ESI MS

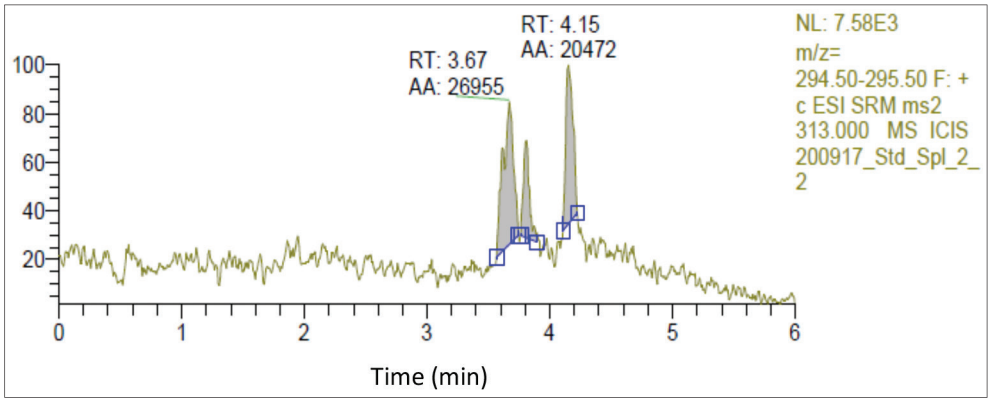

Fig 4. Mass spectrum of progesterone-like compounds after encapsulation in 10% liposome in rat plasma at a retention time of 4.15 min, m/z of 294.50-295.50, and MS-MS of 313.000

Table 1. *The means concentration of PLCs in rats plasma when encapsulated in 10% liposome (trial groups) and not encapsulated in 10% liposome (control group)*

Single Dose Administration Intraperitoneal	Concentration of Progesterone-Like Compounds (µg/mL)				P
	Control (Mean ± SD)		Trial (Mean ± SD)		
	30 min	60 min	30 min	60 min	
3 mg/100 g BW	0.111±0.028[a]	0.245±0.039[a]	0.333±0.015[b]	0.757±0.024[b]	0.08
5 mg/100 g BW	0.250±0.015[a]	0.518±0.009[a]	0.548±0.022[b]	0.936±0.034[b]	
7 mg/100 g BW	0.541±0.013[a]	0.746±0.042[a]	0.928±0.019[b]	2.161±0.042[b]	

[a,b] Values within a row with different superscripts differ significantly at P<0.05

0.05 µg/mL in 98%, 102%, 104% at mean 3.015 %CV; concentrations 0.1 µg/mL in 101%, 111%, 112% at mean 5.632% CV, concentrations 0.9 in 101.3%, 101.4%, 101.6% at mean 0.151%CV. The method had good accuracy as the mean of the values was less than 10% CV. In biological matrices, such as rat plasma, the separation of analytes from impurities is difficult to achieve. However, the analysis can become easier assessing a mass spectrum. All samples had good recoveries, ranging from 80% to 120%. The correlation between serial concentrations and area abundance (AA) at means±SD is presented as follows; 0.03 µg/mL at 83125±876.74 AA; 0.05 µg/mL at 139844±88.255 AA; 0.1 µg/mL at 283111±5.733 AA; 0.9 µg/mL at 2569639±47.137 AA. According to the result at above, the method had good precision as the % CV was less than 3%. This result was used to generate a standard curve to determine the levels of PLCs in the plasma of rats by equation of Y = -2803 + 2858270 X.

The lowest concentrations could be observed in 0.03 µg/mL (83125 AA), then by other concentrations of 0.05 µg/mL (139844 AA) resulted linear regression equation as follows; Y = -1953.5 + 2835950 X. The calculated Limit of Detection (LOD) by inserting the smallest area abundance free from a noise peak (3 × 1000) was resulted 1.746 x 10^{-3}

µg/mL, then was calculated Limit of Quantification (LOQ) from three times of LOD, resulted 5.240 x 10^{-3} µg/mL.

The results showed that the average concentration of progesterone in the trial group administered 3 mg/100 g BW in 30 min and 60 min was 0.333 µg/mL and 0.757 µg/mL, while the control group was 0.111 µg/mL and 0.245 µg/mL. Progesterone concentrations in the trial group administered 5 mg/100 g BW in 30 min and 60 min was 0.548 µg/mL and 0.936 µg/mL, while the control group was 0.250 µg/mL and 0.518 µg/mL. Progesterone concentrations in the trial group giving 7 mg/100 g BW in 30 min and 60 min were 0.928 µg/mL and 2.161 µg/mL, while the control group was 0.641 µg/mL and 0.846 µg/mL. *Table 1* shows the results of the analysis of PLCs encapsulated in liposome correlation to progesterone in the trial and control groups.

Discussion

A new separation technique was used in this study. Briefly, hydrolysis and separation were carried out using 0.5 M HCl in methanol to reduce the interfering substances bound to PLCs, including polyphenols, anthraquinones, and alkaloids, which have terpenoid structures. Many of these substances bind to the stalk of BD, which binds to the leaves. *Fig. 1* shows that the progesterone CRM appeared at a retention time of 7.119 min. Accordingly, the PLCs were collected between retention times of 6.500-8.000 min, and the peak for the BD extract appeared at a retention time of 7.569 min. These findings indicate a change in the pattern of the appearance of progesterone, which originally appeared at a retention time of 6.107 min according to a previous report [18]. As the previous researchers did not perform hydrolysis and separation techniques using SPE, this may justify the differences in the results between the two studies. In the new technique employed in the present study, nuisance materials that often appear between 6.100 and 6.500 min were separated. The removal of these interfering substances yielded very pure PLCs. A new technique, such as that employed herein, requires a large amount of crude extract of BD compared to previous techniques; this is because there are more steps in the separation process than in the process used by previous researchers. *Fig. 2-A,B* shows that the PLCs particles (A) are relatively smaller than the particles for the CRM progesterone (B). *Fig. 2-C* shows that encapsulation in 10% liposomes was ideal to cover the bioactive compounds in the PLCs. Sonification also affected the uniform distribution of particles encapsulated in the liposomes. Thus, the nanoparticle technology using liposome encapsulation encourages agonist bioactive action and induces a controlled release pattern. In this research, very small particles were obtained through nanotechnology, confirming the potential for development on an industrial scale. The fine particles of PLCs obtained are known to be very lipophilic; thus, through liposome encapsulation, the outer part of the envelope component can be hydrophilic [21]. Under such circumstances, the initial action of the injectable drug was almost equivalent to that of the solution dosage form (categorize easy to dissolve at 1:1 as w/v). *Fig. 4* shows that the structure of progesterone in PLCs with 10% liposome could still be observed, with a molecular mass (m/z) between 294.50 and 295.50, which is based on the molecular mass of CRM progesterone as presented in *Fig. 3* white m/z at ESI MS-MS 313.000. Another observation that the active substance progesterone on PLCs is RT at 4.15 min, that is shifted more slowly than standard progesterone at RT 4.18 min. The following parameters of the LC-ESI MS method were assessed to obtained result of the research at SST levels. The precision and accuracy by recovery analysis as a limit of detection (LOD), and limit of quantification (LOQ) were shown sensitive, so that it was very suitable for assaying with small concentrations. Based on our findings, this method was satisfactory for determining drug concentration total at level nanoparticle encapsulated by liposome. In this study, the presence of progesterone in the plasma matrix of rats was determined using MS-MS. The levels of progesterone were also determined using the area and retention time obtained from the chromatogram. Using both chromatograms and molecular mass measurements can demonstrate that the compound being examined is compatible with CRM progesterone [22,23]. As shown in the % recovery ranged from 98.0% to 101.6% with CV ranging from 0.151% to 5.632%. Recovery is defined as the ratio between the observed analysis results and the true value. For example, in samples of biological matrix that contain thousands of metabolites, which serve as impurities, the recovery rates strongly depend on the quality of the sample preparation technique. The lower concentrations of PLCs will yield the lowest precision and a greater uncertainty in the recovery rates. In biological matrices, such as blood plasma, the recovery was found to range from 80% to 120%, with a CV of <7%. Correlations between concentrations versus area abundance were indicated strong relationship on the range of concentrations 0.030 µg/mL to 0.900 µg/mL at equation $Y = -2803 + 2858270\ X$ ($R^2 = 1$). Based on the LOD and LOQ assessed as described at above, the analysis method using LC-ESI MS indicate sensitive to detection at small concentration, especially for metabolite. Concentrations lower than 1.746×10^{-3} µg/mL was not detected, although a signal could still be read by the instrument. By assessing the PLCs encapsulated in 10% liposome, we found that the concentrations of the encapsulated PLCs at 30 min and 60 min were higher in the trial groups than the concentration of pure progesterone in the control groups ($P<0.05$ as described in *Table 1)*. The level of PLCs in each dose (3 mg/kg BW, 5 mg/kg BW, and 7 mg/kg BW) was one-fold higher than the level in the control, indicating that the elimination rate at each time point (30 min and 60 min) differed from that of the control. This is because the peak of PLCs at each time point was higher than that of the control. Such finding aligns with the concept of linear pharmacokinetics, where the rate of elimination is highly dependent on the kinetic

pattern of the drug. Thus, the higher the level of drug in the body, the deeper the spread of the drug, and the longer the drug exists, the lower the clearance value [24]. In such conditions, the drug can remain in the body for a long time and exert an agonist action for an even longer time. This agonist role can occur because of the use of liposomes as a vehicle, which enables the gradual release of bioactive compounds [25,26]. The part of the liposome molecule that is associated with water is hydrophilic while the part that binds to the drug is lipophilic, thereby enabling the quick distribution of the drug preparation in the body. Liposomes protect the molecular structure of progesterone, which is part of cyclopenta[a]phenanthrene that is at risk of rupture when bound by strong electronegative ions from body electrolytes. Thus, PLCs does not have a first pass effect phenomenon due to the body's electrolyte reaction. This novelty proves that the structure of progesterone that is not protected by liposomes is ultimately easily conjugated with electrophilic substances groups, which in turn can decrease the bioavailability of progesterone. Another structure of the progesterone molecule that is also susceptible to electrophilic bonding from body ions is -tetradecahydro bound to cyclopenta-phenanthren-. In conclusion, the encapsulation of PLCs in 10% liposomes increased the spread of progesterone deep within the body of rats. Further, a longer release of progesterone can be achieved with liposomes than without liposomes.

Availability of Data and Materials

The data sets during and/or analyzed during the current study available from the corresponding author (M. Lauzardi) on reasonable request.

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Competing Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

ML: Project manager, preceptors, research coordinators, research analysis, drafting manuscript and writing the manuscript. SS, CHC, JLH, CWL and CKP: writing and editing the manuscript, BH: analysis LC-ESI MS and HPLC; MS: analysis statistic, RS: preparations animal experimental, LM: extraction leaf of *Benalu Duku*.

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RESEARCH ARTICLE

Effects of Dietary Cation-Anion Difference on Milk Performance, Digestion and Blood Parameters in Lactating Cows Under Heat Stress

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Abstract

The current study determined the effects of dietary cation-anion difference (DCAD) on milk performance, total apparent digestibility, and blood parameters in lactating cows subject to heat stress. Eight Chinese Holstein cows (22.04±2.38 kg of milk/d, 512±76 kg of body weight, 219±20 d in milk) at the late stage of lactation were allocated to group 1 or 2. We used a randomized complete block design with a 2x2 factorial arrangement. The experiment consisted of two periods. Each period lasted 21 days, including the first 14 days for adaptation to the diet and the following seven days for trail. During period 1, group 1 fed with DCAD at 335 mEq/kg dry matter (the basal diet=CON) and group 2 fed 507 mEq/kg dry matter (high DCAD). During period 2, group 1 diet (the basal diet = CON) was swapped group 2 diet (high DCAD). The high DCAD had no significant effects on the respiratory frequency, rectal temperature, blood pH value, the acid-base balance, milk yield, milk composition, and feed intake (P>0.05). However, the high DCAD was associated with lower somatic cell count (SCC) in milk (P=0.04) and lower immune cell counts in blood, which was conducive to the improvement of milk quality. The apparent digestibility of dry matter, organic matter, energy, neutral detergent fiber, and ethyl extract was greater in the high DCAD group (P<0.05). In summary, increasing DCAD in the diet could stabilize milk production and feed intake, improve milk quality and apparent digestibility in lactating cows subject to heat stress.

Keywords: *Dietary cation-anion difference, Milk performance, Digestive performance, Blood physiology*

Isı Stresi Altındaki Laktasyon Dönemi İneklerde Diyet Katyon-Anyon Farkının Süt Performansı, Sindirim ve Kan Parametrelerine Etkisi

Öz

Bu çalışmada, ısı stresine maruz kalan laktasyon dönemi ineklerde diyet katyon-anyon farkının (DKAF), süt performansı, toplam görünür sindirilebilirlik ve kan parametreleri üzerine etkileri belirlendi. Geç laktasyon dönemindeki 8 Çin Holstein ineği (22.04±2.38 kg süt/gün, 512±76 kg vücut ağırlığı, 219±20 gün laktasyon süresi) grup 1 veya grup 2 olarak ayrıldı. 2x2 faktöriyel düzenleme ile rastgele bir tam blok tasarımı kullanıldı. Çalışma, her bir grup için ilk 14 gün diyete uyum ve sonraki yedi gün takip olmak üzere 21 gün süren iki periyottan oluşturuldu. Birinci periyotta grup 1, 335 mEq/kg kuru maddeli DKAF (bazal diyet=Kontrol) ile, grup 2, 507 mEq/kg kuru maddeli DKAF (yüksek DKAF) ile beslendi. İkinci periyot boyunca, grup 1 diyeti (bazal diyet=Kontrol) grup 2 diyeti (yüksek DKAF) ile yer değiştirildi. Yüksek DKAF'in, solunum frekansı, rektal ısı, kan pH değeri, asit-baz dengesi, süt verimi, süt bileşimi ve yem alımı üzerine anlamlı bir etkisi yoktu (P>0.05). Fakat, yüksek DKAF, sütte daha düşük oranda somatik hücre sayısı (SHS) (P=0.04) ve kanda daha düşük oranda immün sistem hücre sayısı ile ilişkilendirildi, bu durum süt kalitesinin iyileştirilmesine katkı sağladı. Kuru madde, organik madde, enerji, nötr deterjan lifi ve etil özütünün görünür sindirilebilirliği, yüksek DKAF grubunda daha fazlaydı (P<0.05). Özetle, ısı stresine maruz kalan laktasyondaki ineklerde diyette artan DKAF, süt üretimini ve yem alımını stabilize edebilir, süt kalitesini ve görünür sindirilebilirliği iyileştirebilir.

Anahtar sözcükler: *Diyet katyon-anyon farkı, Süt performansı, Sindirim performansı, Kan fizyolojisi*

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Introduction

The basic metabolic heat production of dairy cows is very sufficient, such as growth and development, lactation, pregnancy, rumen fermentation, which provides a large amount of metabolic heat production, but the ability to dissipate heat is weak [1,2]. In a hot and humid climate, the deep body temperature of cows rises in a short period of time [3]. Dairy cows need to maintain a balance between heat production and heat dissipation. The main way to reduce metabolic heat production is to reduce feed intake [4]. The reduction of DMI will cause insufficient nutrient supply for the physiological activities related to lactation [5], which will further lead to changes in milk production [1,6] and milk composition [7] of dairy cows. The health and animal welfare issues of some dairy cows are related to the hot climate, increase of water consumption, rectal temperature and respiratory rate [8], these would result in the loss of body weight. Clinical mastitis is positively correlated with deep body temperature [9], the somatic cell count (SCC) is an important indicator for monitoring mastitis in dairy cows. Paape et al.[10] and Bouraoui et al.[11] reported that SCC in milk was increased in summer compared with the non-heat stress period. Lying is the most important dairy cow behavior [12]. In a hot and high humidity environment, the cows have poor lying comfort and increased heat dissipation requirements, resulting in increased standing time [13,14].

A decrease in dry matter intake (DMI) in dairy cows under heat stress could lead to a reduction of mineral element intake, as a result of reduced feed consumption. The increase in respiratory frequency of cows could cause a decrease of salivary bicarbonate, which could even lead to a change of blood acidity and alkalinity and affect the cation-anion status in the body. Therefore, manipulating dietary cation-anion difference (DCAD) is an appropriate dietary strategy to meet the increasing demand for mineral elements under heat stress [15,16]. There is evidence showing that the difference between cations and anions in diet has a linear relationship with the 4% fat corrected milk (FCM) yield of early and middle lactation cows in both cold and hot environments. West et al.[17] reported that DMI and milk yield reached the greatest when the DCAD level in diet was at 380 mEq/kg DM.

Previous studies have focused on early and mid-lactation heat-stressed cows, and the effect of higher ion differences on heat stress in late-lactation cows is not clear. Therefore, we conducted this study to explore whether positive DCAD in a high temperature and high humidity environment can increase the DMI and milk production of dairy cows in late lactation.

Material and Methods

Ethical Statement

The use of the animals and the experimental procedures were approved by the Animal Ethics Committee of the College of Animal Science and Technology, Hunan Agricultural University (NO:20180715), in accordance with the China National Standard - Laboratory Animals - Guideline of Welfare and Ethics.

Animals and Experimental Design

Eight lactating Chinese Holstein dairy cows with same parity (2±0), similar milk yield (22.04±2.38 kg/d), body weight (512±76 kg), and days in milk (219±20 d) were allocated into two treatments in a two period cross-over design during the summer season (from August to September). Two treatments were referred to two levels of DCAD in two diets: the basal diet (control=CON) had 335 mEq/kg DM, and high DCAD diet had 507 mEq/kg DM.

The calculation of the DCAD value for the diets was based on the Na-K-Cl formula described by Mongin [18]:

DCAD mEq/kg =10x[(% Na/0.023) + (% K/0.039) - (% Cl/0.035)]

The experiment consisted of two periods. Each period lasted 21 days, including the first 14 days for adaptation to the diet and the following seven days for sampling. Extra three days were allowed between the first and the second period. In the second period, two diets were swapped, so each group received both the diets in two period, and there were eight replicates for each diet.

Two diets were formulated to meet the nutrient requirement for lactating cows according to NRC 2001 [19] recommendations.

The ingredients of two diets, as shown in *Table 1*, were almost identical, except for the changes in the amounts of $NaHCO_3$ and KCO_3 to create the differences in DCAD level between two diets. The ratio of concentrate to roughage was 56:44 (dry matter basis). Total mixed ration (TMR) was prepared daily, and fed three times per day (08:00 h, 14:00 h and 20:00 h). Fresh drinking water was accessible all times.

Temperature-Humidity Index (THI) Monitoring

Two wet and dry bulb thermometers (Tianjin Tianma Instrument Factory. Effective ranges: dry bulb (Td) temperature -10-50°C and, wet bulb (Tw) temperature -10-50°C) were hanged on the feeding bars at 1.5 m above the floor to ensure necessary ventilation, be away from sunlight and rain, and avoid cow's touching. Temperature and humidity were recorded daily at 08:00, 14:00 and 20:00 before the feeding throughout the experimental periods. The THI was then calculated using a formula by National Research Council 2001 [19]:

THI = (Td + Tw) x 0.72 + 40.6

where Td and Tw are the temperature readings on the dry bulb and wet bulb thermometers. The daily averages of THI, Td, and Tw were calculated.

Table 1. *Ingredients and nutrient levels of the diets*

Ingredients (g/kg DM)	Basal Diet CON=Control	High DCAD
Corn silage	420	415
Alfalfa hay	124	123
Oatmeal hay	154	151
Whole cottonseed	185	181
Corn grain, finely ground	180	178
Steam-flaked corn	56.2	53.9
DDGS	68.6	67.3
Soybean meal	81.2	80.0
Extruded full-fat soybean	6.1	6.0
Premix†	31.5	29.6
$NaHCO_3$	2.0	5.8
K_2CO_3	0	8.2
Chemical Composition (g/kg DM)		
OM	908	918
CP	168	161
NDF	454	423
ADF	241	230
EE	72.7	69.6
Ash	91.5	81.7
Ca	4.76	5.23
P	3.94	4.22
Na	3.42	4.48
K	13.0	17.5
Cl	5.51	4.78
DCAD (mEq/kg DM) ‡	335	507

†: One kg premix contains: 400 mg Zn, 100 mg Cu, 200 mg Fe, 3600 mg Mg, 350 mg Mn, 96 mg Cr, 4.0 mg Co, 50 mg Se, 500 mg lysine, 500 mg methionine, 250.0000 IU Vit. A, 100.000 IU Vit. D_3, 4.000 IU Vit. E
‡: DCAD: mEq/kg DM = 10 × [(% Na/0.023) + (% K/0.039)] - [% Cl/0.0355]

Measurement of Rectal Temperature and Respiratory Rate of Cows

The rectum (about 6-8 cm from the annas) temperature (RT) of eight cows was recorded using an electronic thermometer at 08:00, 14:00, and 20:00 every day during the sampling period. The respiratory rate (RR) of the cows was counted by the research officers using a stopwatch and a counter at 08:30, 14:30, and 20:30, each for 60 sec during the sampling period. The RR was calculated by observing thoracoabdominal movements for 1 min and expressed in breaths/minute [20].

Feed and Fecal Sample Collections and Analysis

The mounts of feed offered and refusal were recorded throughout each of the experimental periods to calculate daily feed intake and the average feed intake for the experimental period. The feed and refusal samples were collected on days of 1, 3, 5, and 7 of the sampling period, pooled, and stored at -20°C for analyses later on.

During the sampling period, a 200 g of fecal sample was taken daily from the rectum of each cow. Then 20 mL of 10% sulfuric acid was added into the sample to prevent nitrogen loss. The samples for each cow were pooled, subsampled, and stored at -20°C for analyses of later on.

The feed and fecal samples were determined for dry matter (DM) content first, then ground to pass through a 40 mesh sieve. The dried samples were analyzed using the AOAC [21] procedures for DM, crude ash content, crude protein (2300 Automatic Kjeldahl Nitrogen Analyzer Denmark FOSS Co., Ltd.), crude fat, and gross energy (SDACM3100 calorimeter, Hunan Sande Science and Technology Co., Ltd., China). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the methods described by Van Soest et al.[22] Amylase (Termamyl 120L, TypeS, Denmark) and anhydrous sodium sulfite were added to the analytic processes. Concentrations of Na and K elements were determined using an inductive coupled plasma optical emission spectrometer (ICP-OES) [23]. Water-soluble chlorides were determined by $AgNO_3$ titration [24]. Phosphorus was determined using a vanadium molybdenum yellow colorimetric assay [25], and Ca was determined by complexometric titration with disodium ethylenediamine tetraacetate [26]. The ash undissolved in 2M hydrochloric acid in feed and fecal samples was used as an internal marker for the calculation of the total apparent digestibility [27].

Milk Yield and Milk Composition

Milk yields at 0800 h, 1400 h, and 2000 h were recorded daily throughout the whole experimental period. After milking, a 100 mL of milk sample was collected respectively at 0800 h, 1400 h, and 2000 h on day 7 of the sampling period and pooled at a ratio of 4:3:3. The pooled milk sample was mixed with a drop of potassium dichromate as preservative and then refrigerated at 4°C. Stored 4°C sample was used for determinations of milk fat, lactose, somatic cell count, solid dry matter, and solid content of milk fat by infrared spectrophotometry (Mino-78110 Automatic Milk Composition Analyzer, FOSS Co., Denmark) on the next day.

Blood Samples and Assays

Blood samples were taken from the tail vein into three tubes two hours after the morning feeding on day 7 of the sampling period. One sample with heparin as anticoagulant was sent to Xiangya Hospital of Central South University to analyze gas indicators in blood. The second sample with EDTA as anticoagulant was sent to the Institute of Subtropical Ecology, Chinese Academy of Sciences for hematological analysis. The third sample was centrifuged at 3000 rpm for 15 min at 4°C, plasma was collected, transported in liquid nitrogen, stored in -20°C. The plasma sample was carried out blood biochemical

analysis (Mindray BS-200) at Hunan Co-Innovation Center of Animal Pro Application.

Statistical Analysis

The number of animals was calculated with G*Power Software by using Student *t*-test with P<0.05 and power (1-β) = 0.80.

Statistical analysis of data was performed using SAS 9.4 software (SAS Research Institute Ltd.) in a mixed linear model. In the model, the treatment and period were the fixed factors, and animal as a random factor [5]. The differences in the means with P values <0.05 are declared significant, and difference in the means with P values <0.10 but >0.05 are declared a tendency. GraphPad Prism 16.0 was used to draw graphics.

Results

THI, Respiratory Frequency and Rectal Temperature of Cows

The temperature, humidity, and calculated THI in the cow-shed during the experimental period are shown in *Fig. 1*. The dry bulb thermometer readings ranged from 24.93°C to 34.25°C and the average for the period was at 28.94±2.5°C (SD). The wet bulb thermometer readings ranged from 19.87 to 28.88 and the average was 25.61±2.24°C. The calculated THI ranged 73.94 to 85.00, and the average was 80.12±3.35. The THI decreased slightly from day 34 to day 45 due to the changes of weather. During the whole experiment period, the THI was always greater than 72.

The respiratory rate and rectal temperature of cows are shown in *Table 2*. There were no significant differences in the respiratory rate and rectal temperature between CON and high DCAD groups.

Apparent Digestibility, Milk production Performance, and Efficiency

Dietary intake and apparent nutrient digestibility of the cows are listed in *Table 3*. The difference in DCAD had no significant influence on the intakes DM, OM, CP, energy, ethyl extract, NDF, and ADF (P>0.05). However, the apparent digestibility of those nutrients was all significantly greater in the high DCAD group compared with the basal diet group.

As shown in *Table 4*, the DCAD difference had no significant effect on milk yield and milk compositions, such as protein, fat, lactose, and non-fat milk solids, and milk conversion efficiency (P>0.05). However, SCC was lower in the high DCAD group compared with those in the basal diet group (P<0.05).

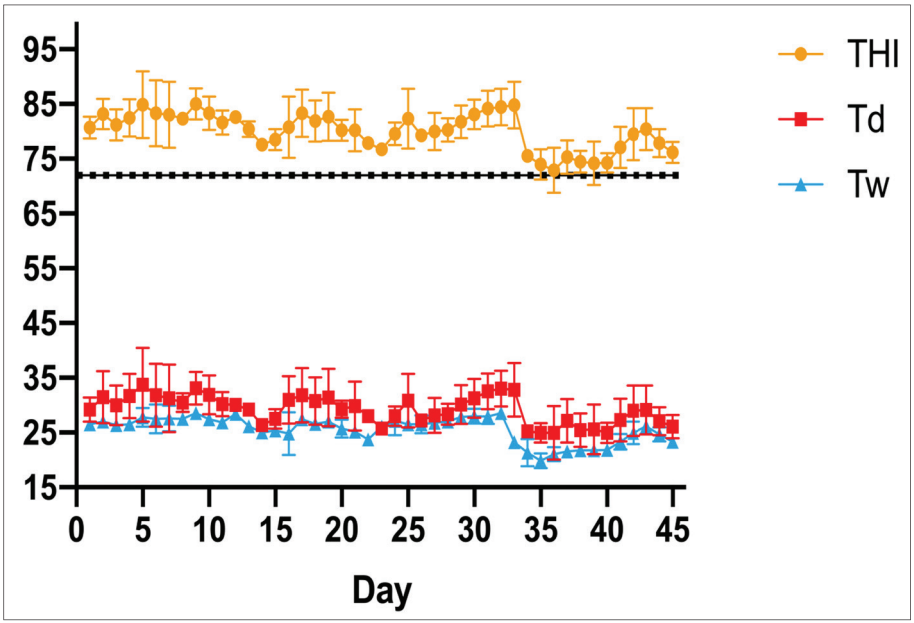

Fig 1. The daily average temperature-humidity index (THI), dry bulb temperatures (Td) and wet bulb temperatures (Tw) during the experimental period (THI:80.15±4.30, Td:29.24±3.86 and Tw:25.64±2.57). The horizontal line at 72 indicates the threshold for heat stress

Table 2. *Effects of dietary cation-anion difference (DCAD) on the respiratory rate and rectal temperatures in lactating cows*

Item	DCAD (mEq/kg DM)		SEM	P Value
	Basal Diet CON=Control	High		
Respiratory rate (the number of breath/min)	64.0	62.0	1.02	0.130
Rectal temperatures (°C)	38.8	38.9	0.09	0.151

The dietary cation-anion difference (DCAD) values were 335 and 507 mEq/kg DM respectively for CON=contorl and High DCAD groups

Table 3. *Effects of dietary cation-anion difference (DCAD) on feed intake and nutrient apparent digestibility in lactating cows*

Item	DCAD (mEq/kg DM)		SEM	P Value
	Basal Diet CON=Control	High		
Intake, kg/d				
DM	21.3	22.0	0.95	0.625
OM	19.5	19.9	0.87	0.763
CP	3.08	3.28	0.140	0.328
Energy (Mcal/kg)	75.3	80.0	3.42	0.340
EE	1.54	1.53	0.091	0.901
NDF	9.26	9.68	0.432	0.500
ADF	4.26	4.13	0.203	0.500
Total apparent digestibility, %				
DM	66.7	76.5	1.03	<.001
OM	69.4	78.0	0.93	<.001
CP	66.8	76.6	1.56	<.001
Energy	64.2	75.6	1.28	<.001
EE	78.5	83.7	1.18	0.008
NDF	58.9	73.2	2.31	0.001
ADF	66.7	76.5	1.03	<.001

The dietary cation-anion difference (DCAD) values were 335 and 507 mEq/kg DM respectively for CON=contorl and High DCAD groups

Table 4. *Effects of dietary cation-anion difference (DCAD) on milk yield, milk compositions, and milk production efficiency in lactating cows*

Item	DCAD (mEq/kg DM)		SEM	P Value
	Basal Diet CON=Control	High		
Milk yield (kg/d)	20.4	20.1	0.75	0.162
FCM (kg/d)	21.1	21.9	0.71	0.260
CP (%)	3.20	3.28	0.039	0.194
Fat (%)	4.52	4.55	0.104	0.840
Lactose (%)	5.07	5.08	0.017	0.685
SNF (%)	9.04	9.10	0.045	0.299
TS (%)	13.7	13.4	0.15	0.191
SCC (cells × 10^3)	158	107	14.5	0.021
Milk production efficiency (kg/kg)	1.37	1.38	0.021	0.748

SCC: *Somatic cell count;* ***SNF:*** *Non-fat milk solids;* ***TS:*** *Total solids*
The dietary cation-anion difference (DCAD) values were 335 and 507 mEq/kg DM respectively for CON=contorl and High DCAD groups

The Gas Indexes in Blood and Hematology

The gas indexes in blood and hematological indexes in lactating cows are shown in *Table 5*. There were no significant differences in blood pH, cCa^{2+}, cK^+, cNa^+, cCl, Hctc, ctHB, $cHCO_3^-$, pCO_2 and pO_2 between two DCAD groups (P=0.05). The SO_2 value in blood was significantly greater in the high DCAD group (P=0.05).

Table 5. *Effects of dietary cation-anion difference (DCAB) on the gas indexes in blood and hematology in lactating cows*

Item	DCAD (mEq/kg DM)		SEM	P Value
	Basal Diet CON=Control	High		
pH	7.39	7.40	0.012	0.646
Hctc (%)	30.9	30.7	0.69	0.798
ctHB (g/dL)	10.0	9.90	0.220	0.744
$cHCO_3^-$t (mmol/L)	24.4	24.4	0.82	0.982
pCO_2 (mmHg)	39.5	37.5	1.04	0.195
pO_2 (mmHg)	58.0	65.1	6.87	0.472
sO_2 (%)	64.1	78.0	6.83	0.050
cCa^{2+} (mmol/L)	1.20	1.19	0.012	0.579
cCl^- (mmol/L)	105	105	0.6	0.972
cK^+ (mmol/L)	3.91	3.85	0.104	0.706
cNa^+ (mmol/L)	140	140	0.4	0.823

BE: *bases excess;* ***HCO^{3-}:*** *bicarbonate;* ***pCO_2:*** *carbon dioxide partial;* ***pO_2:*** *oxygen partial pressure;* ***Hb:*** *hemoglobin;* ***Hct:*** *hematocrit;* ***sO_2:*** *oxygen saturation*
The dietary cation-anion difference (DCAD) values were 335 and 507 mEq/kg DM respectively for CON=contorl and High DCAD groups

Table 6. *Effects of dietary cation-anion difference (DCAD) on the immune cell counts in blood of lactating cows*

Item	DCAD (mEq/kg DM)		SEM	P Value	Normal Range
	Basal Diet CON=Control	High			
Leukocytes (10^9/L)	8.38	6.60	0.382	0.004	5-11.7
Neutrophils (10^9/L)	3.35	2.79	0.196	0.056	1.5-5.2
Lymphocytes (10^9/L)	4.35	3.28	0.241	0.005	1.9-6.4
Monocytes (10^9/L)	0.36	0.28	0.033	0.117	0-0.6
Eosinophils (10^9/L)	0.28	0.22	0.020	0.071	0-3.6
Basophils (10^9/L)	0.04	0.028	0.0048	0.045	0-0.2

The dietary cation-anion difference (DCAD) values were 335 and 507 mEq/kg DM respectively for CON=contorl and High DCAD groups

The Immune Cell Counts in Blood

Table 6 shows the immune cell counts in blood in lactating dairy cows. Cows on the high DCAD diet had relatively lower counts of leukocytes, lymphocytes, and basophils (P≤0.05), and a tendency of low counts of neutrophils and eosinophils (P<0.10) compared with the cows on the basal diet DCAD diet. No significant difference in the monocyte count between two diets was observed (P=0.117). Nevertheless, all those immune cell counts fell into the normal ranges.

Metabolites and Lipids in Plasma

The concentrations of total triglycerides (TG), total cholesterols (TCHO), high-density lipoprotein cholesterols (HDL-C), low-density lipoprotein cholesterols (LDL-C), glucose, and lactic acid, and α-amylase and cholinesterase activities are shown in *Table 7*. There were no significant differences in

Table 7. *Effects of dietary cation-anion difference DCAD on lipids and metabolites in plasma of lactating cows*

Item	DCAD (mEq/kg DM)		SEM	P Value
	Basal Diet CON=Control	High		
TG (mmol/L)	0.232	0.245	0.0090	0.37
TCHO (mmol/L)	6.81	6.70	0.476	0.83
HDL-C (mmol/L)	5.64	5.62	0.345	0.94
LDL-C (mmol/L)	2.76	2.66	0.296	0.74
GLU (mmol/L)	3.62	3.86	0.106	0.03
LAC (mmol/L)	1.28	1.34	0.226	0.85
CHE (g/L)	207.9	210.7	6.55	0.68
AMS (U/L)	28.6	29.4	2.69	0.76

AMS: *α-amylase;* ***CHE:*** *cholinesterase;* ***HDL-C:*** *high-density lipoprotein cholesterols;* ***GLU:*** *glucose;* ***LAC:*** *lactic acid;* ***LDL-C:*** *low-density lipoprotein cholesterols;* ***TCHO:*** *total cholesterols;* ***TG:*** *total triglycerides*
The dietary cation-anion difference (DCAD) values were 335 and 507 mEq/kg DM respectively for CON=contorl and High DCAD groups

those parameters between two DCAD groups (P>0.05) except that glucose concentration in plasma was greater in the high DCAD group compared with that in the CON DCAD group (P<0.05).

Discussion

THI is an objective indicator to thermo and humid environment that can cause a heat stress in animals. When THI exceeds 72, cows begin to suffer heat stress; when THI is between 78 and 89, cows could be under moderate heat stress; once THI exceeds 90, cows are under severe heat stress [28]. The THI in the current study ranged from 73 to 80, indicating that the cows were under moderate heat stress. In the region where this study was conducted, humidity in summer was usually very high, in conjunction with high temperature. High humidity can affect moisture evaporation from the body surface for cooling so that cows cannot emit enough body heat to prevent the increase of the body temperature [29]. As resultant responses to heat stress, respiratory frequency and rectal temperature of animals may increase. In non-heat stress conditions, the respiratory rate of dairy cows ranges from 15 to 35 breaths/minute, and rectal temperature ranges from 38.0°C to 39.0°C [30,31]. We observed high respiratory frequency in the cows in the present study, but the rectal temperature fell in the normal range, indicating that the cows were under heat stress, but the body temperature continued under the control. The change in the DCAD level did not result in any differences in both respiratory rate and rectal temperature.

The changes in DCAD in diet for dairy cows have been found to affect milk production and reduce the productivity loss caused by heat stress. Mallonée et al.[32] reported that milk yield of dairy cows increased with an addition of K^+ in diet. Schneider et al.[33] found when Na^+ in diet increased from 0.18% to 0.55%, DMI and milk yield in dairy cows increased, and the DMI increased linearly with the DCAD level increased from 120 to 460 meq/kg DM. In hot climate conditions, K^+ is lost in sweat of cows, because K^+ is the main cation in sweat [34]. Delaquis and Block [35] enlarged the dietary anion-cation difference, which resulted in an increase in milk production, milk protein and milk fat in early and middle lactating cows in hot weather. However, there is a research report on the dietary anion-cation difference in late lactation dairy cows. The present study showed that when DCAD increased from 335 mEq/kg DM to 507 mEq/kg DM, the milk yield and milk composition had no significant changes. The reason may be that there was adequate K^+ in the feed to compensate the K^+ loss in sweat. West et al.[17] observed that DCAD ranged from 120 to 456 mEq/kg DM with supplementation of $KHCO_3$ in the diet for lactating dairy cows. Chan et al.[36] observed that the DCAD levels at 200, 350, and 500 mEq/kg DM had no significant effect on milk protein and milk fat in early lactating cows. These results are in consistent with our findings in the current study. Although there was no significant difference in dietary intake, we found that the digestibility for the high DCAD diet was greater than those for the basal diet DCAD diet. Wang et al.[37] found that the higher DCAD content significantly enriched the phylum *Fibrobacteres* and genus *Fibrobacter* in the microflora of rumen fluid and elevated the total volatile fatty acid production. This may be responsible for the greater nutrient digestion in the high DCAD group.

Milk is rich in various nutrients, including lactose, protein, fat, and phospholipids. Generally speaking, the concentrations of these nutrients are stable, but could vary among individual animals and with environmental changes, such as ambient temperature. Milk fat is more viable in relevance to milk protein. We did not find any significant change in milk composition in the current study. Solorzan et al.[38] found that an addition of buffers in the diet could increase milk fat, but under heat stress conditions, an increase in dietary DCAD from 220 mEq/kg DM to 470 mEq/kg had no influence on milk yield and milk fat. No significant difference in the milk yield in the present study may be due to the cows in the late stage of lactation, and usually the milk yield is low during this stage. So lactating stage could influence the effect of DCAD on milk production. For example, Wildman et al.[39] reported that high DCAD increased milk yield in cows in the early and middle lactating stages, alleviated the symptoms of heat stress, and reduced the respiratory rate.

Blood pH value is mainly related to the buffer system of HCO_3^- & CO_2. The actual bicarbonate (HCO_3^-) level in blood is mainly dissociated from bicarbonate, and its concentration can directly affect the change in blood pH value. Partial pressure of CO_2 refers to the amount of dissolved CO_2 in plasma, which is an important indicator to the respiratory acid-base balance. When other anions are deficient, an

increase in HCO_3^- can replace other anions and keep the balance with cations. The present study showed that there was no significant difference in the concentrations of different ions and pH value, which indicate that lactating cows maintained the blood acid-base balance through the homeostasis regulation mechanism in the body. Martins et al.[40] found that pCO_2 and tCO_2 in blood increased with an increase of DCAD, while pO_2 decreased, and proposed that the change in the respiratory rate in cows maintained the acid-base balance in blood. The respiratory rate increases in a hot environment, but the concentration of Na^+ and K^+ affect the concentration of HCO_3^- and maintain the stability of pH value in blood.

We found in the current study that the high DCAD was associated with lower SCC. Bouraoui et al.[11] found that under a heat stress condition, SCC could be doubled. An increase in DCAD in diet may reduce SCC in milk of cows, but its mechanism remains unclear. We also noted that most of the immune cells, except for monocytes, in blood were lower in cows fed the high DCAD, indicating the high DCAD may alleviate the innate immune response. Roland et al.[41] assessed the white blood cell populations in healthy dairy cows, and reported that leukocytosis was often associated with stress, excitement, fear and calving. We proposed that high DCAD may relief the symptoms of heat stress in dairy cows by comforting their innate immune systems.

The effect of DCAD on postpartum energy metabolism in dairy cows has been reported [40,42]. In the present study, lipid concentrations were not affected by the DCAD treatments. However, plasma glucose was greater in the high DCAD group, which may be attributed to an improvement of dietary digestion in those cows.

In the present study, an increase in DCAD in diet from 335 to 507 mEq/kg DM for lactating cows subject to moderate heat stress had no significant effect on feed intake, milk yield, and milk composition; However, the apparent digestibility of diet was increased, and SCC in milk was significantly reduced. The respiratory frequency of cows was greater under heat stress but the frequency and rectal temperature were not affected by the difference in DCAD. The blood gas indexes and blood pH value were not affected. An increase in DCAD in diet could stabilize milk production and feed intake in cows under heat stress, improve milk quality, and alleviate heat stress to some extent.

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Conflict of Interest

The authors have declared that they have no conflicts of interest.

Author Contributions

PZ designed the study. XL drafted and wrote the manuscript. XL and ST collected and analyzed the data. XL and BF performed the animal trial and laboratory analysis. ZW, LY and ZT revised the manuscript. All authors gave intellectual input to the study and approved the final version of the manuscript.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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RESEARCH ARTICLE

Role of Dietary Supplementation with Plant Origin Carotenoids (Curcumin and Lutein) for the Control of *Eimeria*-Challenged Broiler Chickens

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Abstract

The influence of anti-coccidial naturally occurring plant origin carotenoids (curcumin and lutein) in *Eimeria*-challenged broiler chicks were tested. A total of 200, day-old commercial Arbor Acres chicks were reared for 42 days. The chicks were weighed individually and randomly assigned into equally 4 treatment groups having 5 replicates. Chicks of control group T_0 were raised on a basal diet with no supplement. The chicks of group T_1, T_2 and T_3 were supplemented with curcumin 300 mg/kg, lutein 300 mg/kg, and curcumin plus lutein 150 mg each with per kg of feed, respectively. On day 21 all the chicks were challenged with *Eimeria maxima*. The findings of this study revealed that among all the groups feed intake, body weight gain and feed efficiency remained statistically unchanged (P>0.05). A significantly higher (P<0.05) liver and spleen weights were observed in the T_0 group as compared to the birds fed lutein supplemented diets whilst the bursa and thymus remained statistically alike and the same trend was observed for bloody diarrhea, intestinal lesion scores except for the oocyte count which was significantly lower in the T_3 group. On day 20, T_3 dietary groups exhibited numerically increased antibody titers against Newcastle disease virus (NDV) and Avian influenza virus (AIV), additionally T_3 also exhibited the same trend on day 30. The serum malondialdehyde (MDA) and aspartate aminotransferase (AST) levels were at a lower rate in the T_3 group while the liver MDA was statistically lower in the T_1 (P<0.05) group as compared to other groups. The alanine aminotransferase (ALT) remained unchanged within the groups. Compared to T_0 (control), all the carotenoid supplemented groups exhibited increased (P>0.05) shank color and skin b* value (yellowness). Our conclusion showed that application of curcumin single or in combination with lutein may promote skin pigmentation, lowered liver injury, and exhibit a better anti-coccidial impact.

Keywords: *Broilers, Carotenoids, Curcumin, Lutein, Eimeria, Feed efficiency*

Eimeria İle Enfekte Edilmiş Etlik Piliçlerde Bitki Kökenli Karotenoidleri (Curcumin ve Lutein) İçeren Diyet Takviyesinin Enfeksiyonun Kontrolündeki Rolü

Öz

Doğal olarak oluşan bitki kökenli karotenoidlerin (kurkumin ve lutein), *Eimeria* verilmiş etlik piliçlerde anti-koksidiyal etkinliği test edildi. Toplam 200 adet bir günlük ticari Arbor Acres civcivine 42 gün boyunca bakım uygulandı. Civcivler tek tek tartıldı ve 5 tekrarlı olmak üzere rastgele 4 eşit sağaltım grubuna ayrıldı. Kontrol grubu T_0'a ait civcivler, ek besin içermeyen temel bir diyetle beslendi. T_1, T_2 ve T_3 grubundaki civcivlere, her bir kg yem içerisinde sırasıyla 300 mg/kg kurkumin, 300 mg/kg lutein ve 150 mg kurkumin artı 150 mg lutein içeren diyet uygulandı. 21. günde tüm civcivlere *Eimeria maxima* verildi. Çalışma bulguları, tüm gruplar arasında yem tüketimi, canlı ağırlık artışı ve yemden yararlanmanın istatistiksel olarak değişmediğini ortaya koydu (P>0.05). Lutein takviyeli diyetlerle beslenen piliçlere kıyasla, T_0 grubunda karaciğer ve dalak ağırlıklarında anlamlı derecede (P<0.05) artışa rastlanırken, bursa ve timus istatistiksel olarak benzerliklerini korudu ve T_3 grubunda anlamlı olarak daha düşük olan oosit sayısı dışında kanlı ishal ve bağırsak lezyon skorları için de aynı eğilim gözlendi. 20. günde, T_3 diyet grupları Newcastle hastalığı virüsüne (NDV) ve Avian influenza virüsüne (AIV) karşı sayısal olarak artan antikor titreleri sergiledi, aynı eğilim T_3'te 30. günde de saptandı. Diğer gruplara göre serum malondialdehit (MDA) ve aspartat aminotransferaz (AST) düzeyleri T_3 grubunda daha düşük oranda bulunurken, karaciğer MDA'sı T_1 grubunda istatistiksel olarak daha düşüktü (P<0.05). Alanin aminotransferaz (ALT), gruplar içerisinde değişim göstermeden kaldı. T_0 (kontrol) ile karşılaştırıldığında, tüm karotenoid takviyeli gruplar artmış gövde rengi (P>0.05) ve cilt b* değeri (sarılık) sergiledi. Sonuç olarak, kurkuminin tek başına veya lutein ile kombinasyon halinde uygulanmasının cilt pigmentasyonunu artırabileceği, karaciğer hasarını azaltabileceği ve daha iyi bir koksidiyal etki sergileyebileceği sonucuna varıldı.

Anahtar sözcükler: *Etlik piliç, Karotenoidler, Kurkumin, Lutein, Eimeria, Yemden yararlanma*

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Introduction

World poultry production plays a significant role in the nutritional security of human beings but this industry is under threat of disease outbreaks. The infectious origin diseases of poultry may be effectively controlled by applying vaccines, however, a protozoal disease i.e coccidiosis caused by several different *Eimeria* species is still seriously impairing poultry development [1]. The species of *Emeria* destroy the intestinal lumen, exhibit a negative impact on the immunity and nutrient absorption mechanism of broiler chickens. Moreover, the cost of prophylactic measures, professionals, and low production efficiency resulting from coccidiosis further aggravate the condition, leading to a huge global economic loss [2].

The conventional coccidiosis control approaches heavily rely on chemoprophylaxis and vaccination, which seemed to be operative during the past decade, however, the widespread occurrence of anti-microbial resistance to anti-coccidial drugs has raised serious concerns for the poultry industry with a renewed challenge. Further, the legislative limitations from the European Union regarding the inclusion of anti-microbial drugs and increasing consumers' preference for organic food have compelled professionals to explore new alternative natural feed additives that may serve the purpose successfully [3].

Published literature indicates that negative consequences of coccidial infection may be reduced by the inclusion of natural antioxidants, herbs, essential oils, and anti-microbial in the avian feed [4]. Recently, plant-origin carotenoids have gained much attention as feed ingredients due to their high nutraceutical activities. Moreover, these promote bird's health and performance by beneficially modulating gut morphology, digestion, immunity, pigmentation of broiler and layer chicks [5-8]. Hence, the current study was carried out to evaluate the potential beneficial impact of curcumin and lutein (individual and in combination) against the *Eimeria maxima* infected broiler chicks.

Material and Methods

The recommendation of the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, People's Republic of China) was used for the execution of this trial. A total of 200 1-d old broiler chicks (Arbor Acres) were run randomly into 4 groups (each group with 5 replicates;10 chicks per each). Four nearly *iso-nitrogenous* CP (21.19%) and *isocaloric* (ME= 2998 kcal/kg) broiler starter diets *(Table 1)* were prepared and fed from 1-21 days of age. The T_0 was (control) diet, whilst the diets T_1, T_2 and T_3 were enriched with curcumin 300 mg/kg, lutein 300 mg/kg and curcumin and lutein 150+150 mg/kg, respectively. Similarly, four nearly *iso-nitrogenous* CP (19.20%) and *iso-caloric* (3023, 90 kcal/kg) finisher diets *(Table 1)* encompassing the similar treatment as in starter diet were fed from 22-42 days of age. The carotenoids were supplied from Guangzhou Leader Biotechnology Co Ltd, Guangzhou, China with 98% purity. The basal diet was prepared with a major portion of the rice-soybean meal *(Table 1)* under the guideline of the NRC 1994 [9]. The birds were reared according to the managed metal procedures of Arbor Acres and kept in a triple-stack cage in an environmentally controlled poultry house. A specific amount of sporulated oocysts *Emeria maxima* were harvested from the Laboratory of Veterinary Parasitology, Nanjing Agricultural University, Nanjing, China. A weighed quantity of feed was provided to chicks however water was *ad libitum*. On the 21st day, all the chicks were challenged orally with pure *Emeria maxima* containing 1×10^4 sporulated oocysts which were diluted in distilled water (0.5 mL) [10]. Roche color fan score was used to measure the strength of shank skin color with the help of Konica Minolta chromameter CR-400 (Japan) method was used for yellowness measurement of breast skin color after 45 min of slaughtering. The relative organ weight was found by equation i.e =100 × (organ weight/total live body weight) *(Table 2)*. The Mc Master counting chamber was used for counting the fecal oocysts [11] *(Table 3)*. The 15 mL of concentrated salt (NaCl) solution was homogenized for one gram of the fecal sample and filtered via muslin cloth. From sterile plastic tubes, the liquid was collected and the solid material was discarded. Then on the Mc-Master counting chamber, a specific volume was loaded for 15 min, which cause the oocysts to settle upon the surface, and counting was done under the microscope easily. One mL of solution per total number of *Eimeria* oocysts were

Table 1. *Composition (g/kg) of experimental starter and finisher diets*

Ingredients	Starter	Finisher
Rice broken	602.5	654.7
Soybean meal 45%	325.0	280.0
Corn gluten meal 60%	30.0	25.0
Lime stone	12.0	12.0
Di calcium phosphate	20.0	18.0
NaCl	3.0	3.0
Vitamin mineral premix[1]	5.0	5.0
L- lysine HCl	1.0	1.0
DL- Methionine	1.5	1.3
Percent calculated nutrient composition		
ME (kcal/kg)	2998	3023.9
Crude Protein	21.19	19.2
L-lysine	1.21	1.1
Methionine	0.496	0.4
Methionine and cystine	0.80	0.7
Calcium	0.988	0.9
Available P	0.531	0.5

Supplied the following per kg fed: Vit. B_{12}, 0.012 mg; Fe 82 mg; Cu 7.5 mg; Mn 110 mg; Zn, 64 mg; Calcium iodate, 1.1 mg; Se 0.28 mg; Vit. B_1, 2.3 mg; Vit. B_2 8 mg; Vit. B_3 42 mg; Biotin, 0.04 mg; Folic acid, 1 mg; Vit. B_5, 10 mg; Pyridoxine HCl, 4 mg; Choline chloride, 400 mg; Vit. A, 25 mg; Vit. D_3, 6 mg; Vitamin K_3, 1.2 mg

Table 2. *The Influence of natural carotenoids supplementation on the relative organ's weight in Eimeria challenged broiler chicks*

Parameters	Diets[1]			
	T_0	T_1	T_2	T_3
Liver	2.57±0.60^{a}	1.96±0.34^{ab}	1.84±0.19^{b}	2.27±0.41^{ab}
Spleen	0.06±0.01^{a}	0.05±0.01^{ab}	0.04±0.01^{b}	0.05±0.01^{ab}
Bursa	0.08±0.02	0.08±0.05	0.06±0.02	0.07±0.04
Thymus	0.05±0.01	0.06±0.01	0.05±0.01	0.05±0.01

[1] T_0 (Control), T_1 contained (curcumin 300 mg/kg), T_2 (lutein 300 mg/kg), T_3 contained a mixture of curcumin + lutein (150+150 mg/kg); ^{abc} Mean values with common superscript did not differ (P>0.05)

Table 3. *The Influence of natural carotenoid supplementation on the intestinal lesion score, bloody diarrhea, and fecal oocyst count score in Eimeria infected broiler chicks*

Item	Diets[1]			
	T_0	T_1	T_2	T_3
Bloody diarrhea	3.75 ±0.50^{a}	3.00±0.81^{ab}	2.17±0.57^{b}	2.08±0.83^{b}
Lesion score	2.17±0.18^{a}	1.66±0.24^{b}	1.94±0.06^{ab}	1.87±0.01^{ab}
Oocyst count	104.4×10^{3}±8.87^{a}	64.96×10^{3}±6.30^{bc}	83.11×10^{3}±8.03^{b}	49.55×10^{3}±5.48^{c}

[1] T_0 (Control), T_1 contained (curcumin 300 mg/kg), T_2 (lutein 300 mg/kg), T_3 contained a mixture of curcumin + lutein (150+150 mg/kg); ^{abc} Mean values with common superscript did not differ (P>0.05)

used for counting and reading. The number of oocysts was estimated by the formula i.e Oocysts per gram (OPG) of a fecal sample of broiler = oocysts count x dilution factor x (volume of fecal sample/volume of counting chamber/ number of broiler chicks per treatment group were determined. On 3-10 (dpi) intensity of bloody diarrhea was observed twice a day. According to Youn and Noh [12], the score was assigned from 0 to 4 scale. The zero value was recognized as the normal condition, however, the values of 1, 2, 3, and 4 exhibited the percentage as 1-25, 26-50, 51-75, or over 75 bloody diarrhea in the total fecal samples. During the 2nd week of production, birds were vaccinated for the Newcastle disease virus NDV and AIV.

Data Collection

The data regarding the growth performance including feed consumption, body weight gain was recorded to estimate feed conversion ratio (FCR). Then after 6 h of feed deprivation, fresh samples of blood (10 mL) were collected by randomly selecting one bird for each replicate. The birds were slaughtered and the de-feathered carcass was then eviscerated. The weight of the visceral organ (liver and spleen) for each group was individually found. To measure the shade of shank skin color three birds per replicate were selected on the 28th day, whilst for examination breast skin color one bird from each replicate was selected. After a post-infection period, the collection of feces was done twice a day. The collected pooled feces of each replicate of all treated groups were measured for the *Eimeria* oocyst shedding.

Further, on the 7th day (dpi), 5 birds/treatment groups were selected randomly for intestinal lesions scores determination as suggested by Johnson and Reid [13]. A volume of 2 mL blood was collected from the wing vein of 2 birds per pen (10 birds/treatment group) to measure antibody titers against NDV and AIV twice at 20 and 30 days of age. The samples of the blood were centrifuged at 3000×g for 10 min at 4°C to isolate serum aliquots and further stored at -20°C temperature. Then by using the protocol of haemagglutination inhibition (HI) tests the antibody titer against NDV and AIV was measured. The plasma concentrations of enzymes, AST and ALT were observed by a corresponding test. Thio-barbituric acid reactive substances (TBARS) as the concentration of MDA (from liver tissue and blood plasma) were measured by the protocol of Zhang et al.[14].

Additionally, fecal samples were observed for continuously 6 days (3 dpi) for spots of bloody diarrhea. The component of blood was visually detected (4 dpi) in almost all of the treatment groups, and scores were assigned based on the severity of bloody appearance.

Statistical Analysis

The statistical *software* SPSS version (SPSS 15.0 K for Windows, Chicago, IL) was used to perform statistical analysis and all calculated values were presented as means ± SEM. One-way analysis of variance technique was applied to compare the mean. By using Tukey's method at (P<0.05) the significant variations among the mean values were evaluated. The lesion scores were assessed from the mean score of one bird per replicate. Bloody diarrhea and oocysts count were observed by a pooled fecal sample of each replicate and the mean value of 5 replicate/treatment groups were considered bloody diarrhea and oocysts count.

Results

Growth Performance

Concerning the influence of dietary supplementation of curcumin, lutein, or as a mixture of both investigated in this study failed to exhibit significant change (P>0.05) on the feed consumption, body weight gain, and FCR as presented in *(Table 4)*. A significantly better (P<0.05) weight of liver and spleen was found in the T_0 (control) group as compared to other counterparts. Moreover, the relative weight of the thymus and bursa remained statistically unchanged as illustrated in *(Table 2)*.

Fecal Oocysts Count, Intestinal Lesion Score, and Bloody Diarrhea

Table 3 illustrates the findings regarding the oocyst count, intestinal lesion score, and bloody diarrhea of broilers. The results revealed that T_2 and T_3 dietary groups exhibited significantly decreased bloody diarrhea values. The carotenoid inclusion controls the harmful effects of *Eimeria* concerning intestinal lesion scores. Lesion score of intestine was significantly low in T_1 group followed by T_3, T_2 and T_0 dietary groups. A significantly lower (P<0.05) fecal shedding of oocyte count was recorded in the T_3 dietary group followed by T_1, T_2 and T_0 dietary group.

Antibody Titers Against Newcastle Disease Virus and Avian Influenza Virus

The antibody titers against NDV and AIV have been presented in *Table 5*. It could be observed that birds of treatment group T_1 and T_3 produced slightly greater antibodies against NDV as compared to T_0 and T_2 counterparts (at day 20). Moreover, the highest (P<0.05) antibody titers were in the T_3 dietary group however, the T_2 dietary group produced the lowest (P>0.05) antibody titers against NDV. The T_0 and T_1 group exhibited statistically similar immunogenic responses.

Shank and Skin Color

The results of shank and skin color are presented in *(Fig. 1)*. Dietary supplementation with lutein produced a darker yellow color on the broiler shank skin. The highest intensity of the shank skin color *(Fig. 1)* was observed in the broilers supplemented with a combination of both carotenoids (C+L) followed by LTN, curcumin CRM and control groups. While, the skin color was significantly higher in LTN and CRM than L+C and Control as measured by chroma meter.

Plasma Alanine Aminotransferase, Aspartate, Aminotransferase

Fig. 2 indicates the plasma AST and ALT concentrations. The AST (U/L) was significantly higher (P<0.05) in T_2 diets whilst T_3 exhibited a minimum level of AST. Moreover, birds reared with diet T_0 and T_1 groups exhibited statistically similar results. Similarly, the T_2 group produced a higher level of ALT among all other counterparts whilst the T_3 group exhibited a minimum level.

Plasma and Hepatic Thiobarbituric Acid Reactive Substances (TBARS) Activities

As shown in *(Fig. 3)* the hepatic and plasma TBARS were

Table 4. *Impact of natural carotenoids supplementation on growth performance in Eimeria challenged broiler chicks*

Parameters	Diets[1]			
	T_0	T_1	T_2	T_3
Feed intake(kg/bird)	2.20±0.18	2.16±0.59	2.18±0.42	2.04±0.24
Bodyweight(kg/bird)	1.15±0.13	1.25±0.34	1.28±0.27	1.22±0.17
FCR	1.75±0.03	1.72±0.04	1.70±0.05	1.67±0.04

[1] T_0 (Control), T_1 contained (curcumin 300 mg/kg), T_2 (lutein 300 mg/kg), T_3 contained a mixture of curcumin + lutein (150+150 mg/kg)

Table 5. *The Influence of natural carotenoid supplementation on the serum Antibody titer against Newcastle Disease Virus (NDV)) and Avian Influenza Virus (AIV) and against Eimeria infected broiler chicks*

Item	Diets[1]			
	T_0	T_1	T_2	T_3
20 d				
ND	5.60±0.36	5.80±0.34	5.40±0.52	5.80±1.05
AI	5.67±1.15	5.67±0.06	6.33±1.15	6.00±1.00
30 d				
ND	$4.50±0.52^{ab}$	$4.20±0.95^{ab}$	$3.80±0.26^{b}$	$5.40±0.26^{a}$
AI	4.33±0.58	4.67±0.57	5.00±1.00	4.67±0.57

[1] T_0 (Control), T_1 contained (curcumin 300 mg/kg), T_2 (lutein 300 mg/kg), T_3 contained a mixture of curcumin + lutein (150+150 mg/kg); ^{abc} Mean values with common superscript did not differ (P>0.05)

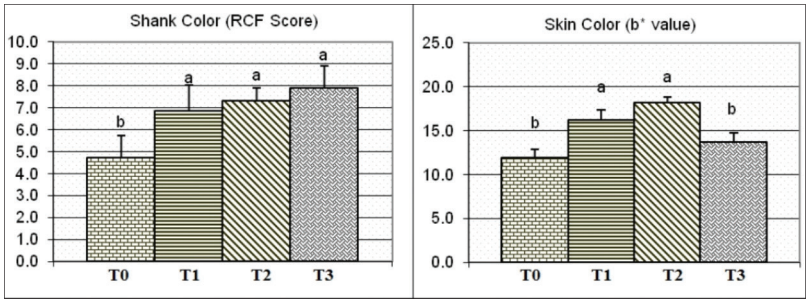
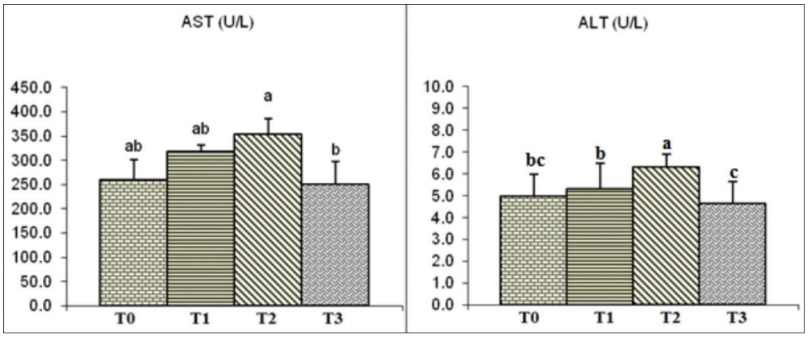


Fig 1. Shank and Skin color in *Eimeria* infected broiler chicks

Fig 2. Plasma Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) concentrations in *Eimeria* infected broiler chickens



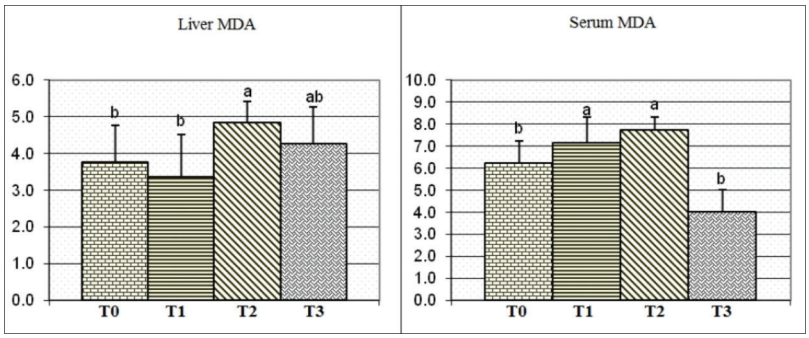


Fig 3. The *Malondialdehyde level (MDA)* infected broiler chicks

recorded by MDA concentrations. The T_2 dietary group ($P<0.05$) produced the highest values for hepatic MDA followed by the T_3 group. The broiler chicks reared with T_0 and T_1 diets exhibited similar results. The serum MDA concentration was statistically similar in the T_2, T_1, and T_0 dietary groups. The T_3 dietary group exhibited minimum values.

Discussion

Phyto-chemicals including curcumin and lutein are extracted from natural sources. According to Partovi et al.[15], these natural phytogenics have a wide range of therapeutic properties and may elevate the growth potential of birds. Further, carotenoids rich feeds improve the anti-oxidant status and immunogenic response of broilers [5,7]. Among the group's optimum performance was shown in T_2 and these results correlate with the conclusion of Rajput et al.[7], who recorded no significant changes in the performance traits of broiler subjected to a diet containing carotenoids. The outcomes of the study showed that the body weight was found to be higher in the T_1 group after the challenge of coccidiosis. The turmeric *(Curcuma longa* L.) supplementation promoted the live body weight of *Eimeria*-infected broiler chicks, these findings align with our results [16]. Besides, low-level contamination of oocysts and improved body weight gain show that animals are resistant to *Eimeria*, but interestingly, these parameters have not always been documented in review [17]. Likewise, in our study, curcumin supplementation failed to produce a significant change in growth performance relative to the control group, however, curcumin supplementation lowered the level of oocyst shedding. In the present study, dietary alone supplementation of lutein seems inefficient whereas, curcumin supplementation showed more promising effects towards the promotion of feed efficiency. Moreover, this study found that livers' relative weights were increased in the lutein and control groups as compared to other groups. Significant changes in the organ weight of broilers were recorded by the inclusion of 5 g/kg *Curcuma longa* [18]. The experiment also revealed an increase in the liver and spleen weights by the treatment of the curcumin+lutein mixture. A significantly increased liver weight might be associated with the doses of the compound, which may cause hepatotoxicity. Further, overall carotenoid supplementation posed no adverse effects on

the organ's weight of the broiler. After inoculation avian *Eimeria* sporozoites appear to pass through mucosa and travel to the spleen and liver then leave these tissues very shortly and this action may reveal morphological modifications in the liver and spleen tissues [19]. According to this study, at 4-6 (dpi) signs of intestinal coccidiosis including bloody diarrhea were visible in nearly all groups, whilst the reduced bloody diarrhea was in the chickens reared on individual lutein and with a mixture of curcumin and lutein enriched diets. After the coccidiosis challenge, a lower value fecal oocysts count was recorded in the group of birds supplemented with carotenoid. More encouraging results were in the birds reared on basal diet added with curcumin and a mixture of curcumin and lutein than the diets of control and alone lutein group. Although, both the plant phytochemicals prevented the infection of intestinal tissue as detected by intestinal lesion scores. Moreover, broilers fed a diet enriched with carotenoid showed a lower rate of lesion scores than reared with a carotenoid-free diet. Both the bioactive nutrients exerted protective effects against coccidiosis because they are being known as anti-oxidant and anti-radical activities.

Another report by Allen et al.[16], demonstrated that turmeric *(Curcuma longa* L.) lowered small intestinal lesion scores and fecal oocyst counts in meat birds. Antibody titers against the disease are a biomarker of explicit humoral immunity. Before the onset of coccidiosis, both carotenoids slightly raised the antibody titer at the 20th d of age, as in this study. The inclusion of 2 mL/L herbal mixture improved the immunity of coccidiosis challenged broiler by regulating the mRNA expression [20].

Further, Ntrallou et al.[21], observed that food colorants derived from natural sources enhance antibody production. But, here diets supplemented with curcumin, lutein and their mixture could not raise antibody concentrations against (AIV) at 30 d (10 dpi). The current study opposes our earlier observations, which confirmed that antibody titers were increased in curcumin and lutein treated, Lipo-polysaccharide (LPS)-induced broiler chickens augmented with the increase of age [7], which align the hypothesis that coccidiosis decreases immunity. According to our findings, all the tested diets enriched with carotenoids promoted the skin color of broilers and these findings are verified by Karadas et al.[22], who recorded that a dose of 25 mg/kg keto-carotenoid pigment i.e canthaxanthin promoted the leg skin color. Moreover, the author also observed that other carotenoids sources also improved color parameters on an overall basis. Further, carotenoids promoted the shank color of broilers. The tissues of chickens were saved from stress as zeaxanthin and β-carotene contents maintain immunity [8].

Certain environmental factors and metabolic pathways generate free radicals which cause oxidative stress, which is measured as an important cause of various liver diseases. Hepato-cellular injuries cause the production of enzymes aspartate aminotransferase AST and alanine aminotransferase ALT into the rotation and promote in the plasma levels of AST indicate hepatic injuries likely to hepatitis and muscular damages [23,24]. The plasma AST concentrations were high among all the groups but the plasma ALT level was not significantly high in any of the experimental groups. The reason for this is not clear, and the normal levels may be deceptive because significantly injured hepatic cells do not generate ALT enzyme. This study found that AST concentrations and hepatic structures reflected the hepatocellular damage. Furthermore, plasma ALT and AST concentrations are associated with the reduction of plasma antioxidant levels, mainly when carotenoids are supplemented. The addition of β-carotene was many operatives in reducing the liver infection and generated a concomitant reduction in plasma AST level [25]. Further, carotenoids reduced the level of ALT and AST in mule ducklings stimulated by aflatoxin [26]. At present, this finding of peroxidation MDA levels, histopathology, and ALT and AST concentrations highlighted the beneficial effects and potential anti-oxidative role of natural carotenoids in the liver tissue of *Eimeria* affected broiler chicks. The previous finding of Rajput et al.[6], was also observed carotenoids numerically recorded the capability of improving growth even in challenging conditions. All challenged birds showed a high level of AST compared with the previous observation of Partovi et al.[15], in which broilers were challenged with *LPS*. No influence of *Eimeria* infection and tested diets on the ALT concentration in birds at (42 dpi).

Zhang et al.[3] and Zhai et al.[27] reported that curcumin may positively impact liver and kidney activity. Moreover, lutein may behave as a powerful antioxidant carotenoid; but, our results found that lutein created an adverse effect on the biochemical analysis and liver histology. The deterioration of the liver may be associated with relatively higher (300 mg/kg) supplementation of lutein which may exhibit clinically toxic effects on the liver. But interestingly, Rajput et al.[7], recorded that 200 mg/kg lutein exhibited beneficial effects on *LPS*-infected chickens.

The current experiment revealed that a dose of 300 mg/kg lutein and curcumin failed to express any significant change in the growth performance of the coccidiosis-infected broiler. Supplementation of 300 mg/kg curcumin lowered lesion score, oocyst shedding and decreased coccidial effect. Similarly, lutein reduced bloody diarrhea but produced adverse effects on the liver at the same dose. Additionally, a mixture of both the doses of carotenoids not only elevated the skin pigmentation but also exhibited a positive impact on the humoral and cell-mediated immunity of the broiler chicks.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author and can be provived on your request.

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Competing Interests

The authors whose names are listed in the manuscript certify that they have NO conflicts of research in the subject matter or materials discussed in this manuscript.

Authors' Contributions

WT and NR, designed the project. The sampling, data collection, processing and interpretation of results were made by NR, SA, and MN. The data analysis was made by MN, SA and the manuscript was written and reviewed by NR, RMB and WT. All the authors read the manuscript and approved the contents.

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RESEARCH ARTICLE

Histopathology and Tumor Necrosis Factor-α Expression in The Kidney of an Asphyxial Cardiac Arrest Rat Model

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Abstract

Multiple organ injuries in patients with post cardiac arrest syndrome (PCAS) after cardiac arrest (CA) is associated with mortality. Among multiple organ injuries after return of spontaneous circulation (ROSC), renal dysfunction can lead to acute kidney injury, which is known to be associated with high mortality. This renal injury is associated with a systemic inflammatory response syndrome mediated by ischemia reperfusion by ROSC following CA. However, the mechanism remains unclear. Therefore, the objective of this study was to determine, the relationship between the expression of tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, and renal injury in PCAS. In the present study, asphyxial CA was induced in Sprague-Dawley rats with normothermia and the survival rate was measured at two days after ROSC. The rats in each group (n=6) were sacrificed at 6 h, 12 h, 1 day, and 2 days after ROSC. Renal injury was analyzed with by Masson's trichrome stain, TNF-α immunohistochemistry (IHC) and western blot. The mortality was 72% at 12 h after ROSC and the survival rate of rats was decreased to 24% 2 days after ROSC. Histopathological score in the renal tissue after CA showed a significant increase at 6 h than sham group. The expression level of TNF-α in the renal cortex tissue was also increased at 6 h after CA based on both IHC and western blot results. After CA, the renal histopathological injury was significantly increased at 6 h after ROSC with a proportional increase of TNF-α expression in the kidney tissue leading to rapid injury to the kidney.

Keywords: *Post cardiac arrest syndrome, Asphyxial cardiac arrest, kidney, Histopathology, TNF-α*

Asfiksiyal Kardiyak Arrestli Rat Modelinde Böbrek Histopatolojisi ve Tümör Nekrozis Faktör-α Ekspresyonu

Öz

Kardiyak arrest (KA) sonrası post-kardiyak arrest sendromu (PKAS) olan hastalarda çoklu organ hasarı mortalite ile ilişkilidir. Spontan dolaşıma dönüş (SDD) sonrası çoklu organ hasarları arasında böbrek fonksiyon bozukluğu, yüksek mortalite ile ilişkili olduğu bilinen akut böbrek hasarına yol açabilir. Bu böbrek hasarı, KA'yı takiben SDD tarafından iskemi-reperfüzyonun aracılık ettiği sistemik bir inflamatuar yanıt sendromu ile ilişkilidir. Ancak, bunun mekanizması belirsizliğini korumaktadır. Bu nedenle, bu çalışmanın amacı, bir proinflamatuar sitokin olan tümör nekrozis faktör-α (TNF-α) ekspresyonu ile PKAS'ta böbrek hasarı arasındaki ilişkiyi belirlemekti. Çalışmada, normotermik Sprague-Dawley ratlarda asfiksiyal KA indüklendi ve ratların hayatta kalma oranı SDD'den iki gün sonra ölçüldü. Her gruptan ratlar (n=6), SDD'den 6 saat, 12 saat, 1 gün ve 2 gün sonra sakrifiye edildi. Böbrek hasarı Masson trikrom boyama, TNF-α immünohistokimyasal (IHC) yöntem ve western blot ile analiz edildi. SDD'den 12 saat sonra mortalite %72 iken, SDD'den 2 gün sonra ratlarda hayatta kalma oranı %24'e düştü. KA sonrası böbrek dokusundaki histopatolojik skor, kontrol grubuna göre 6. saatte anlamlı bir artış gösterdi. Renal korteks dokusundaki TNF-α ekspresyon seviyesi, hem IHC hem de western blot analiz sonuçlarına göre KA'dan 6 saat sonra arttı. KA'dan sonra, böbrek histopatolojik hasarı, böbrek dokusunda orantılı bir TNF-α ekspresyon artışı ile birlikte böbrekte hızlı hasara yol açan SDD'den 6 saat sonra önemli ölçüde arttı.

Anahtar sözcükler: *Post cardiac arrest syndrome, Asphyxial cardiac arrest, kidney, Histopathology, TNF-α*

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Introduction

Cardiac arrest (CA) is a sudden stop of normal blood flow. It is a major cause of death in Europe and United States [1]. Over the last few decades, many researchers have conventionally tried to improve the rate of return of spontaneous circulation (ROSC) for CA, achieving remarkable outcomes [2]. Even after ROSC, mortality rates are still high. Overall survival rates after CA have been reported to be less than 1% worldwide [3]. The poor prognosis after ROSC is explained by a pathophysiologic process called post cardiac arrest syndrome (PCAS). PCAS is clinically manifested as brain injury, myocardial dysfunction, ischemia/reperfusion (IR) injury and persistent precipitating pathology [4]. According to Roberts et al.[5], dysfunction of multiple organs is common after ROSC following CA and that is associated with PCAS.

Chronic kidney disease and poor renal function with heart failure have been associated with poor outcomes and high mortality [6]. Even small changes in serum creatinine (Cr) can be associated with high systemic mortality rates [7]. Acute kidney injury(AKI) after ROSC following CA is quite common, with an incidence of over 40% [8]. However, most recent studies have focused on brain or heart injury after ROSC. Despite extensive renal impairments, research has been barely conducted on kidney injury after CA. The PCAS causes systemic IR-mediated injury, inducing systemic inflammatory response syndrome. While inflammatory reactions are usually caused by certain infections, "sterile inflammation" occurs during ischemic insults [9]. As systemic inflammatory response syndrome, IR injury after CA can triggers inflammation. Several studies have reported that tumor necrosis factor- (TNF-α) is increased in the heart, lung and brain after CA in animal models, although the exact mechanisms have not been clearly understood [10-12]. Previous studies have reported that AKI by IR is associated with pro-inflammatory cytokines such as TNF-α [13]. However, there is no previous study on the CA-induced kidney injury.

The present study hypothesized that IR after ROSC causes a sterile inflammatory response in the kidney, leading to renal injury and production of pro-inflammatory cytokines. We attempted to investigate the relationship between low survival rate and renal injury in the early an inflammatory stage after ROSC. The aim of present study was to understand the mechanism of renal injury by the changes of TNF- α as an inflammatory cytokine in the asphyxial CA rat model.

Material and Methods

Animals and Ethical Approval

Male Sprague-Dawley rats (9 weeks, 280~310g) were obtained from the Experimental Animal Center of Jeonbuk National University (Iksan, South Korea). Rats were housed in a rat isolator with a 12 h light/dark cycle and maintained on standard laboratory chow *ad libitum*. All experimental animals used in this study were maintained under the protocol approved by the Institutional Animal Care and Use Committee (Approval no. JBNU 2019-005) of Jeonbuk National University. The rats were randomly divided into two groups as follows: Sham group (n=6), which was not subjected to CA operation, and CA-operated group (n=24), which was subjected to CA. The rats in each group were sacrificed at 6 h (CA-6 h, n=6), 12 h (CA-12 h, n=6), 1 d (CA-1 d, n=6), and 2 d (CA-2 d, n=6), respectively, after ROSC.

Induction of CA, and Cardiopulmonary Resuscitation (CPR)

CA induction and CPR were performed according to published protocols [14]. In brief, the rats were anesthetized with 2% to 3% isoflurane and mechanically ventilated with a rodent ventilator (Harvard device, Massachusetts Holly Stern material, USA). We monitored peripheral oxygen saturation (SpO_2) using an oxygen saturation probe (Nonin Medical, Plymouth, MN, USA) attached to the left hind foot of each rat. The electrocardiographic probes (GE Healthcare, Milwaukee, WI, USA) were attached to limbs, and the electrocardiogram (ECG) was recorded ceaselessly. The left femoral artery was cannulated under monitoring the mean arterial pressure (MAP) (MLT 1050/D, AD Instruments, Bella Vista, Austria), and the right femoral vein was cannulated for injection. After 5 min of the stabilization period, we administered 2 mg/kg of vecuronium bromide (GensiaSicor Pharmaceuticals, Irvine, CA, USA) intravenously, and stopped anesthesia and mechanical ventilation. About 3-4 min after vecuronium injection, CA was defined when MAP was less than 25 mmHg and pulseless electrical activity occurred. CPR was initiated at 5 min after CA by a bolus injection of epinephrine (0.005 mg/kg; Yeongdeungpo-gu, Seoul, Korea) and sodium bicarbonate (1 mEq/kg; Sungnam, Kyunggi-do, Korea). Mechanical ventilation (VentElite; Havard apparatus, Holiston, MA, USA) with 100% oxygen was followed. Mechanical chest compression was given at a rate of 300/min until the MAP reached 60 mmHg, as well as electrocardiographic activity, was observed. When the animals were hemodynamically stable and spontaneously breathable they were extubated 2 h after resuscitation and monitored for outcome evaluation.

Measurement of Serum Blood Urea Nitrogen (BUN), Creatinine (Cr)

Rats were anesthetized with 30% urethane (1.5 g/kg, i.p.; Daejung, Gyeonggi-do, Korea) and 3 mL blood was collected from inferior vena cava. Thereafter, it was centrifuged to 4000 rpm for 10 min and serum was obtained. The serum was used for the analysis of BUN and Cr with Automatic Analyzer 7020 (Hitachi, Japan).

Histopathologic Assessment

The kidneys were collected and fixed with 4% paraformaldehyde (PFA) at room temperature for 24 h, and paraffin-embedded. Tissues were cut into 7 μm thick sections, which were stained with Masson's trichrome (Scytek, West Logan, UT, USA) at room temperature and visualized under a light microscope at ×400 magnifications (Leica DM 2500; Leica Microsystems GmbH, Germany). Masson's trichromic method was used for defining tubular injury, with tubular dilatation, tubular atrophy, tubular cast formation, vacuolization, degeneration and sloughing of tubular epithelial cells, or thickening of the tubular basement membrane. In brief, only cortical tubules were included in the following scoring system in all cortex regions each sample: 0 = no tubular injury; 1≤9% of tubules injured; 2 = 10-25% of tubules injured; 3 = 26-50% of tubules injured; 4 = 51-75% of tubules injured; 5≥76% of tubules injured [15,16].

Immunohistochemistry (IHC)

The expression and localization of TNF-α in renal tissue were detected immunohistochemically with a rabbit polyclonal antibody against TNF-α (Abcam Incorporated, Cambridge, MA, USA). After deparaffinization, tissue sections were treated using a microwave antigen-retrieval procedure in 10 mM sodium citrate buffer pH6.0 (Sigma-Aldrich, Sigma Aldrich, Burlington, MA, USA) After blocking endogenous peroxidases, sections were incubated with a non-immune serum to block non-specific staining. To assess alterations of TNF-α immunoactivity levels, tissue sections were incubated with anti-TNF-α (diluted 1:500, Abcam, Cambridge, UK), anti-rabbit secondary antibody (biotinylated anti-rabbit IgG(H+L); Vector Laboratories, Burlingame, CA, USA), and using 3,3'-diaminobenzidine (DAB; Sigma Aldrich, Burlington, MA, USA). After DAB the sample slide counterstained the hematoxylin. A light microscope was used to make images at fixed x 400 magnifications. For quantitative analysis of densities of TNF-α immunoreactivities, relative optical density percentage (ROD %) was measured using image-J threshold analysis software [IJ172-win-Java1.8.0, Bethesda, MD, USA].

Western Blot Analysis

To examine change in the level of TNF-α protein in the kidney after CA, western blot analysis was performed according to a previously published method [17]. Kidney tissues were homogenized with proteinase and phosphatase inhibitors in a protein extraction solution (Pro-Prep; Intron). The homogenates, which contained 20 μg of protein, were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The blot was probed with primary antibody against TNF-α (diluted 1:500, Abcam, Cambridge, UK), and β-actin (diluted 1:1000, Cell Signaling Technology, Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology, CA, USA) was used as a secondary antibody. The band images were detected with a Las-4000 imager (GE Healthcare Life Science, Pittsburgh, PA, USA).

Statistical Analysis

All data were entered into Graph Pad Prism 5.0 and presented as means ± standard error of the mean (S.E.M.). Survival was analyzed using Kaplan-Meier statistics and log-rank test. Statistical analyses were used one-way analysis of variance (ANOVA) and Masson's trichrome staining was analyzed Kruskal Wallis analysis followed by Bonferroni's multiple comparison tests. Differences were considered significant when P-value was less than 0.05.

Results

Physiological Variables and Survival Rate

We estimated the survival rate 2 d after CA. Survival rate of CA group was significantly reduced *(Fig. 1)*. A mortality rate of 75% occurred at 12 h after ROSC and the survival rate of rats decreased to 24% 2 days after ROSC (P<0.05). Before CA operation, all the rats in the sham and CA-operated groups showed physiological values of body weight, body temperature, and heart rate *(Table 1)*. There was no significant (P>0.05) difference among groups for baseline characteristics, including body weights, body temperature, and heart rate. The induction of CA was 2-3 min after the intravenous injection of vecuronium bromide (2 mg/kg). CA was confirmed with isoelectric electrocardiogram (ECG), SpO_2, and MAP. ECG, SpO_2 and MAP were changed as expected according to the experimental protocol. Body temperature was the same as that at the baseline or after ROSC.

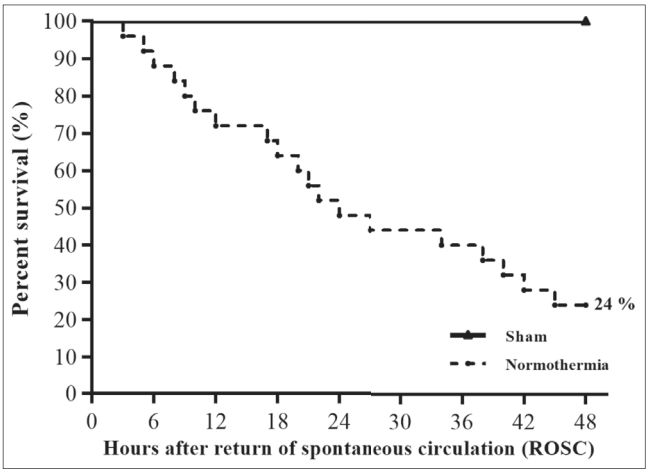

Fig 1. Survival rate. Cumulative survival rate analyzed by a Kaplan-Meier analysis in the sham- and cardiac arrest (CA) groups 2 days after return of spontaneous circulation (ROSC). The survival of rats in the CA group had significantly different from that in the sham-CA group (log-rank test, P<0.05)

Table 1. *Physiological variables in the sham and CA groups. Schematic representation of the asphyxial CA model in rats and measurements obtained at animal stabilization period (baseline), induction of CA, CPR time, Heart rate, and sacrifice time*

Parameters	Sham	CA-6h	CA-12h	CA-1d	CA-2d
Body temperature (°C)	36.88±0.04	37.06±0.05	36.75±0.06	36.60±0.10	36.33±0.03
Asphyxial time to CA (second)	-	163.2±4.66	163.75±5.54	170.00±5.40	154.75±4.92
CPR time (minute)	-	1.23±0.06	1.26±0.07	1.22±0.09	1.26±0.07
Heart rate (beat/min)	337.83±2.09	347.83±3.06	342.67±2.95	336.17±2.91	343.67±2.23

CA: *Cardiac arrest,* ***CPR:*** *Cardiopulmonary resuscitation*

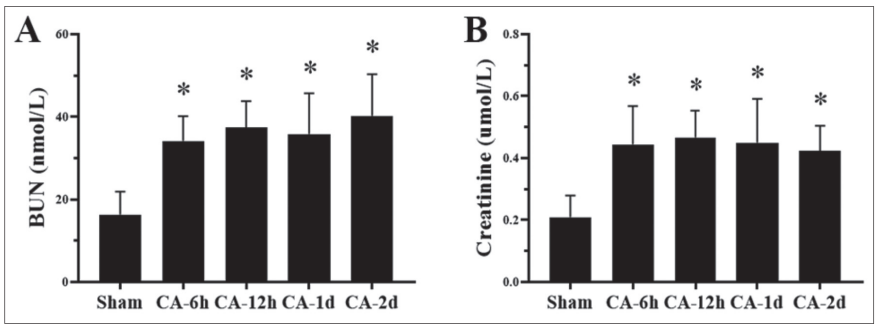

Fig 2. Blood urine nitrogen (BUN) and creatinine (Cr). BUN **(A)** and Cr **(B)** were significantly higher in the CA groups compared to the sham group. Data are expressed as mean ± standard error means (SEM). * P<0.05 compared with the sham group and CA groups at 6 h, 12 h, 1 d, and 2 d groups respectively in a time-dependent manner

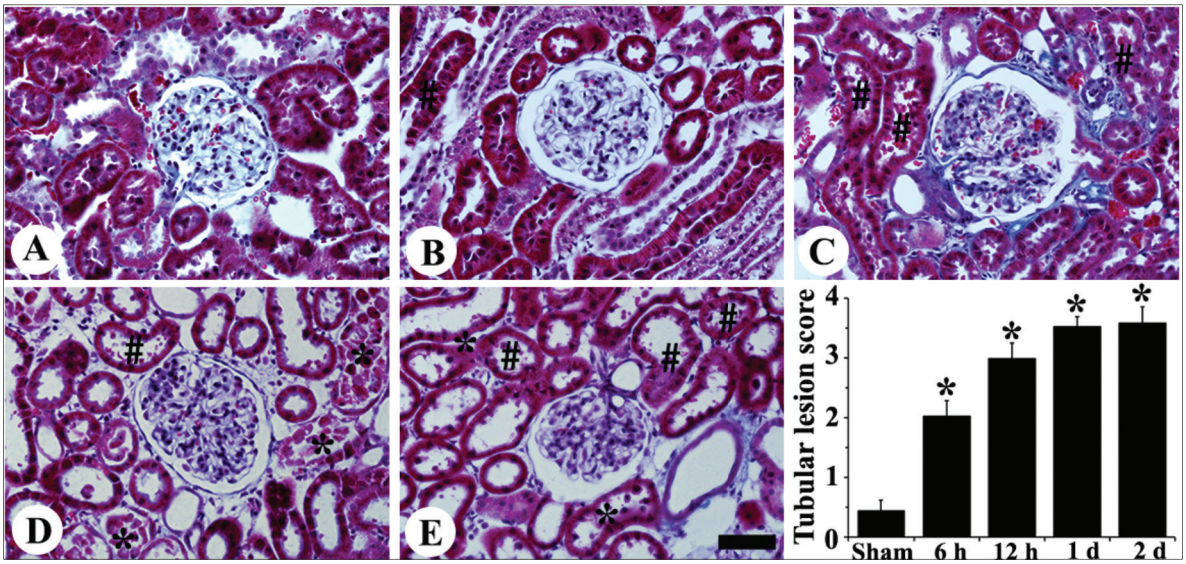

Fig 3. Masson's trichrome stain. Masson's trichrome staining of the sham group **(A)** has shown no tubular injury. In CA groups [B-6h, C-12h, D-1d, E-2d], the tubular injury score is markedly increased at 6 h **(B)** and maintained until 2 d **(E)**. Renal tubules showed severe dilatation(star), loss of brush border with necrosis (hash). Data are expressed as mean ± standard error means (SEM). * P<0.05 compared with the sham and CA-operated groups at 6 h **(B)**, 12 h **(C)**, 1 d **(D)**, and 2 d **(E)**. Scale bar: 200 μm (400x)

Renal Function

The serum level of BUN and Cr was measured to assess the effect of CA in kidney *(Fig. 2)*. One day after CA, serum BUN and Cr levels remarkably increased in CA group compared with the sham group (P<0.05). The results were maintained until 2 days after ROSC.

Renal Histopathological Changes

Masson's trichrome staining was performed to observe the extent of renal injury. It was shown that glomeruli and tubular injury was increased with the increase of time after ROSC. As indicated in *Fig. 3-A,B,C,D,E* severe architectural disruptions of the kidney were triggered by CA, including glomerular capillaries dilation, brush border loss. Tubular lesion scores were significantly increased in the CA group (P<0.05) at 6 h after CA *(Fig. 3-B)*. Tubular injury lesions at 6 h after ROSC were found to increase time-dependently. Glomeruli injury of the kidney increased at 2 d after ROSC as compared to that in the sham group *(Fig. 3-A,E)*.

Immunohistochemical Analysis

Immunohistochemical analysis was performed to examine the expression and localization of TNF- α *(Fig. 4)*. TNF-α

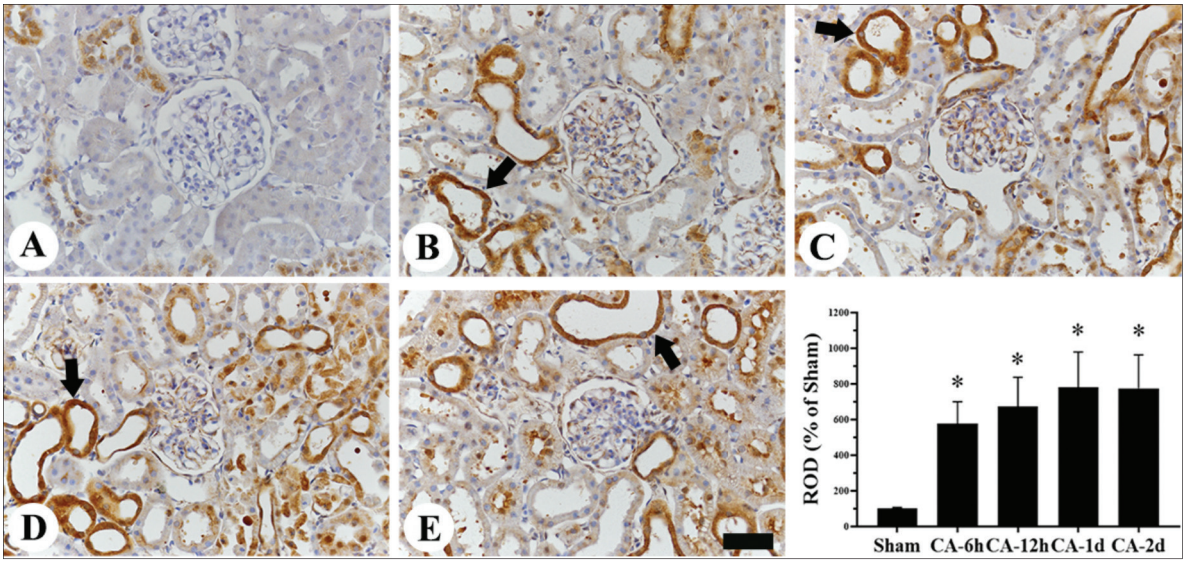

Fig 4. TNF-a IHC. IHC of TNF-α in the kidney of the sham **(A)** and cardiac arrest (CA) operated [B-6h, C-12h, D-1d, E-2d] groups. Relative optical density (ROD%) of TNF-α expression is significantly increased in proximal convoluted tubules (PCT) and some glomeruli at 6 h **(B)**, 12 h **(C)**, 1 d **(D)**, and 2 d **(E)** after ROSC in CA-operated groups compared to the sham (arrow). Data are expressed as mean ± standard error means (SEM). * P<0.05 compared with the sham and CA groups at 6 h **(B)**, 12 h **(C)**, 1 d **(D)**, and 2 d **(E)** groups. Scale bar: 200 μm (400x)

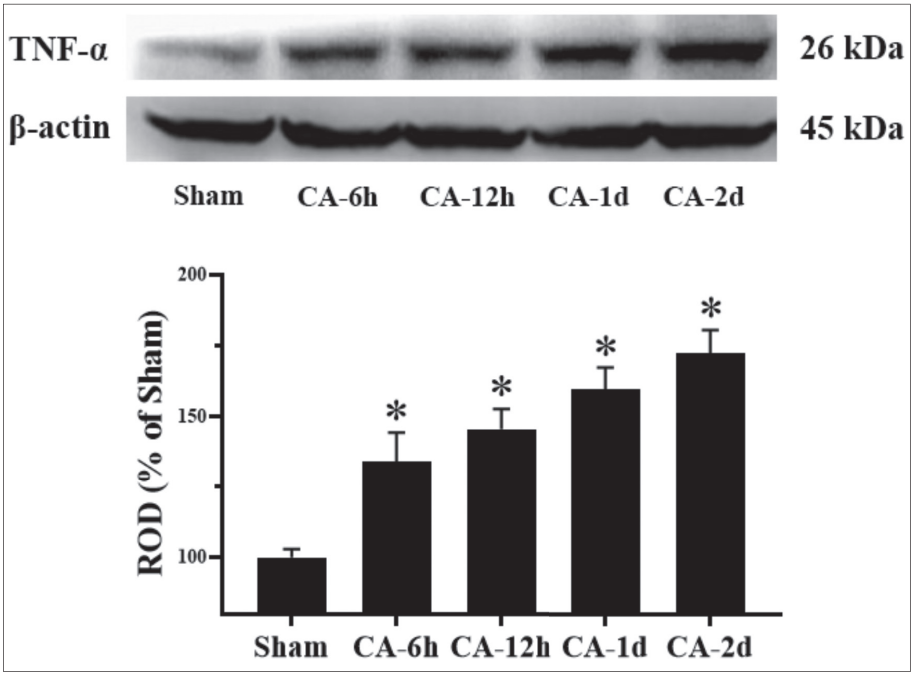

Fig 5. Western blot of TNF-a level. Western blot analysis of TNF- α in the renal cortex of the sham and cardiac arrest (CA) operated groups. TNF- α protein level was significantly increased at 6 h and increased by 2 d as compared to the sham. TNF- α protein levels were measured relative to β-actin. All the data are expressed as mean ± standard error means (SEM)

expression significantly increased at 6 h after ROSC. TNF-α was highly expressed at 1 d in CA renal cortex sections compared with the sham group. These expressions appeared almost on the proximal convoluted tubules (PCT) and some expression was found in the glomeruli.

Western Blot Analysis

TNF-α levels in the kidney of CA-operated group changed in a time-dependent manner after ROSC *(Fig. 5)*. TNF-α level significantly increased at 6 h after ROSC compared with the sham-operated group. Two days after ROSC, TNF-α expression showed the peak level. TNF- α expression was upregulated in a time-dependent manner after 6 h ROSC, but it was not significant among the groups.

Discussion

In the present study, we studied the time-dependent inflammatory injury of the kidney after ROSC in an asphyxial CA rat model. A cohort study of survival rates in patients with CA showed a survival rate of 18.6% after 1 d of ROSC [18] and Lei et al.[19] reported that survival rates

was 25% in asphyxial CA rat model at 48 h. In the present study, the survival rate after 2 d of ROSC was 11%. As such, present study showed similarly low survival rates after ROSC in CA patients and in previous CA model [19]. AKI caused by CA occurs in 12 to 81% of patients [20-22]. The amount of serum BUN and Cr in the present CA model increased significantly after 6 h of ROSC, and this result is related to renal injury. Six hours after ROSC, tubular epithelium cell degeneration and infiltration of inflammatory cells in the renal cortex were observed. This injury is similar to renal tubular necrosis and endothelial cell injury in AKI patients due to renal IR [23]. Therefore, the renal injury pattern caused by the asphyxiation CA in this study was similar to the injury pattern of AKI patients caused by ROSC, thus we thought that the present asphyxia CA model was suitable for AKI of PCAS.

ROSC following CA causes IR injury and it induce AKI, which is associated with inflammation of the renal tubular epithelial [23]. This was consistent with the PCT injury to the loss of tubular epithelial cells of the renal cortex, brush border loss and as shown by Masson's trichrome stain. This tubular injury is derived from an inflammatory response, and IR-induced inflammation is regulated by many pro-inflammatory cytokines [24-26]. TNF-α is one of pro-inflammatory cytokines known to play a role in inflammation. Interleukin (IL)-1b, IL-6, and IL-12 also belong to pro-inflammatory cytokines [27]. TNF-α is produced in a variety of cells, including macrophages, lymphocytes, fibroblasts, and keratinocytes, and is known to regulate inflammation by immunocytes [27,28]. In the present study, time-dependent changes in TNF-α in the kidneys were confirmed by IHC and western blot analysis. TNF-α expression was significantly increased in the tubular epithelial cells of PCT 6 h after ROSC in the renal cortex and this increase was at peak in 1 d and maintained until 2 d after ROSC. Based also on the western blot analysis, TNF-α expression in the renal cortex significantly increased at 6 h, being the highest at 2 d after ROSC. These results are similar to the increase in TNF-α mRNA expression in renal tissue qPCR in the rat renal IR injury model [29], and suggests that CA-induced IR injury can increase TNF-α in the kidney. In the present study, TNF-α expression in the kidney increased accordingly to the time after CA. Furthermore, Nagata et al.[30] reported inhibition of TNF-α reduced inflammation caused by renal IR injury in rats. Therefore, it is suggested that an increased TNF-α would be injury to the kidney through an inflammatory response.

After ROSC, PCAS causes a systemic inflammatory response and the production of pro-inflammatory cytokines, leading to an early inflammatory response that is closely related to the systemic inflammatory response [31]. In addition, an increase in TNF-α in vital organs is observed after CA [31,32]. TNF-α level was shown to be significantly increase at 6 h after ROSC in the CA1 region of the hippocampus of the brain and maintained until 2 d after ROSC, however, brain injury was reported to be not observed histopathologically [32]. TNF-α was also shown significantly increase in the heart 12 h after ROSC and it was maintained until 2 d [10], moreover, Myocardial injury and inflammatory cells infiltration were also observed 12 h after ROSC [10]. Therefore, TNF-α expression increased in the brain and heart early of ROSC, it may be related to tissue injury through an inflammatory response. In the present study, TNF-α expression in the PCT region of the renal cortex significantly increased at 6 h after ROSC. Moreover, a significant cellular injury was observed at 6 h after ROSC. Although it is hard to directly compare heart and brain, these phenomena were faster than the brain and heart of a similar asphyxial CA model [10,32], thus we hypothesized that increasing TNF-α in the kidney induces a rapid inflammatory response and induces renal injury, which is associated with a low early survival rate. After CA, a time-dependent increase TNF-α was confirmed in the kidney however, the exact mechanism was not confirmed in the present study. These were the potential limitations of the present study. Expression levels of other pro-inflammatory cytokines in an asphyxial CA rat model also need to be evaluated in the future and it may be related to the high mortality in the early period after CA.

In conclusion, ROSC after CA increased TNF-α expression in renal tubular epithelial cells and induced inflammatory response. This leads to rapid injury to the renal tubules, which may be associated with low survival rates. Moreover, we suggest that TNF-α level in the kidney might be an indicator of early survival rate in the CA.

Availability of Data and Materials

The datasets during and/or analyzed during the current study are available from the corresponding authors (JC Yoon and H Tae) on reasonable request.

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Competing Interest

The authors declare that they have no competing interest.

Author Contributions

YJY, JHL and HJT were responsible for the experimental design, data acquisition, data analysis and manuscript writing. YJY, JCY, ISK, DHY, YH and JCY performed the experiments and data analysis. JCY, JHL, ISK, DHY and YH performed data analyses and made critical comments on the entire process of the study. All of the authors read and approved the final version of manuscript. JCY and HJT confirm the authenticity of the raw data.

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RESEARCH ARTICLE

Evaluation of Acute Phase Response in Blood and Milk Samples of Healthy Holstein Cattle in the Postpartum Period

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Abstract

Normally, birth triggers an acute phase response (APR). In particular, interleukins and proinflammatory cytokines released from activated leukocytes at the site of tissue damage stimulate APR. In the liver, these cytokines also stimulate acute phase proteins (APPs). APPs are one of the options used in monitoring the health status of animals. This study was evaluated in 12 healthy Holstein cattle, 3-6 years old, who calved at least once. Blood and milk samples were collected from the animals' immediately after calving (0th h) and on the 7th, 14th, and 21st days postpartum. Haptoglobin (Hp), serum amyloid A (SAA), ceruloplasmin (Cp), milk amyloid A (MAA), albumin, total protein (TP) and globulin levels were determined in blood serum and milk serum samples. In the findings; when the measurements of blood and milk Hp, Cp and SAA and TP values were compared; there was a statistically significant difference (P<0.05) between 0th h and 21st day measurements. In the correlation findings, a relationship was found between APP's in blood and milk. In conclusion, this study revealed that APR develops after calving and in the postpartum 21-day period, and the developing APR can clearly be seen in blood and milk. In addition, it was shown that the APR can be traced from milk in dairy cattle, in the present study.

Keywords: *Acute phase response, Acute phase protein, Ceruloplasmin, Dairy cattle, Haptoglobin, Milk amyloid A, Postpartum period, Serum amyloid A*

Postpartum Dönemde Sağlıklı Holstein Sığırların Kan ve Süt Örneklerinde Akut Faz Yanıtın Değerlendirilmesi

Öz

Normalde doğum bir akut faz yanıtını (AFY) tetikler. Özellikle doku hasarı bölgesinde aktive edilmiş lökositlerden salınan interlökinler ve proinflamatuar sitokinler AFY'yi uyarır. Karaciğerde bu sitokinler ayrıca akut faz proteinlerini (AFP) de uyarır. AFP'ler, hayvanların sağlık durumunun izlenmesinde kullanılan seçeneklerden biridir. Bu çalışma en az bir kez doğum yapmış, 3-6 yaşlı, 12 adet sağlıklı holstein ırkı sığırda değerlendirildi. Hayvanlardan doğumdan hemen sonra (0. saatte) ve doğum sonrası 7, 14, ve 21. günlerde kan ve süt örnekleri alındı. Elde edilen kan ve süt serum örneklerinde haptoglobin (Hp), serum amiloid A (SAA) seroplazmin (Cp), süt amiloid A (MAA), albümin, total protein (TP) ve globulin seviyeleri belirlendi. Bulgularda; Kan ve süt Hp, Cp ve SAA ve TP değerlerinin ölçümleri karşılaştırıldığında; 0. saat ile 21. gündeki ölçümler arasında istatistiksel olarak anlamlı fark (P<0.05) vardı. Korelasyon bulgularında ise kan ve sütteki AFP'leri arasında ilişki bulunmuştur. Sonuç olarak bu çalışma ile doğum sonrasında ve postpartum 21 günlük periyotta AFY'ın geliştiği ve gelişen AFY'ın kanda ve sütte açık bir şekil görüldüğü ortaya konmuştur. Ayrıca bu çalışma sütçü sığırların sütlerinden de AFY'ın takip edilebileceğini göstermiştir.

Anahtar sözcükler: *Akut faz yanıt, Akut faz proteini, Doğum sonrası dönem, Haptoglobin, Seroplazmin, Serum amiloid A, Süt amiloid A, Süt sığırı*

INTRODUCTION

The transition period in dairy cattle is known as the period from 3 weeks before calving to 3 weeks after calving. The first 3 weeks of this period before calving is the prepartum period, and the first 3 weeks after calving is the postpartum period [1,2]. There are many factors that predispose dairy cattle to infections and metabolic diseases in the transition

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period. Among these factors; decreased feed consumption, negative energy balance due to high energy loss, lipolysis, weight loss in early lactation, hypocalcaemia following calving and suppression of the immune system that begins 1-2 weeks before calving and continues for 2-3 weeks after calving are among the important metabolic and immune causes. Bacterial contamination of the uterus, which continues for 2-3 weeks after delivery, is among the causes of microbial origin [2-4].

Acute phase response (APR) is a reaction that develops with the disruption of homeostasis in an organism. In particular, interleukins and proinflammatory cytokines released from activated leukocytes at the site of tissue damage stimulate APR. In the liver, these cytokines also stimulate the production of glycoproteins known as acute phase protein (APP) [5,6]. APP differs significantly between species. In cattle, haptoglobin (Hp) and serum amyloid A (SAA) are considered important acute phase proteins. Ceruloplasmin (Cp) is also one of the acute phase proteins considered to be of medium or low importance in cattle [7]. In ruminants, APPs are used to detect inflammation early and definitively [6-9]. In cattle, APPs are widely used in monitoring therapeutic efficacy in diseases [7] and as predictors of retained placenta or metritis [9] and as markers of mastitis [9,10].

Since APPs are one of the options used in monitoring the health status of animals, interest in this field has increased recently [10,11]. For this reason; in this study, it was aimed to evaluate APR in blood and milk samples of healthy Holstein cattle in the postpartum period.

Material and Methods

Ethical Statement

This study was approved by the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (MAKUHAD-YEK) (Approval no: 20/05/2021-89/774).

Study Design

In this study, 12 healthy Holstein cattle, 3-6 years old, who calved at least once, were evaluated. Blood (from vena jugularis) and milk samples were taken from the animals immediately after calving (0^{th} h) and on the 7^{th}, 14^{th}, and 21^{st} days of postpartum. Blood samples were centrifuged at 3000 rpm for 15 min and serum was obtained. Due to the accumulation of milk fat in the milk samples, the samples were placed in the centrifuge upside down, centrifuged at 3000 rpm for 20 min and stored at -25°C until analysis. In the obtained blood and milk serum samples; Hp, SAA, milk amyloid A (MAA) (Tridelta Development Ltd, Ireland) and Cp were determined by the colorimetric method based on the p-phenylenediamine oxidase activity described by Colombo and Richterich [12]. Albumin and total protein (TP) were determined with a commercial test kit (Biolabo, France) (Epoch, Biotek, USA). The globulin value was determined by subtraction of the albumin from the total protein according to Doumas et al.[13].

Statistical Analysis

The findings were evaluated using the IBM SPSS 22.0 for Windows package program. Shapiro-Wilk test was used to determine whether the data were normally distributed. As a result of the Shapiro-Wilk test, it was seen that all data were normally distributed. Due to the normal distribution of the data, repeated measurement comparisons within the group were made using one-way repeated measure ANOVA test and Benferoni corrected multiple comparison tests were used. Pearson Correlation analysis was used to determine the correlation between variables.

Results

The results and statistical evaluations of the Hp, Cp and SAA values in blood serum and milk serum performed at the 0^{th} h and on the 7^{th}, 14^{th} and 21^{st} days are given in *Table 1*. When the measurements of blood and milk Hp, Cp and SAA (MAA in milk) values at 0^{th} h and 7^{th}, 14^{th} and 21^{st} days were compared; a statistically significant differences ($P<0.05$) were determined between the measurements of both blood Hp and SAA and milk Hp and MAA values at the 0^{th} h and on the 14^{th} and 21^{st} days. On the other hand, statistically significant differences ($P<0.05$) were observed between the measurements of both blood and milk Cp values only at the 0^{th} h and the 21^{st} day. In addition, a statistically significant differences ($P<0.05$) were detected between the 7^{th} day and the 14^{th} and 21^{st} days of the milk Hp values. Furthermore, statistically significant differences ($P<0.05$) were found between the measurements of milk Cp and MAA values on the 7^{th}, 14^{th} and 21^{st} days. It was also revealed that there was a statistically significant difference ($P<0.05$) between the measurements of milk TP values at the 0^{th} h and the 21^{st} day. The correlation analysis findings of blood and milk Hp, Cp, SAA, MAA, albumin, TP and globulin values are given in *Table 2*. Hp, Cp, SAA, MAA, albumin, TP, globulin levels in blood and milk at the 0^{th} h and on the 7^{th}, 14^{th} and 21^{st} days are given as *Fig. 1-A,B,C,D,E,F*.

Discussion

In high milk yielding cows, the stress of birth and the onset of milk production after calving cause a large metabolic load and stress on the animals and adversely affect the metabolism [4]. Normally, birth triggers an acute phase response. Jawor et al.[14] reported that APR increased after calving. In parallel with this information, in our study, it is seen that APPs in blood and milk were higher and statistically significant ($P<0.05$) after calving (0^{th} h) compared to 7^{th}, 14^{th} and 21^{st} days *(Table 1)*. These findings demonstrated once again that the acute phase response clearly developed after calving.

Table 1. *Evaluation of acute phase response in blood and milk immediately after calving (0th h) and on days 7, 14 and 21*

Parameters	Measurement Time			
	After Calving (0th Day) X±Sd	7th Day X±Sd	14th Day X±Sd	21th Day X±Sd
Hp (Blood) (mg/L or µg/mL)	167.83±59.27^{a}	117.08±44.59^{ab}	73.33±28.35^{b}	79.91±30.68^{b}
Hp (Milk) (mg/L or µg/mL)	16.4±02.61^{a}	12.36±2.71^{b}	6.45±2.40^{c}	5.68±2.12^{c}
SAA (mg/L)	47.13±11.53^{a}	36.77±7.09^{ab}	33.43±4.39^{b}	28.66±5.87^{b}
MAA (mg/L)	13.03±4.27^{a}	9.73±2.61^{a}	5.67±1.13^{b}	3.70±0.89^{c}
Cp (Blood) (mg/dL)	19.16±2.81^{a}	16.94±3.26^{ab}	17.14±4.94^{ab}	15.42±2.66^{b}
Cp (Milk) (mg/dL)	6.30±1.93^{abc}	6.11±2.49^{b}	3.46±1.87^{cd}	2.75±0.89^{d}
Albumin (Blood) (g/dL)	3.56±0.23^{a}	3.63±0.49^{a}	3.57±0.34^{a}	3.73±0.45^{a}
Albumin (Milk) (g/dL)	2.12±0.37^{a}	2.40±0.73^{a}	2.47±0.44^{a}	2.63±0.47^{a}
TP (Blood) (g/dL)	6.94±0.39^{a}	7.07±0.34^{a}	7.21±0.55^{a}	7.18±0.62^{a}
TP (Milk) (g/dL)	3.04±0.46^{a}	3.34±0.69^{ab}	3.41±0.44^{ab}	3.59±0.23^{b}
Globulin (Blood) (g/dL)	3.37±0.52^{a}	3.44±0.48^{a}	3.64±0.68^{a}	3.45±0.77^{a}
Globulin (Milk) (g/dL)	0.92±0.39^{a}	0.93±0.36^{a}	0.94±0.40^{a}	0.9±50.47^{a}

^{a,b,c,d} Values within a columns with different superscripts differ significantly at P<0.05
***Hp:** Haptoglobin; **SAA:** Serum amyloid A; **Cp:** Ceruloplasmin; **MAA:** Milk amyloid A; **TP:** Total protein.*

Table 2. *Correlation findings between Hp, Cp, SAA, MAA, albumin, TP and globulin values in blood and milk*

Variables		Hp (Blood)	SAA (Blood)	Cp (Blood)	Albumin (Blood)	TP (Blood)	Globulin (Blood)	Hp (Milk)	MAA (Milk)	Cp (Milk)	Albumin (Milk)	TP (Milk)	Globulin (Milk)
Hp (Blood)	Pearson Correlation	1	.468**	.266	-.071	-.168	-.088	.615**	.702**	.328*	-.332*	-.144	.250
	Sig. (2-tailed)		.001	.068	.632	.253	.551	.000	.000	.023	.021	.328	.086
SAA (Blood)	Pearson Correlation	.468**	1	.355*	-.238	-.124	.052	.558**	.707**	.281	-.284	-.186	.135
	Sig. (2-tailed)	.001		.013	.104	.401	.725	.000	.000	.053	.050	.207	.361
Cp (Blood)	Pearson Correlation	.266	.355*	1	.105	-.404**	-.385**	.266	.314*	.192	-.433**	-.175	.344*
	Sig. (2-tailed)	.068	.013		.476	.004	.007	.068	.030	.191	.002	.235	.017
Albumin (Blood)	Pearson Correlation	-.071	-.238	.105	1	.023	-.614**	-.023	-.059	-.187	.137	.127	-.017
	Sig. (2-tailed)	.632	.104	.476		.879	.000	.875	.690	.204	.353	.389	.908
TP (Blood)	Pearson Correlation	-.168	-.124	-.404**	.023	1	.776**	-.237	-.224	-.009	.204	.096	-.145
	Sig. (2-tailed)	.253	.401	.004	.879		.000	.105	.126	.953	.164	.517	.326
Globulin (Blood)	Pearson Correlation	-.088	.052	-.385**	-.614**	.776**	1	-.172	-.139	.111	.075	-.005	-.104
	Sig. (2-tailed)	.551	.725	.007	.000	.000		.242	.345	.453	.614	.976	.484
Hp (Milk)	Pearson Correlation	.615**	.558**	.266	-.023	-.237	-.172	1	.772**	.598**	-.239	-.319*	-.095
	Sig. (2-tailed)	.000	.000	.068	.875	.105	.242		.000	.000	.101	.027	.521
MAA (Milk)	Pearson Correlation	.702**	.707**	.314*	-.059	-.224	-.139	.772**	1	.462**	-.228	-.097	.174
	Sig. (2-tailed)	.000	.000	.030	.690	.126	.345	.000		.001	.120	.511	.236
Cp (Milk)	Pearson Correlation	.328*	.281	.192	-.187	-.009	.111	.598**	.462**	1	-.216	-.316*	-.121
	Sig. (2-tailed)	.023	.053	.191	.204	.953	.453	.000	.001		.141	.029	.412
Albumin (Milk)	Pearson Correlation	-.332*	-.284	-.433**	.137	.204	.075	-.239	-.228	-.216	1	.702**	-.412**
	Sig. (2-tailed)	.021	.050	.002	.353	.164	.614	.101	.120	.141		.000	.004
TP (Milk)	Pearson Correlation	-.144	-.186	-.175	.127	.096	-.005	-.319*	-.097	-.316*	.702**	1	.359*
	Sig. (2-tailed)	.328	.207	.235	.389	.517	.976	.027	.511	.029	.000		.012
Globulin (Milk)	Pearson Correlation	.250	.135	.344*	-.017	-.145	-.104	-.095	.174	-.121	-.412**	.359*	1
	Sig. (2-tailed)	.086	.361	.017	.908	.326	.484	.521	.236	.412	.004	.012	

** Statistically significant correlation is found;. ** Statistically significant correlation is found*
***Hp:** Haptoglobin; **SAA:** Serum amyloid A; **Cp:** Ceruloplasmin; **MAA:** Milk amyloid A; **TP:** Total protein.*

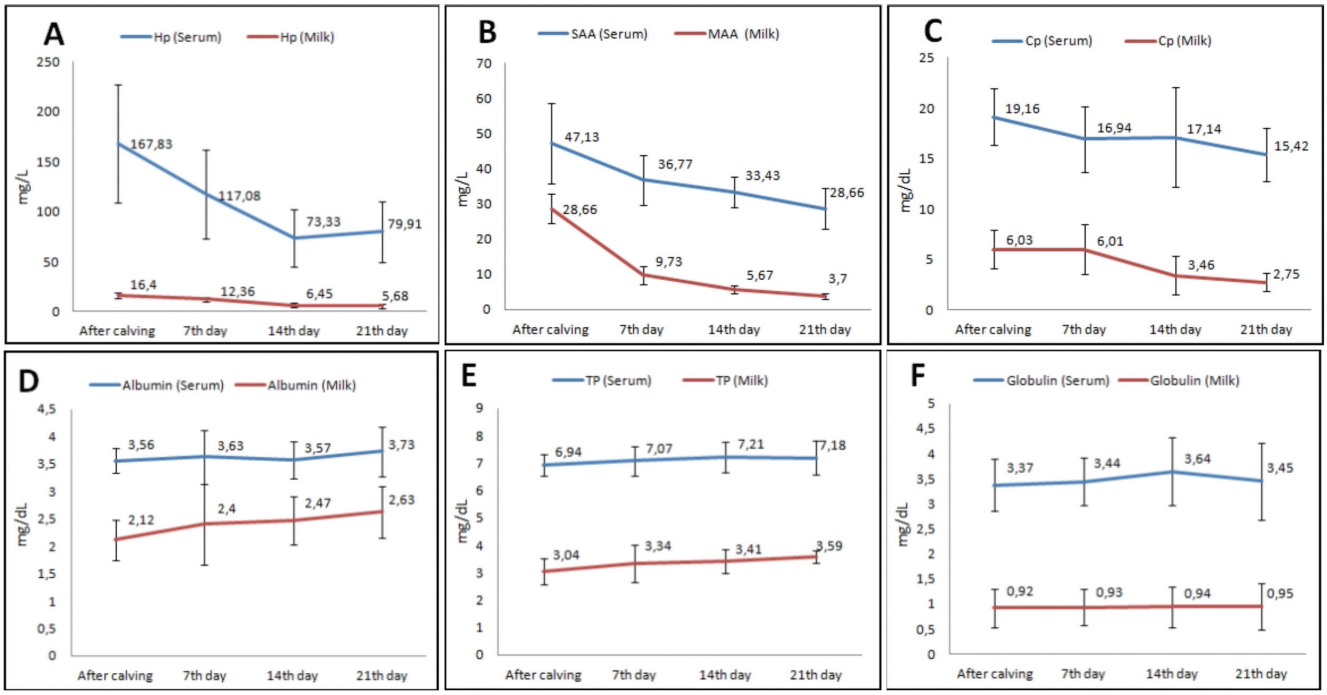

Fig 1. Hp (**A**), Cp (**B**), SAA and MAA (**C**), albumin (**D**), TP (**E**), globulin (**F**) levels in blood and milk at the 0th h and on the 7th, 14th and 21st days. **Hp:** Haptoglobin; **SAA:** Serum amyloid A; **Cp:** Ceruloplasmin; **MAA:** Milk amyloid A; **TP:** Total protein

Bertoni et al.[11] and Humblet et al.[10] found that serum Hp levels were high in dairy cows, especially in the first 7-day period after calving. Trevisi et al.[15] determined that serum Hp levels increased in dairy cows within the first 10 days after calving, and decreased after the 10th day. Nightingale et al.[16] recorded that serum Hp levels increased in 240 dairy cows between 2 and 8 days postpartum. Uchida et al.[17] and Chan et al.[18] showed that serum Hp concentration increased after calving in healthy cows and the highest Hp concentration occurred 2-3 days after calving. In addition, Bossaert et al.[19] found that there was an increase in serum Hp levels especially in the first 3 days after calving and gradually decreased after the first week. In our current study, it was observed that the Hp value in milk and blood was at the highest level after calving, and the Hp value continued to decrease on the 7th and 14th days. A slight increase in serum Hp was observed on day 21. On the other hand, the decrease in milk Hp value continued on the 21st day. It is extremely interesting that while the Hp value in the blood increases on the 21st day, the decrease in milk continues *(Table 1, Fig. 1-A)*. How this develops should be investigated in future studies.

Another important positive APP in cattle is SAA [20] Uchida et al.[17] reported that the increased SAA concentration after calving reached its highest level in 2-3 days. But, Chan et al.[18] determined that SAA levels in healthy cows decreased to normal levels within the first week after calving. On the other hand, in our study, it was observed that SAA concentration was at the highest level after calving, and decreased dramatically on the 7th, 14th and 21st days *(Table 1, Fig. 1-B)*.

Cp is one of the parameters used in the evaluation of animal health and welfare [21]. Studies in cattle have revealed that Cp can be used for diagnostic purposes in various diseases and conditions [22]. Trevisi et al.[15] reported that the Cp value increased after calving and in the first week after calving in high milk yielding cows with low and high LFI index. Furthermore, Bossaert et al.[19] determined that the level of Cp increased after calving and remained high for 42 days after calving. In supporting this, Hussein et al.[23] found that there was a significant increase in serum Cp levels in the first week after calving in cows. In our study, it was observed that the serum Cp value increased after calving and decreased to the normal limits within a week. It was also observed that the milk Cp value increased after calving, and decreased dramatically on the 7th, 14th and 21st days *(Table 1, Fig. 1-C)*.

Bayyit and Merhan [24] determined serum Hp concentration as 0.176±0.007 g/L, Cp concentration as 15.68±0.83 mg/dL, and serum albumin concentration as 3.15±0.08 g/dL in cows with normal calving. In parallel with this information, in our study, Hp concentration was determined as 0.167±0.059 g/L, Cp concentration as 19.16±2.81 mg/dL, and serum albumin concentration as 3.56±0.23g/dL after calving in dairy cows *(Table 1)*.

Chan et al.[18] reported serum Hp and SAA concentrations as 630±302 µg/mL and 66±15 µg/mL on days 0-3, 380±250 µg/mL and 48±20 µg/mL on days 4-7, 310±197 µg/mL and 42±18 µg/mL on day 14, and 86±73 µg/mL and 37±19 µg/mL on day 21 respectively in healthy dairy cattle. In our study; serum Hp and SAA concentrations were determined

as 167.83±59.27 µg/mL and 47.13±11.53 µg/mL after calving, 117.08±44.59 µg/mL and 36.77±7.09 µg/mL on day 7, 73.33±28.35 µg/mL and 33.43±4.39 µg/mL on day 14, 79.91±30.68 µg/mL and 28.66±5.87 µg/mL on day 21 respectively *(Table 1)*.

The synthesis of plasma proteins is primarily made in the liver. Especially in some diseases, analysis of TP concentrations and percentage of protein fractions is important [7,24] Albumin, one of the plasma proteins, is the most osmotically active serum protein and is involved in the transport of many substances. Globulins are a heterogeneous group of proteins. They include antibodies and other inflammatory molecules, haemostatic and fibrinolytic proteins, lipid transporters, vitamins, and hormones. Albumin and globulin concentrations shift during physiological or pathological conditions [25]. Total serum globulin concentrations are component of the organism's defence system. Therefore, it has the property of an indicator of humoral immune status or response. It has been reported that there is decrease in serum globulin concentration in cattle in the peripartum period [26]. After calving, plasma volume increases and albumin synthesis decreases. Therefore, albumin remains at a low level for 2 weeks after calving, and the clinical use of albumin as APP decreases during lactation [27]. Negative APPs are important for albumin in cattle [7]. The amount of APPs produced by each species during its inflammatory response is unique. However, it is speculated that the serum albumin level decreases between 10-30% in all mammalian species [28,29]. In parallel with this information, in our study; although there was no statistical difference, it was observed that the albumin value in blood and milk was at the lowest level at the 0th h and increased on the 7th, 14th and 21st days. It was observed that serum globulin and TP values were low at 0th h, increased on 7th and 14th days, and decreased on 21st day. It was observed that milk albumin, globulin and TP values were low at the 0th h and continued to increase on the 7th, 14th and 21st days. In the measurements taken on the 21st day; while the serum TP value decreased, the milk TP value continued to increase. In addition, there was a statistically significant difference (P<0.05) between 0th h and 21st day in the measurements of milk TP values. It is extremely interesting that the TP value decreases in blood serum and increases in milk, and it should be further investigated *(Table 1, Fig. 1-D,E,F)*.

Hp, SAA, Cp and c-reactive protein (CRP) are among the APPs identified in milk. These APPs have been reported to have the potential to be biomarkers in cases of mastitis [30,31]. The main isoforms of SAA are SAA1, SAA2 and SAA3. SAA1 and SAA2 are produced in the liver. SAA3 is produced in extra hepatic regions. It is more commonly known as mammary-associated amyloid A (M-SAA3), especially since it is predominantly found in milk [32,33]. Cp, which is mainly synthesized from hepatocytes, is also synthesized in the mammary gland and increases in case of infection and tissue damage [34]. In normal milk, the Hp value is 0.32 µg/mL [35], MAA 3.58 mg/L [36] and Cp levels 0.5 mg/dL [30]. In parallel with this information, in our study, milk Hp, milk Cp and MAA concentrations were determined as 16.40 µg/mL, 13.03 µg/mL, and 6.30 mg/dL at 0th h, 12.36 µg/mL, 9.73 µg/mL, and 6.11 mg/dL on 7th day, 6.45 µg/mL, 5.67 µg/mL, and 3.46 mg/dL on 14th day, 5.68 µg/mL, 3.70 µg/mL, and 2.75 mg/dL on 21th day respectively. In addition, there was a statistically significant difference (P<0.05) between 0th h and 21st days in the measurements of milk Hp, milk Cp and MAA values *(Table 1, Fig. 1-A,B,C)*. These findings reveal that the acute phase response after calving clearly occurs in milk as well. While SAA is produced in the liver, MAA is produced in non-hepatic regions (mammary tissue) [32,33]. The increase in MAA may be related to the mammary specificity of MAA and postpartum mammary oedema and inflammation. An increase in the SAA (synthesized from the liver) may also be associated with calving. Since there is a strong and statistically significant positive correlation between blood SAA and MAA, it should be investigated in more detail to determine the acute phase response developing in the mammary tissue.

Among the APPs, Hp and SAA increase in parallel in serum and milk [37,38]. Dalanezi et al.[39] reported that there is a positive correlation between milk Hp and SAA values in their study. Similarly, in our study, a moderate and statistically significant positive correlation was found between blood Hp value and SAA value (r=0.468; P=0.001). A strong and statistically significant positive correlation was found between milk Hp value and milk MAA value (r=0.772; P<0.001) *(Table 2)*. Increased activity of Cp in blood and milk has been reported in cases of mastitis in cattle [22,34,40]. Bertoni et al.[11] determined a weak, but significant and linear relationship between serum Cp and serum Hp. Parallel to this information, in our study, a weak positive correlation was found between blood Hp value and blood Cp value (r=0.266; P=0.068). A moderate and statistically significant positive correlation was found between milk Hp value and milk Cp value (r=0.598; P<0.001) *(Table 2)*. Gürler et al.[41] found that there was a significant positive correlation between milk TP and albumin values in subclinical mastitis cases in buffaloes. In our study, a strong and statistically significant positive correlation was found between milk, albumin value and milk TP value (r=0.702; P<0.001) *(Table 2)*. According to these results, it was clearly demonstrated that negative and positive acute phase proteins increase in parallel in serum and milk.

The high amount of MAA in colostrum binds to Gram-negative bacteria with high affinity, as well as initiating or increasing mucin secretion by stimulating neonatal intestinal cells to secrete mucus, which will reduce bacterial colonization and increase resistance to disease in newborn calves [32,38]. Similarly, the antioxidant activity of high Hp in colostrum will be beneficial in countering

bacterial invasion of the intestine [38]. Since Cp in colostrum is an extracellular antioxidant, it will be necessary for the anti-oxidative defence of the organism [42,43]. The results obtained in this study once again demonstrate the importance of APPs in a passive transfer of colostrum-induced immunity in newborns calves.

In conclusion, this study revealed that APR develops after calving and in the postpartum 21-day period, and the developing APR can clearly be seen in blood and milk. In addition, it was shown that the APR can be traced from milk in dairy cattle, in the present study.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (K. Varol) on reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Design of the study: VK. EEH. MO. BK. Data collection: VK. Data analysis; EEH. MO. BK. Article writing VK. EEH. MO. BK.

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RESEARCH ARTICLE

Protective Effect of Melatonin and Mycophenolate Mofetil Against Nephrotoxicity Induced by Tacrolimus in Wistar Rats

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Abstract

Although Tacrolimus (TAC) is a potent and well-tolerated drug, it has some side effects. Melatonin and mycophenolate mofetil (MMF) have some protective properties against drug-induced damage. We aimed to evaluate TAC-induced nephrotoxicity and the protective effect of melatonin and MMF against this injury in rats. The animals were divided into five equal groups (n=6): Control group (untreated), group II TAC, group III as the TAC + melatonin, group IV as the TAC + MMF, and group V as the TAC + melatonin + MMF. TAC was applied orally, 2 mg/kg once daily. Melatonin and MMF were applied orally 10 mg/kg once and 40 mg/kg once daily, respectively. In the TAC group, kidney tissue malondialdehyde (MDA), total oxidative status (TOS), interleukin-1, and tumor necrosis factor-alpha levels were higher, and catalase and total antioxidant status (TAS) levels were lower. Severe histopathologic changes such as glomerular congestion, intertubular hemorrhage, hyaline formation, degenerative-necrotic tubules epithelium, and mononuclear cell infiltration were seen in the TAC group. There was a clear improvement in the groups in which melatonin and MMF were used together with TAC. It was shown that TAC causes nephrotoxicity through oxidative stress. Melatonin and MMF together or separately protect the kidney against oxidative stress damage caused by TAC.

Keywords: *Tacrolimus, Melatonin, Mycophenolate mofetil, Nephrotoxicity, Oxidative stress*

Melatonin ve Mikofenolat Mofetilin Wistar Sıçanlarında Takrolimus Tarafından İndüklenen Nefrotoksisiteye Karşı Koruyucu Etkisi

Öz

Takrolimus güçlü ve iyi tolere edilen bir ilaç olmasına rağmen; bazı yan etkileri vardır. Melatonin ve mikofenolat mofetil (MMF), ilaca bağlı hasara karşı bazı koruyucu özelliklere sahiptir. Biz bu çalışmada sıçanlarda Takrolimus kaynaklı nefrotoksisiteye karşı melatonin ve MMF'nin koruyucu etkisini değerlendirmeyi amaçladık. Hayvanlar beş eşit gruba ayrıldı (n=6): Kontrol grubu (tedavi edilmemiş), grup II Takrolimus, grup III Takrolimus +melatonin, grup IV Takrolimus + MMF ve grup V Takrolimus + melatonin + MMF. Takrolimus, günde bir kez 2 mg/kg oral olarak uygulandı. Melatonin ve MMF, sırasıyla günde bir kez 10 mg/kg ve 40 mg/kg oral olarak uygulandı. Takrolimus grubunda böbrek dokusu malondialdehit (MDA), toplam oksidatif stress (TOS), interlökin-1 ve tümör nekroz faktör-alfa düzeyleri daha yüksek, katalaz ve toplam antioksidatif stress (TAS) düzeyleri daha düşüktü. Takrolimus grubunda glomerüler konjesyon, intertübüler kanama, hiyalin oluşumu, dejeneratif-nekrotik tübül epiteli ve mononükleer hücre infiltrasyonu gibi ciddi histopatolojik değişiklikler görüldü. Takrolimus ile birlikte melatonin ve MMF kullanılan gruplarda belirgin bir iyileşme oldu. Takrolimusun oksidatif stres yoluyla nefrotoksisiteye neden olduğu gösterilmiştir. Melatonin ve MMF birlikte veya ayrı ayrı böbreği Takrolimusun neden olduğu oksidatif stres hasarına karşı korumaktadır.

Anahtar sözcükler: *Tacrolimus, Melatonin, Mycophenolate mofetil, Nefrotoksisite, Oksidatif stres*

INTRODUCTION

The prevalence of chronic renal failure increases daily, leading to a cause of health problems worldwide. Kidney transplantation (KT) is the most effective and advanced treatment option for these patients [1]. However, graft rejection is still a major problem and sometimes can result in graft loss. During the rejection, the recipient

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immune system cells attack and destroy the graft [2]. Immunosuppressive agents such as tacrolimus (TAC), cyclosporine, mycophenolate mofetil (MMF) are used alone or combined to prevent rejection after transplantation [3]. Thanks to these immunosuppressive agents, success rates in the field of organ transplantation have increased significantly. TAC is an important immunosuppressive agent and greatly improves grafts and patients' survival rates after solid-organ transplantation [4]. Although TAC has strong potency and is well tolerated, serious side effects such as nephrotoxicity, hepatotoxicity, neurotoxicity, glucose intolerance, gastrointestinal toxicity, posttransplant lymphoproliferative disease have been reported in previous studies [5]. Among them, nephrotoxicity and hepatotoxicity continue to be serious problems [6,7]. A previous study found that TAC caused glomerular damage by disrupting the balance in the oxidant-antioxidant defense system [8,9].

Melatonin, a protein hormone, plays a role in many hormone-related reactions endocrine functions such as sleep, sexual development and reproduction, mood, immune functions, and aging [10,11]. Although melatonin has been found in different parts of the body, such as the gastrointestinal tract, retina, bile, skin, bone marrow, and some leucocytes, it is mainly concentrated in several brain areas [12]. Melatonin not only acts as an antioxidant by stimulating antioxidant enzymes but also has free radical scavenging properties. It also has antiapoptotic and anti-inflammatory effects [13].

Mycophenolate mofetil (MMF) inhibits inosine monophosphate dehydrogenase and, consequently, blocks T and B's proliferation and clonal expansion [14]. MMF is one of the most common immunosuppressive agents used in combination therapy, mostly with TAC [15]. During the last years, it has been proven that MMF has a cytoprotective effect by reducing the production of free radicals [16-18].

This study's main objective is to investigate the role of TAC in kidney injury and the protective effect of melatonin and MMF against this damage. Specifically, the TAC-induced nephrotoxicity rat model was constructed, and each kidney sample was divided, and histopathological changes were evaluated by using light microscopy. Furthermore, the biochemical analyses were performed for measuring superoxide dismutase (SOD), catalase (CAT), malonyl dialdehyde (MDA) total antioxidant status (TAS), total oxidative status (TOS), interleukin-1 (IL-1), and tumor necrosis factor (TNF) alpha, contents in kidney tissues and blood urea nitrogen (BUN), creatinine, albumin, total protein, and uric acid levels in serum.

Material and Methods

Animals

For this experimental animal study, approval was provided from Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (decision no.65202830-050.04.04-310). All the experimental procedures were performed following the institution's ethical standards at which the studies were conducted. We used 30, 220-300 g, 10-12-week-old male Wistar rats purchased from the Sivas Cumhuriyet University Experimental Animal Production Application and Research Center (Sivas, Türkiye). Before starting experimental work, all animals were preserved for 14 days under the same laboratory conditions of a 12 h day -12 h night period, temperature (22±3°C), relative humidity (55±5%) and fed a standard diet (DSA Poultry, Kırıkkale, Türkiye).

Experimental Design

The subjects were classified into five groups, 6 in each group, for 14-day regimens of control or experimental groups. Control group: animals were untreated. The treatments in the experimental groups were as follows: TAC group: 2 mg/kg once daily TAC (Prograf®; Astellas Phama Inc. Tokyo, Japan) was given by gavage for 14 days starting on day 1; TAC + melatonin group: TAC as above plus 10 mg/kg/day melatonin (Bio Basic, Canada) was given by gavage for 14 days; TAC + MMF group: TAC as above plus 40 mg/kg once daily MMF (Cellcept® 250 mg tablets; Roche) were administered by gavage for 14 days starting on day 1; and the TAC + melatonin + MMF group were given TAC as above plus melatonin and MMF as described previously. The choice of medication, dosage, and administration was done under the guidance of previous studies [18-20]. At the end of the study, prior to euthanasia, blood samples were obtained by cardiac puncture, then all the animals were sacrificed using anesthesia overdose ketamine HCl (Ketalar®; Eczacibasi Warner-Lambert, Levent, Istanbul, Türkiye), and tissue samples were obtained. Blood was centrifuged at 2.058xg at 4°C for 15 min to obtain serum, which then was stored at -80°C. Each kidney tissue was prepared for histopathologic evaluation by light microscopy, and biochemical analyses were performed for measuring SOD, CAT, MDA, TAS, TOS, IL-1, and TNF alpha contents.

A priori power analysis was conducted using G-Power Version 3.1.9.7 to test the difference between five independent groups using a two-tailed test, a medium-large effect size (f=0.70), and an alpha of 0.05. Results showed that a total sample of 30 participants with five equal-sized groups of 6 rats was required to achieve a power of 0.80.

Histopathologic Evaluations

Samples were taken from the kidneys of the animals in all groups and fixed in 10% formalin. The specimens were processed in an auto-technician device, later embedded in paraffin blocks. The blocks were cut at 5 µm thickness, deparaffinized, rehydrated using standard techniques, and sections were stained with Haemotoxylen-Eosin (H&E) stain using standard protocols for analysis by light microscopy (Eclipse E 600; Nikon, Tokyo, Japan). The histopathological

scoring method was modified and used in histopathological examinations, and changes in kidney tissues were graded [21,22]. The kidney's main histopathological lesions that were considered include glomerular congestion, intertubular hemorrhage, hyaline formation, degenerative-necrotic tubules epithelium, mononuclear cell infiltration. The histopathological harm's degree was evaluated for each kidney group *(Table 1, Table 2)* is classified according to the severity.

Biochemical Analyses

- Preparation of Kidney Tissue Homogenates

The tissue samples were mixed with a cold phosphate-buffered saline solution (PBS, pH: 7.4) and homogenized using a mechanical homogenizer (Analytic Jena speed mill plus, Jena, Germany). The homogenates were centrifuged at 2.957xg for 10 min at a temperature of 4°C. Then, the supernatants were received and preserved in a glacial environment until evaluation. Bradford protein assay kit (Cat no:39222.03, Serva, Heidelberg, Germany) was used to determine the amount of protein in samples [23].

- Measurement of SOD, CAT, MDA, IL-1, and TNF alpha

SOD, CAT, MDA, IL-1, and TNF-alpha levels from kidney supernatants were measured using rat ELISA commercial kits (Sunred Biological Technology, Shanghai, China). Standard and tissue samples were added to the plate and incubated at 37°C for 60 min. Plates with standard and tissue samples added were incubated at 37°C for 60 min. Following the washing process, the staining solution was added and incubated in the same conditions (37°C for 60 min). Finally, a stop solution was added and read at 450 nm. The coefficients of variation between and within plates were found below 10%. During these measurements, the manufacturer's guidelines were followed.

Table 1. *Histopathological scoring for renal cortex damage among groups*

Pathological State	Score	Definition
Glomerular congestion	0	No
	1	1 of 10 glomeruli
	2	1 to 3 out of 10 glomeruli
	3	4 or more of 10 glomeruli
Intertubular hemorrhage	0	No
	1	2 or less of 10 tubules
	2	3 to 5 out of 10 tubules
	3	5 or more of 10 tubules
Hyaline formations	0	No
	1	2 or less of 10 tubules
	2	3 to 5 out of 10 tubules
	3	5 or more of 10 tubules
Degenerative-necrotic tubules epithelium	0	No
	1	10 to 20% of the tubules
	2	20 to 50% of 10 tubules
	3	50% or more of 10 tubules

The degree of kidney damage was rated according to a grading system described as follows; 0: None, 1: mild, 2: moderate, 3: severe

Table 2. *Histopathological scoring for renal medulla damage among groups*

Pathological State	Score	Definition
Intertubuler hemorrhage	0	No
	1	2 or less of 10 tubules
	2	3 to 5 out of 10 tubules
	3	5 or more of 10 tubules
Mononuclear cell infiltration	0	No
	1	2 or less of 10 tubules in the intertubular area
	2	Between 3 and 5 of 10 tubules in the intertubular area
	3	5 or more of 10 tubules in the intertubular area

The degree of kidney damage was rated according to a grading system described as follows; 0: None, 1: mild, 2: moderate, 3: severe

- Measurement of TAS and TOS

The rate of TAS in the kidney supernatants was determined according to the novel assay method. This method is based on finding the reaction rate during the free radical reaction by measuring the absorption of colored dianisidyl radicals [24]. The results were expressed in micromolar Trolox equivalents per milligram tissue protein (µmol Trolox Eq/mg protein).

The amount of TOS in the kidney tissue was measured using the automated assay method of Erel [25]. This method is based on quantifying TOS levels by measuring tissue ferric ion concentration using xylenol orange. The ferrous ion is oxidized to ferric ion when enough oxidants are in the medium. Calibration of the assay results was performed using hydrogen peroxide [25]. The assay data were expressed with micromolar hydrogen peroxide equivalents per milligram tissue protein (µmol H_2O_2 Eq/mg protein).

- Measurement of Serum Biochemical Parameters

Serum BUN, creatinine, albumin, total protein, and Uric acid levels were measured with the spectrophotometric method (Roche Cobas 8000, Germany, Mannheim).

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) version 20.0 was used for the analysis of the data obtained. One-way analysis of variance (ANOVA) was applied to compare the groups' laboratory parameters ($P<0.001$). The Kruskal-Wallis test was applied for histopathologic statistical evaluation to determine all groups' effects on each experimental parameter. $P<0.05$ was considered to indicate a statistically significant difference among groups.

Results

Effects of TAC on Oxidative Stress

We measured biochemical parameters such as SOD, CAT, MDA, TAS, and TOS in kidney tissues to evaluate oxidative stress-mediated nephrotoxicity. A comparison of laboratory parameters among groups is shown in *Table 3*. No statistically significant differences were found when comparing group I (control) to group II (TAC) in SOD levels. Increased SOD levels were determined in group V compared to group I and group II. The difference between group III and group IV was not statistically significant in terms of SOD. It was observed that CAT, which is an endogenous antioxidant, significantly decreased in the TAC group. Higher CAT activity was found in group III (TAC + melatonin), group IV (TAC + MMF), and group V (TAC + melatonin + MMF) compared to group II.

The MDA level was measured to evaluate the lipid peroxidation status. In the TAC group, kidney tissue MDA level was higher than group II, III, IV, and V ($P<0.05$). MDA level was significantly decreased in rats treated with combined drugs, especially in the group where TAC, melatonin, and MMF are applied together. Decreased TAS and increased TOS levels were found in group II concerning group I. Increased TAS and decreased TOS levels were detected in groups III, IV, and V compared to group II.

We examined IL-1 and TNF-alpha to determine whether melatonin and MMF could reduce inflammation caused by TAC. As shown in the results, the expressions of these different cytokines were elevated in the TAC-treated groups. Increased IL-1 and TNF-alpha levels were determined in group II compared to group I ($P<0.001$). Compared to group II, decreased IL-1 and TNF-alpha levels were determined in group IV and group V ($P<0.001$) *(Table 3)*. There was no significant difference between the groups in terms of the serum BUN, creatinine, albumin, total protein, and uric acid results *(Table 4)*.

Histopathologic Findings

Light microscope examination of group I showed normal renal cortex architecture. Group II (TAC) revealed significant renal cortex changes with severe glomerular congestion, intertubular hemorrhage, hyaline formation, degenerative-necrotic tubules epithelium, and mononuclear cell infiltration. These changes were found to be moderate in TAC + Melatonin and TAC + MMF groups. On the other hand, histopathological examination of group V (TAC + melatonin + MMF) had a near-normal appearance. Only milddegenerative-necrotic tubule epithelium was seen in group V. Histopathological examination findings of the renal medulla were similar to those of the renal cortex mentioned above. While histopathological findings of both renal cortex and renal medulla were severe in the TAC group, these findings

Table 3. *Comparison of kidney homogenates oxidative-antioxidative enzyme activity between groups*

Parameters	Control (n=6)	TAC (n=6)	TAC+Melatonin (n=6)	TAC+MMF (n=6)	TAC+Melatonin+MMF (n=6)	P
SOD/TP (U/mg protein)	11.56±4.86^a	9.65±1.04^a	8.22±1.56^b	8.28±1.66^b	15.08±1.38^c	P<0.001
CAT/TP (U/mg protein)	20.81±2.44^a	8.82±1.44^b	14.98±1.17^c	20.57±2.43^a	28.48±1.68^d	P<0.001
MDA (nmol/mg protein)	8.56±0.54^a	12.44±0.55^b	9.98±1.29^c	7.92±0.81^a	8.68±0.71^a	P<0.001
TAS (µmol Trolox Eq/mg protein)	0.68±0.03^a	0.48±0.03^b	0.48±0.05^b	0.55±0.03^c	0.55±0.02^c	P<0.001
TOS (µmol H_2O_2 Eq/mg protein)	2.93±0.59^a	7.92±1.76^b	3.10±0.44^a	3.93±0.51^a	3.00±0.23^a	P<0.001
IL-1/TP (pg/mg protein)	1113.47±91.12^a	1537.74±228.73^b	1261.66±78.22^a	1222.51±122.06^a	1154.61±104.51^a	P<0.001
TNF-alpha/TP (pg/mg rotein)	385.23±22.17^a	474.65±25.06^b	380.33±60.55^a	381.55±23.19^a	382.70±44.71^a	P<0.001

SOD: *Superoxide dismutase,* ***CAT:*** *Catalase,* ***MDA:*** *Malonyl dialdehyde,* ***TAS:*** *Total antioxidative status,* ***TOS:*** *Total oxidative status,* ***IL-1:*** *Interleukin-1,* ***TNF:*** *Tumor necrosis factor-alpha,* ***TAC:*** *Tacrolimus,* ***MMF:*** *Mycophenolate mofetil. Results were given as mean±standard deviation. Different upper superscripts indicate statistical differences among groups*

Table 4. *Comparison of laboratory parameters among groups*

Parameters	Control (n=6)	TAC (n=6)	TAC+Melatonin (n=6)	TAC+MMF (n=6)	TAC+Melatonin+MMF (n=6)	P
BUN	18.41±2.25^a	25.81±4.74^a	17.65±2.43^a	16.38±3.56^a	22.85±10.72^a	NS
Kreatinin	0.39±0.05^a	0.41±0.03^a	0.38±0.06^a	0.33±0.02^a	0.32±0.07^a	NS
Albumin	39.71±1.91^a	40.81±2.48^a	40.16±0.99^a	39.18±2.26^a	37.75±4.33^a	NS
T.Protein	68.23±2.06^a	68.46±3.57^a	66.61±1.53^a	59.15±6.36^b	58.50±5.56^b	P<0.001
Uric acid	1.06±0.37^a	1.40±0.35^a	1.13±0.36^a	0.88±0.24^a	0.96±0.19^a	NS

BUN: *Blood urea nitrogen,* ***ALT:*** *Alanine aminotransferase,* ***ALP:*** *Alkaline phosphatase,* ***TAC:*** *Tacrolimus,* ***MMF:*** *Mycophenolate mofetil,* ***NS:*** *Not significance. Results were given as mean±standard deviation. Different upper superscripts indicate statistical differences among groups*

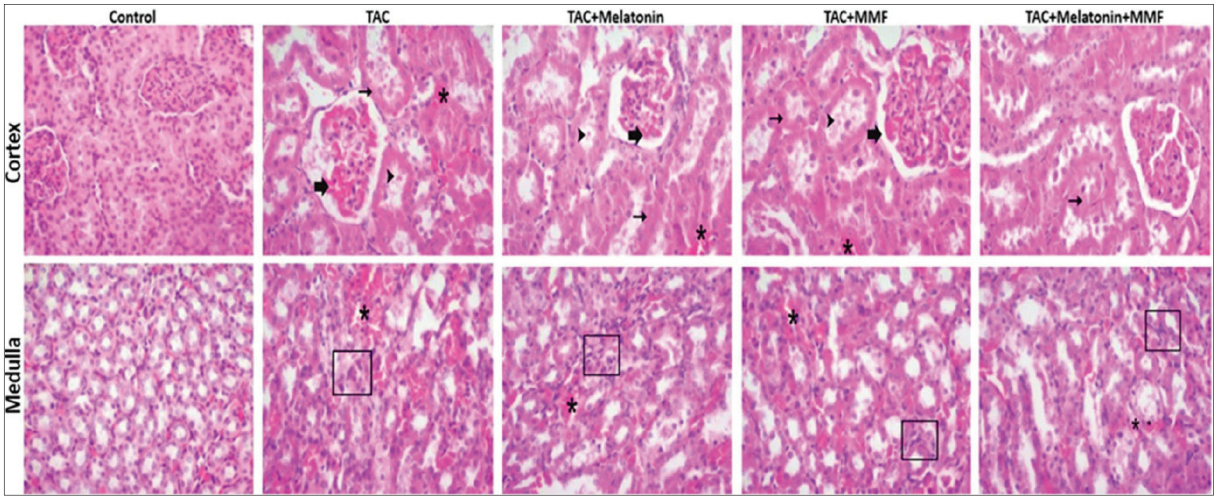

Fig 1. Renal cortex. A photomicrograph of sections in the renal cortex of different studied groups. In the control group, normal renal cortex appearance. In group II (TAC group), severe glomerular congestion (thick arrow), intertubular hemorrhage (*), hyaline formation (arrowhead), and degenerative-necrotic tubules epithelium *(arrow)* were observed. In group III (TAC + melatonin) and group IV (TAC+ MMF), moderate glomerular congestion, intertubular hemorrhage, hyaline formation, and degenerative-necrotic tubules epithelium were observed. Only mild degenerative-necrotic tubules epithelium was observed in group V (TAC + melatonin + MMF) (H&Ex40); **Renal medulla.** A photomicrograph of sections in the renal medulla of different studied groups. In the control group, normal renal medulla appearance. In group II (TAC group), severe intertubular hemorrhage (*) and mononuclear cell infiltration (□) were observed. In group III (TAC + melatonin) and group IV (TAC+ MMF), moderate intertubular hemorrhage and mononuclear cell infiltration were observed. In group V (TAC + melatonin + MMF), only mild intertubular hemorrhage was observed (H&Ex40). TAC: Tacrolimus, MMF: Mycophenolate mofetil

Table 5. *Comparison of histopathological changes in renal cortex among groups*

Parameters	Control (n=6)	TAC (n=6)	TAC+Melatonin (n=6)	TAC+MMF (n=6)	TAC+Melatonin+MMF (n=6)
Glomerular congestion	0.16±0.40^{a}	2.83±0.40^{b}	1.83±0.40^{c}	2.16±0.40^{c}	0.33±0.51^{a}
Intertubuler hemorrhage	0.16±0.40^{a}	2.83±0.40^{b}	2.00±0.63^{c}	2.33±0.51^{c}	0.33±0.81^{a}
Hyaline formation	0.33±0.51^{a}	3.00±0.00^{b}	2.00±0.63^{c}	2.00±0.63^{c}	0.33±0.81^{a}
Degenerative-necrotic tubulus epithelium	0.33±0.51^{a}	3.00±0.00^{b}	2.16±0.40^{c}	2.16±0.40^{c}	1.16±0.40^{d}

TAC: *Tacrolimus,* ***MMF:*** *Mycophenolate mofetil. Results were given as mean±standard deviation. Different upper superscripts indicate statistical differences among groups (P<0.05)*

Table 6. *Comparison of histopathological changes in renal medulla among groups*

Parameters	Control (n=6)	TAC (n=6)	TAC+Melatonin (n=6)	TAC+MMF (n=6)	TAC+Melatonin+MMF (n=6)
Intertubuler hemorrhage	0.33±0.51^{a}	3.00±0.00^{b}	1.83±0.40^{c}	1.66±0.51^{c}	0.83±0.40^{d}
Mononuclear cell infiltration	0.33±0.51^{a}	2.83±0.40^{b}	2.83±0.40^{b}	1.66±0.51^{c}	1.66±0.51^{c}

TAC: *Tacrolimus,* ***MMF:*** *Mycophenolate mofetil. Results were given as mean±standard deviation. Different upper superscripts indicate statistical differences among groups (P<0.05)*

were significantly regressed in group V *(Fig. 1)*. Comparison of histopathological parameters among groups was demonstrated in *Table 5* and *Table 6*. The histopathological changes such as glomerular congestion, intertubular hemorrhage, hyaline formation, degenerative-necrotic tubule epithelium, mononuclear cell infiltration were more pronounced in group II than group III, IV and V (P<0.05 in all cases). These changes were significantly improved in group V.

Discussion

Living or cadaveric kidney transplantation is the gold treatment for chronic kidney failure due to various reasons [1]. Thanks to immunosuppressive agents such as TAC, which are used to prevent rejection, good progress has been made in organ transplantation in the last few decades. Unfortunately, the use of TAC has side effects such as nephrotoxicity, hepatotoxicity, neurotoxicity, glucose intolerance, and gastrointestinal toxicity [26].

The mechanism of TAC-induced nephrotoxicity remains unclear. Therefore, we aimed to biochemically and histopathologically investigate the toxic effects of TAC on the kidney and examine the cytoprotective effect of melatonin and MMF against TAC-induced kidney injury. Our study demonstrated that melatonin and MMF effectively reduced

TAC-induced histopathological changes such as glomerular congestion, intertubular hemorrhage, hyaline formation, degenerative-necrotic tubule epithelium, mononuclear cell infiltration in the kidney tissue. Moreover, activation and up-regulation of pro-inflammatory cytokines such as IL-1 and TNF-alpha and the generation of oxidative stress products were reduced. Our study revealed that melatonin and MMF could protect against oxidative stress-mediated acute kidney injury in a rat model induced by TAC.

Our findings in this study revealed that TAC alone caused a significant increase in MDA, TOS, and pro-inflamatuar cytokines such as IL-1 and TNF alpha in rats' kidneys. We thought that increased MDA production due to TAC nephrotoxicity in kidney tissues of rats might be accompanied by induction of lipid peroxidation. The obtained results are parallel to the results of lipid peroxidation caused by TAC reported in previous studies [27-30]. Besides, CAT activity was significantly reduced after treatment with TAC alone. Hence, decreased CAT activities in renal tissues may be due to ROS/lipid peroxidation's excess production. Disruption in the oxidative system has been reported as one of the main causes of TAC-induced renal dysfunction [9,27,31,32].

Melatonin, thanks to its antioxidant and cell-protective properties, prevents the formation of free radicals and neutrophil accumulation during oxidative damage induced by various events, such as drug-induced injury, ischemia-reperfusion, and prevents the destruction of kidney tissue [33-35]. Our study results revealed that melatonin has antioxidant potential to prevent oxidative stress and lipid peroxidation, which likely contributed to its protection against TAC-induced kidney injury in rats. This protective effect of melatonin may be due to suppressing lipid peroxidation and activation of SOD and CAT. These findings suggest that MMF could effectively treat the tissue damage induced by TAC.

Combination therapy is an ideal treatment option for providing adequate immunosuppression after organ transplantation and minimizing graft rejection. MMF, a novel immunosuppressive drug, is often used in combination therapy with TAC [15]. The previous study showed that MMF has a protective effect against drug-induced renal injury [18]. In this study, we investigated the protective effect of MMF against TAC-induced oxidative damage by the measure of MDA level, biomarkers of the prooxidant system, and CAT activities, an indicator of the antioxidant system in the kidney of rats. Oxidative stress and lipid peroxidation mediated by oxygen free radicals are important causes of damage to the cell and mitochondrial membranes [29,36,37]. The histological examination of the kidney sections confirmed the results mentioned above; MMF co-administration with TAC can restore the kidney's nearly normal cellular architecture and reverse TAC-induced histopathological effects *(Fig. 1)*. These results demonstrated the potential beneficial effects of MMF to counteract the oxidative stress induced by TAC administration.

We also used histological scoring methods to evaluate the histopathological changes, which are oxidative stress injury features increased due to TAC-induced kidney damage. These two features were decreased after both melatonin and MMF treatment in the TAC-treated groups. Moreover, histopathological examination of the kidney of group V (TAC+melatonin+MMF) was close to normal appearance, with only mild degenerative-necrotic tubules epithelium in the cortex and mild intertubular hemorrhage in the renal medulla. These findings suggest that melatonin and MMF together can protect effectively against oxidative stress -mediated kidney injury in a rat model induced by TAC.

Although TAC, which is used for immunosuppressive purposes after organ transplantation, prevents rejection, it also causes nephrotoxicity through oxidative stress. This kidney injury can be assessed by demonstrating an increase in ROS and lipid peroxidation marker MDA levels and decreased TAS. We have seen that melatonin and MMF, especially together or separately, protect the kidney against oxidative stress damage caused by TAC not only biochemically but also histopathologically by reducing glomerular congestion, intertubular hemorrhage hyaline formation, degenerative-necrotic tubules epithelium, mononuclear cell infiltration in the kidney tissue.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (S. Koc) on reasonable request.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

SK, AA, BS and MO conceived and supervised the study. SK, AA and MO collected and analyzed data. AA and BS made laboratory measurements. MO applied the histopathological examination of the study. The first draft of the manuscript was written by SK, and all authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Studies on Overwintering Behavior and Cold Stress Related Unigenes of *Wohlfahrtia magnifica*

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Abstract

The overwintering behavior and unigenes related to cold stress were studied in this paper. The pupae of *Wohlfahrtia magnifica* were placed at room temperature, 4°C, -5°C, -10°C, -15°C and -24°C respectively, and the recovery experiment after low temperature induction was carried out. The hatching of the pupae was counted, the shell interior pupae at -5°C, -10°C, -15°C and -24°C were photographed and recorded. Transcriptome sequencing was performed on the pupae at room temperature (PA), -5°C (PA1) and -10°C (PA2). The results were analyzed by Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), and the HSP67Bc, HSP23, HSP27, HSC70-4 and HSP70Ba were verified by Q-PCR. The results showed that the expression level of heat shock proteins (HSPs) in PA1 and PA2 were significantly higher than in PA, and HSP23, HSP27, HSP67Bc, HSP70Ba, HSP60, HSP83 and heat shock protein homologous (HSCs) such as HSC70-4, HSC7-5 were highly expressed in the pupae under low temperature stress. 13168 and 11161 entries were annotated in GO and KEGG, respectively. Q-PCR result showed that except HSP67Bc, the analysis results of the other four unigenes were consistent with the data of transcriptome analysis. Therefore, the overwintering behavior of *Wohlfahrtia magnifica* was in the form of pupa. HSPs played an important role in the overwintering process of the *Wohlfahrtia magnifica* pupa.

Keywords: *GO, HSPs, Low temperature stress, Transcriptome, Wohlfahrtia magnifica*

Wohlfahrtia magnifica'nın Kışlama Davranışı ve Soğuk Stresiyle İlgili Unigenleri Üzerine Çalışmalar

Öz

Bu çalışmada, *Wohlfahrtia magnifica*'nın kışlama davranışı ve soğuk stresi ile ilgili unigenleri incelenmiştir. *Wohlfahrtia magnifica*'nın pupaları sırasıyla 4°C, -5°C, -10°C, -15°C ve -24°C'de oda sıcaklığına yerleştirildi ve düşük sıcaklık indüksiyonundan sonra geri kazanım deneyi yapıldı. Pupalardan çıkan erişkin sinekler sayıldı, -5°C, -10°C, -15°C ve -24°C'deki kabuk iç pupalar fotoğraflandı ve kaydedildi. Pupalarda, oda sıcaklığında (PA), -5°C (PA1)'de ve -10°C (PA2)'de transkriptom sekanslama yapıldı. Sonuçlar, Gen ontolojisi (GO) ve Kyoto Gen ve Genom Ansiklopedisi (KEGG) ile analiz edildi ve HSP67Bc, HSP23, HSP27, HSC70-4 ve HSP70Ba, Q-PCR ile doğrulandı. Sonuçlar, PA1 ve PA2'deki ısı şoku proteinlerinin (HSP'ler) ekspresyon seviyelerinin PA'ya göre önemli ölçüde yüksek olduğunu ve HSP23, HSP27, HSP67Bc, HSP70Ba, HSP60, HSP83 ve HSC70-4 ve HSC7-5 gibi ısı şok protein homologlarının (HSC'ler) düşük sıcaklık stresi altındaki pupalarda yüksek oranda eksprese edildiğini gösterdi. GO ve KEGG'de sırasıyla 13168 ve 11161 kayıtlarına açıklama yapıldı. Q-PCR sonucu, HSP67Bc dışındaki diğer dört unigenin analiz sonuçlarının, transkriptom analiz sonuçları ile tutarlı olduğu saptandı. Bu nedenle, *Wohlfahrtia magnifica*'nın kışlama davranışı pupa formunda olmuştur. HSP'ler, *Wohlfahrtia magnifica* pupalarının kışlama sürecinde önemli rol oynamıştır.

Anahtar sözcükler: *GO, HSP, Düşük sıcaklık stresi, Transkriptom, Wohlfahrtia magnifica*

INTRODUCTION

Wohlfahrtia magnifica is the main pathogen causing hemorrhagic trauma and natural openings of human and animals. In the Mongolian plateau, *Wohlfahrtia magnifica* is the only pathogen causing Bactrian camel vaginal myiasis. The 1st, 2nd and 3rd instar larvae of *Wohlfahrtia magnifica* parasitize in the camel vaginal, causing vaginal

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diseases of Bactrian camel [1]. The annual vaginal myiasis of Bactrian camel incidence rate is 20%~30%, and the mortality rate is about 2% [2], it has caused great economic losses to local camel farming. It has been reported that *Wohlfahrtia magnifica* maggot may infect foot of diabetic patients causing muscle trauma and may also cause periungual myiasis in girls [3,4].

Chen et al.[5] and Reynolds et al.[6] compared brain proteome differences between diapause and non-diapause musca domestica Linnaeus pupae, and found high expression of HSPs in diapause, but phosphoenolpyruvate synthase, fatty acid-binding protein and endonuclease expression levels decreased.

Heat shock proteins (HSPs) are highly conserved heat stress proteins that widely exist in prokaryotes and eukaryotic cells. They can assist in protein refolding to maintain protein stability and cell integrity, and acting as essential regulators of diverse constitutive metabolic processes [7-9]. Many animals have HSPs, and insects produce HSPs to maintain cell homeostasis when they are stimulated by cold and heat, including non-ATP-dependent small heat shock proteins and large ATP-dependent heat shock proteins, such as HSP70, HSP90 and HSP60 [10]. Small HSPs are the most least conservative of all HSPs [11]. At present, more and more HSPs have been proved to be responsive to temperature stress, and the expressions of HSP70, HSP90 and HSP60 were increased after low-temperature induction of insects [12-15]. The expression of HSP26 in the brain of *Sarcophage crassipalpis* was up-regulated after cold treatment [16].

Some freeze-tolerant insects produce antifreeze proteins (AFPs) for self-adaptation to resist cold. AFPs are specific proteins, carbohydrates and polypeptides produced by different organisms that enable cells to survive in subzero conditions [17]. It is the earliest biological antifreeze material found in polar fish [18], which mainly in hemolymph, intestinal juice and intracellular fluid. It can bind to the surface of ice crystals and inhibit the growth of ice crystals [19]. It has the function of thermal hysteresis, inhibiting the recrystallization of ice crystal and strengthening the interaction between cell membrane and membrane proteins [20]. AFPs exist in fish, insects, plants and micro-organisms [21], and the antifreeze ability of arthropods is higher than that of fish. AFPs have potential application value in gene transformation and tumour freezing, and it can be used to produce economical and efficient fish, animals and plants in agriculture, and it also can be used to resist extreme low temperatures [22].

Different insects have different cold-resistant strategies. In behavior, some insects overwinter with one insect state pupae, others with multiple insect state; physiologically, they can improve their cold tolerance by reducing water content and storing more cold-resistant substance [23], such as the physiological mechanism of protecting diapause larvae from dehydration may also increase their cold tolerance [24]. Although the overwinter morphology of insects is different, winter with pupae is the most secure and safest, which may be due to the metabolic inhibition of diapause pupae [25].

The overwintering behavior and unigenes related to cold stress were studied in this paper, further reveal the overwintering behavior and molecular mechanism of *Wohlfahrtia magnifica*.

Material and Methods

Ethical Statement

All experimental procedures were approved by the Animal Protection and Use Committee of Inner Mongolia Agricultural University and strictly followed animal welfare and ethical guidelines.

Studies on the Overwintering Behavior of Wohlfahrtia magnifica

The adult flies and each stage larvae of *Wohlfahrtia magnifica* were collected, and some of the 3rd instar larvae were put into culture medium to pupate. Putting the pupae, adult flies and remaining each stage larva into 4°C to observe. When their activity weakens to no activity, they were removed and returned to room temperature, then observed for their survival.

Emergence Rate of the Pupae of Wohlfahrtia magnifica at Different Ambient Temperature

Collecting the larvae of *Wohlfahrtia magnifica* and cultured them to mature the 3rd instar larvae, and then put them into culture medium to pupate. After 2~3 days, the pupae were cultured at room temperature, 4°C, -5°C, -10°C, -15°C and -24°C, and 80 pupae were used in each temperature gradient. Five groups of pupae except the room temperature group were slowly let hypothermia in refrigerator to induce low temperature stress. After falling to the target temperature, the pupae were kept for 5 days, then they were returned to room temperature and the hatching of them were counted. The shell interior morphology of the pupae was photographed and recorded by superview imager and anatomical microscope at target temperatures.

Studies on Transcriptome Sequencing of Wohlfahrtia magnifica at Room Temperature and Low Temperature Stress at -5°C and -10°C

The 3rd instar larvae of *Wohlfahrtia magnifica* were divided into three groups after pupation. One group was preserved at room temperature, and the others were kept at -5°C and -10°C for 5 days, respectively. Then the three group samples were put into liquid nitrogen quickly for transcriptome sequencing.

According to the reagent instruction, Trizo reagent (Invitrogen, CA, USA) was used to extract total RNA. The quantity and purity were analyzed by Bioanalyzer 2100 that RIN >7 and RNA 1000 Nano LabChip kits (Angelen, California, USA). Using magnetic beads that adhered to poly-T oligonucleotides, poly(A) RNA was purified from total RNA through two rounds of purification. After purification, the mRNA sections were transformed into small sections using bivalent cations at high temperature, and the lytic RNA sections were reverse transcribed according to the procedure of mRNASeq sample preparation kit (Illumina, San Diego, USA) to establish the final cDNA library. The average insert size of the match terminal library was 300bp (±500bp). Then the pairing end sequencing was performed on Illumina Hiseq4000 from LC Sciences in the USA according to the vendor recommended protocol.

Unigene Annotation and Functional Classification

First, entrails Cutadapt and perl libretto to delete reads that contain adapter pollution, inferior base pairs and indefinite base pairs, then FastQC to verify sequence quality, including Q20, Q30 and GC content of clean data were used. All downstream analyses were based on high-quality clean data. All the packaged unigenes were compared with SwissProt's non-redundant proteins and GO, KEGG and eggNOG used the data base of DIAMOND and threshold Evalue <0.00001.

Differential Unigene Expression Analysis

Salmon [26] performed the expression level of unigenes by calculating TMP [27]. The differential expression unigenes were selected by edgeR [28], where log2 >1 or log2 <-1, and the difference was statistically significant (P<0.05). Then the differential expression unigenes were analyzed by GO and KEGG enrichment analysis by entrails perl libretto.

Statistical Analysis of HSP23, HSP27, HSP70Ba, HSP67Bc and HSC70-4 Unigenes Expression Under Different Temperature Stress

All the selected HSPs and their homologues were chosen, and four highly expressed and significantly different HSPs (HSP23, HSP27, HSP70Ba, HSP67Bc) and a HSP homologue unigene (HSC70-4) were chosen from the HSPs and their homologues, then they were verified by FQ-PCR.

Results

Studies on the Overwintering Behavior of Wohlfahrtia magnifica

Through the experiment, it was found that the 1st instar larvae and the 2nd instar larvae were hardened and stiffen on the day when the temperature dropped to 4°C, and after recovered to room temperature, they could not survive. The 3rd instar larvae hardened and stiffened at 4°C after one and a half day and did not survive after recovery to room temperature. The adult flies showed no signs of life after surviving at 4°C for 4 days, and there was no resuscitation when they were recovered to room temperature. The pupae were placed at 4°C for 7 days and then restored at room temperature, it was observed that flies began to hatch on the 7th day after being taken out from 4°C. The results of the experiment showed that the pupa has the property of cold-tolerance ability.

Emergence Rate of the Pupae of Wohlfahrtia magnifica at Different Ambient Temperatures

The emergence rate at room temperature, 4°C, -5°C, -10°C, -15°C and -24°C are 75%, 50%, 48.75%, 47.5%, 3.75% and 0%, respectively *(Table 1)*. The pupae peeled off their shell cuticle and found that interior morphology was intact after low temperature stress at -5°C *(Fig. 1)* and -10°C *(Fig. 2)*. While at the temperature of -15°C, it was found that most of the pupae blackened and suppurated *(Fig. 3)*, a small part of the pupae developed to the third stage but failed to emerge from shell, and at the temperature of -24°C, all pupae wizened and died *(Fig. 4)*. It can be concluded that the emergence rate of *Wohlfahrtia magnifica* is affected by ambient temperatures.

Studies on Transcriptome Sequencing of Wohlfahrtia magnifica *at Room Temperature and Under Low Temperature Stress at -5°C and -10°C*

- Analysis of Overall Unigenes Expression Level

In order to make the data more reliable, three biological replicates were used for transcriptome sequencing in each group *(Fig. 5)*. It was found that the upper quartile, median and lower quartile of unigene expression level in each group were basically at the same level, and it shows

Table 1. *Number of emergence and emergence rate of the pupa of* Wohlfahrtia magnifica *after different temperature stresses*

Temperature (°C)	Number	Number of Emerge	Emergence Rate (°C)
-24	80	0	0
-15	80	3	3.75
-10	80	38	47.50
-5	80	39	48.75
4	80	40	50.00
Room temperature	80	60	75.00

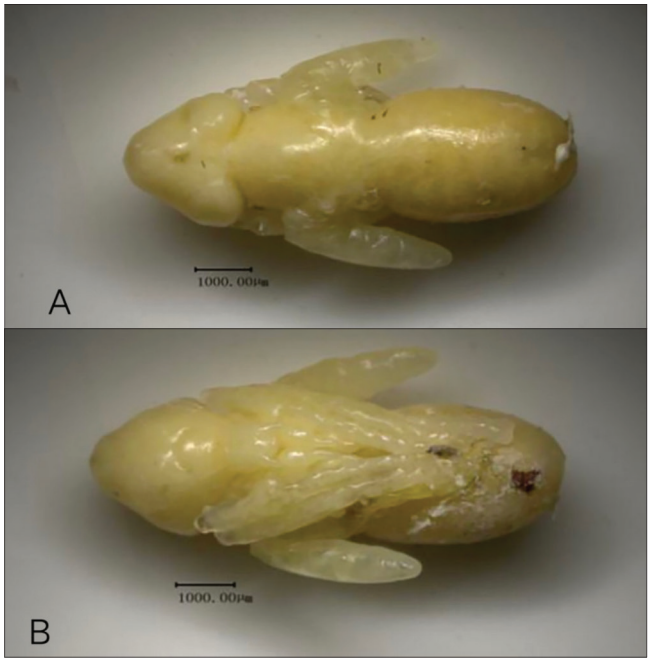


Fig 1. Morphology of the pupa after low temperature stress at -5°C in the shell (A, B)

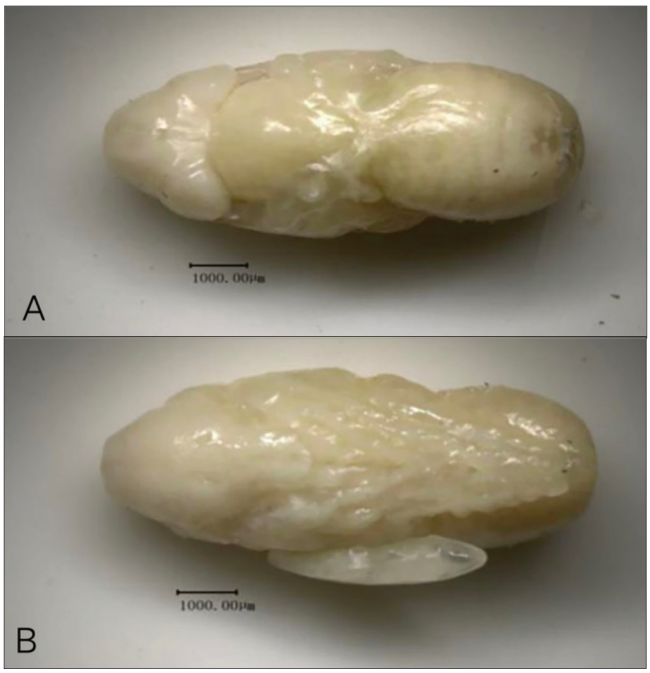


Fig 2. Morphology of the pupa after low temperature stress at -10°C in the shell (A, B)

that the repeatability of the sample is good and the next experimental analysis can be carried on.

Differential Unigene Expression Analysis

- Differential Unigene Expression

Differential unigene expression profile analysis showed that the transcripts were compared with each other (blue

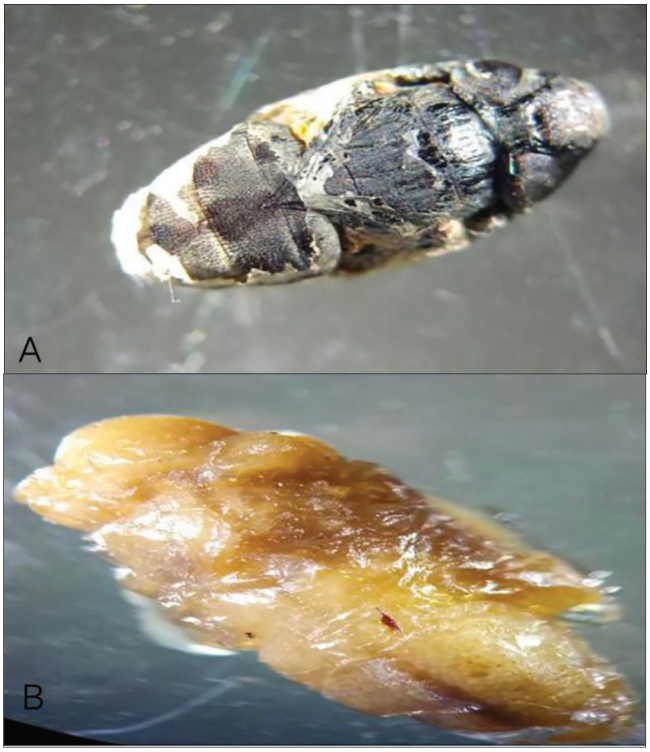


Fig 3. Morphology of the pupa after low temperature stress at -15°C in the shell (A, B)

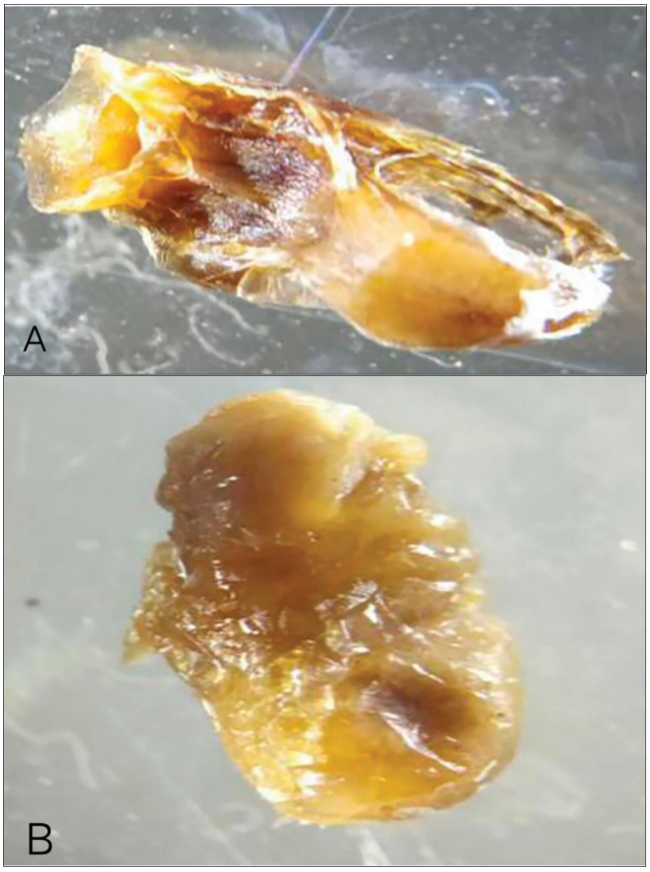


Fig 4. Morphology of pupa after low temperature stress at -24°C in the shell (A, B)

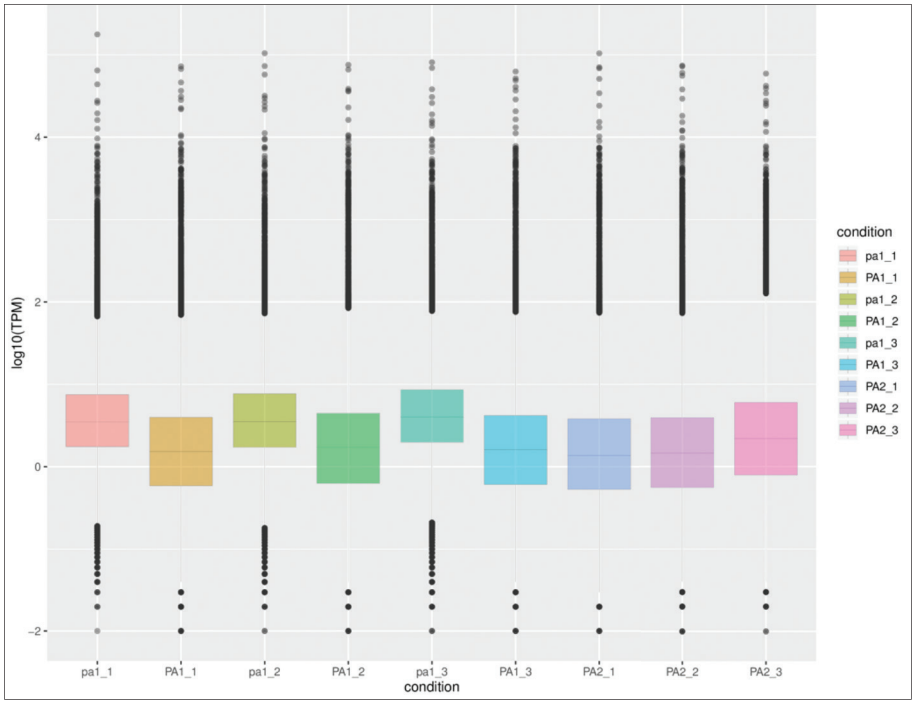
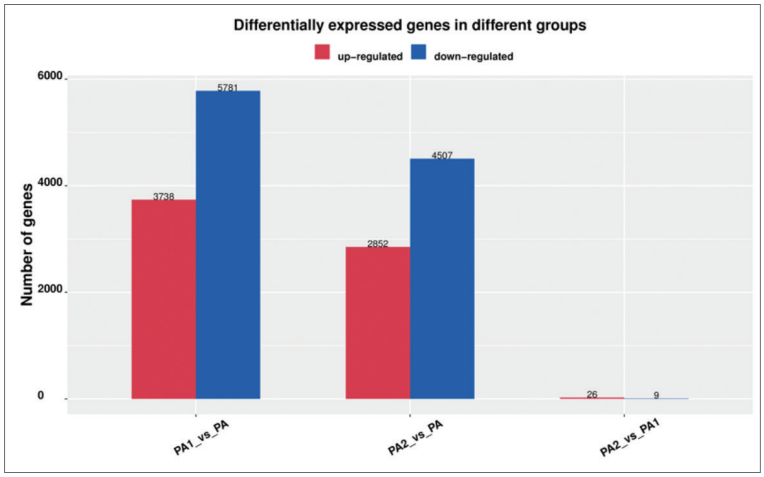


Fig 5. Box plot of unigene expression of *Wohlfahrtia magnifica* at -5°C, -10°C and room temperature. In the figure, PA1_1, PA1_2 and PA1_3 are the three results of room temperature group

Fig 6. Histogram of differential unigene expression in different groups



column for down-regulation, red column for up-regulation *(Fig. 6)*. Compared with PA (at room temperature), 9519 and 7359 unigenes were found to be significantly different in the PA1 and PA2 (at -5°C, -10°C), including 3738 and 2852 up-regulated unigenes, and 5781 and 4507 down-regulated unigenes, respectively; 35 unigenes difference were found between PA1 and PA2, of which 26 different unigenes were up-regulated and 9 different unigenes were down-regulated.

By processing the differential unigenes among PA, PA1 and PA2 groups and making the Venn diagram *(Fig. 7)*, it was found that PA1, PA2 and PA contained 9159 and 7359 differential unigenes respectively, and PA1 and PA2 contained 35 unigenes. PA1 and PA2 contained 3026 and 872 unigenes separately from PA; PA1 and PA2 separately contained 7 unigenes; Together PA1_vs_PA with PA2_vs_PA contains 6469 unigenes; Together PA1_vs_PA with PA1_vs_PA2 contains 24 unigenes,together PA2_vs_PA with PA1_vs_PA2 contains 18 unigenes and together PA1_vs_PA with PA2_vs_PA and PA1_vs_PA2 contains 7 unigenes.

- GO Enrichment Analysis of Differential Unigenes

PA1 and PA2 separately were enriched 286 and 223 unigenes in biological process, 678 and 452 unigenes in cytoplasm, 611 and 402 unigenes in nucleus, 419 and 314 unigenes in molecular function, and 386.258 unigenes in protein binding from PA *(Fig. 8, Fig. 9)*.

- The Analysis of HSPs and HSCs Unigene

Through the GO enrichment, it was found that HSPs and HSCs were enriched in biological process, molecular function and cellular component respectively, in which HSPs participated in cold acclimation, cold translation, protein binding transport, ATP binding transport and other 177

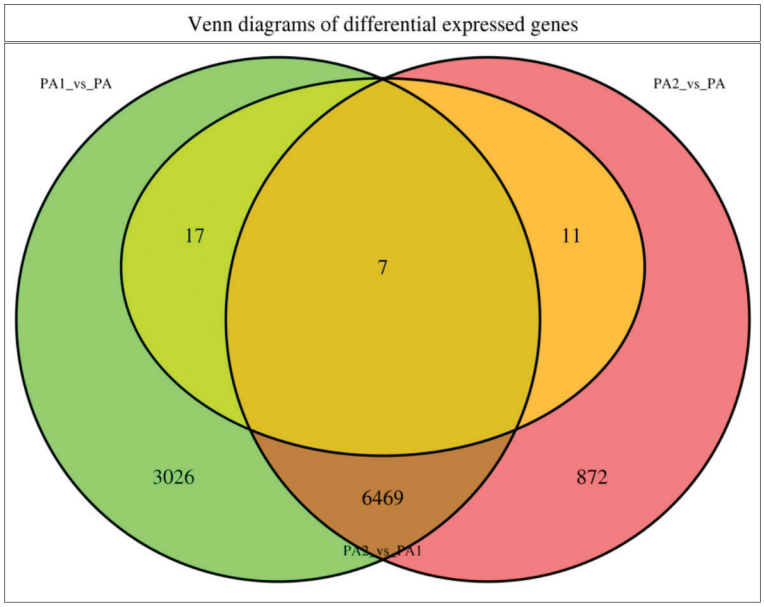
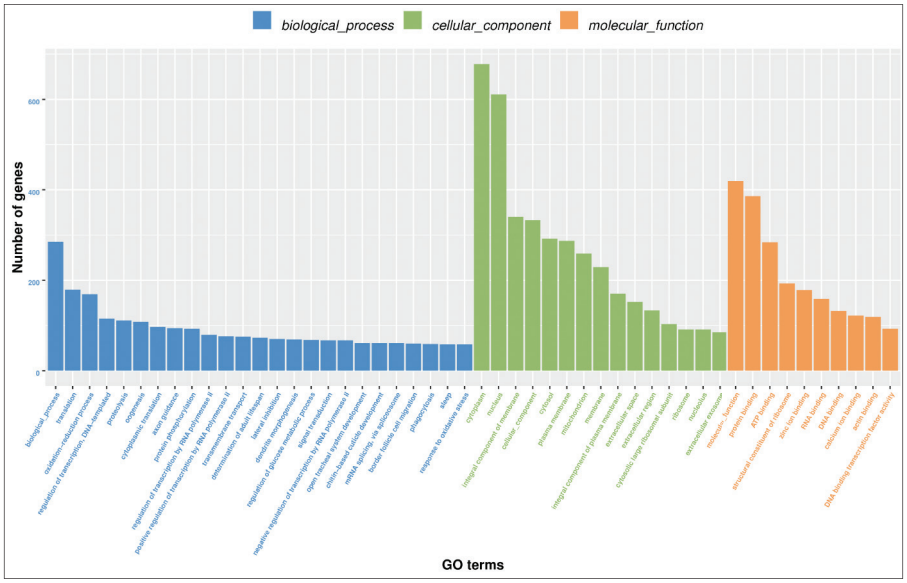


Fig 7. Venn diagram of differential unigene expression

Fig 8. GO enrichment map of PA1_vs_PA differential unigene



small items; while HSCs participated in 47 small items such as chaperones mediated protein folding, cell response to heat, protein renaturation and TAP binding.

The results of sequencing showed that the HSPs were highly expressed in pupae under low temperature stress, in which 4 unigenes of HSP23 and 1 unigene of HSP83 were involved in cold acclimation of pupae of *Wohlfahrtia magnifica*. Differential HSCs were found in both -5°C and -10°C treatment gruops: HSC70, HSC70-1, HSC70-2, HSC70-3, HSC70-4, HSC70-5 and HSCB; of which HSC70 and HSC70-1 were down-regulated, the rests were up-regulated. The HSC70-4 and HSC70-5 with P<0.05 and log2FC>2 were chosen, and there were 52 and 58 differential HSPs in the -5°C and -10°C treatment groups compared with the room temperature group, respectively. Among them, 2 unigenes were down-regulated, 50 unigenes were up-regulated at -5°C; and 13 unigenes were down-regulated, and 45 unigenes were up-regulated at -10°C. There were 22 unigenes among the HSPs which chosen for log2FC>2 and P<0.05 *(Fig. 10, Fig. 11)*.

The HSP67Bc, HSP23, HSP27, HSC70-4 and HSP70Ba were selected, and the unigenes expression TMP of their transcriptome sequencing data were performed variance analysis. It was found that the unigene expression levels of HSP67Bc(P=0.002), HSP23 (P=0.003), HSP27 (P=0.00), HSC70-4 (P=0.00) and HSP70Ba (P=0.00) were significantly different between pupae under low temperature stress and pupae at room temperature *(Fig. 12)*.

Statistical Analysis of HSP23, HSP27, HSP70Ba, HSP67Bc and HSC70-4 Unigenes Expression Under Different Temperature Stress

The mRNA treatment results of candidate unigenes under

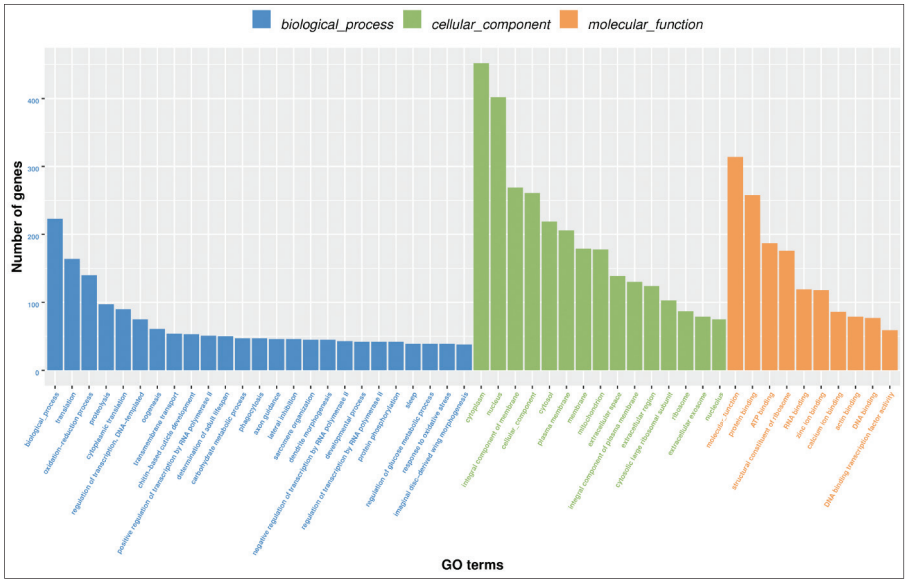
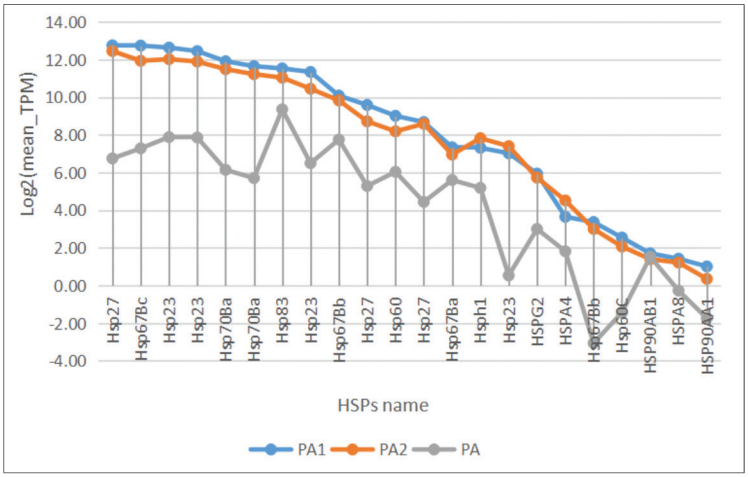


Fig 9. GO enrichment map of PA2_vs_PA differential unigene

Fig 10. Line graph of HSPs expression



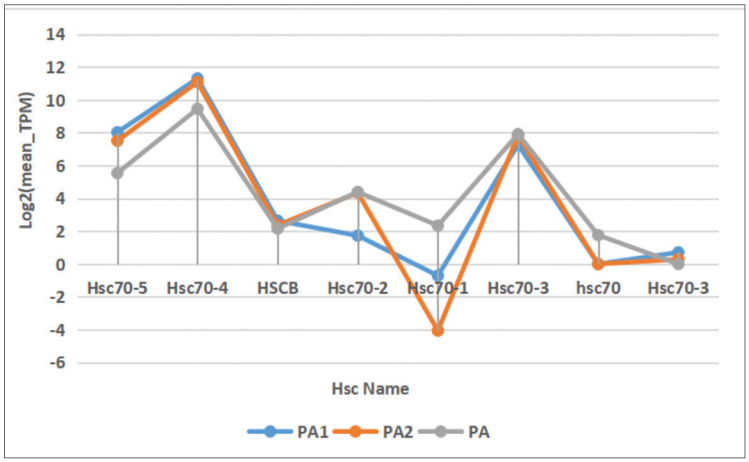


Fig 11. Line graph of HSCs expression. The two HSC70-3 in the figure represent two different sequences respectively

different treatment conditions were shown in *Fig. 13*. PASWStatistics18 was used for variance analysis of the relative expression levels of the same unigene under different temperature conditions, and Duncan's new multiple range test (MRT) was used for pairing test of mean difference. Through the analysis and detection of the expression profiles of five HSPs of the screened cold-tolerance related unigenes of pupae at different temperatures, and the results showed the screened five HSPs were expressed to different degrees: The expression of HSP27 was significantly different ($P=0.009<0.01$) and the expression of HSP23 was significantly different ($P=0.037<0.05$)

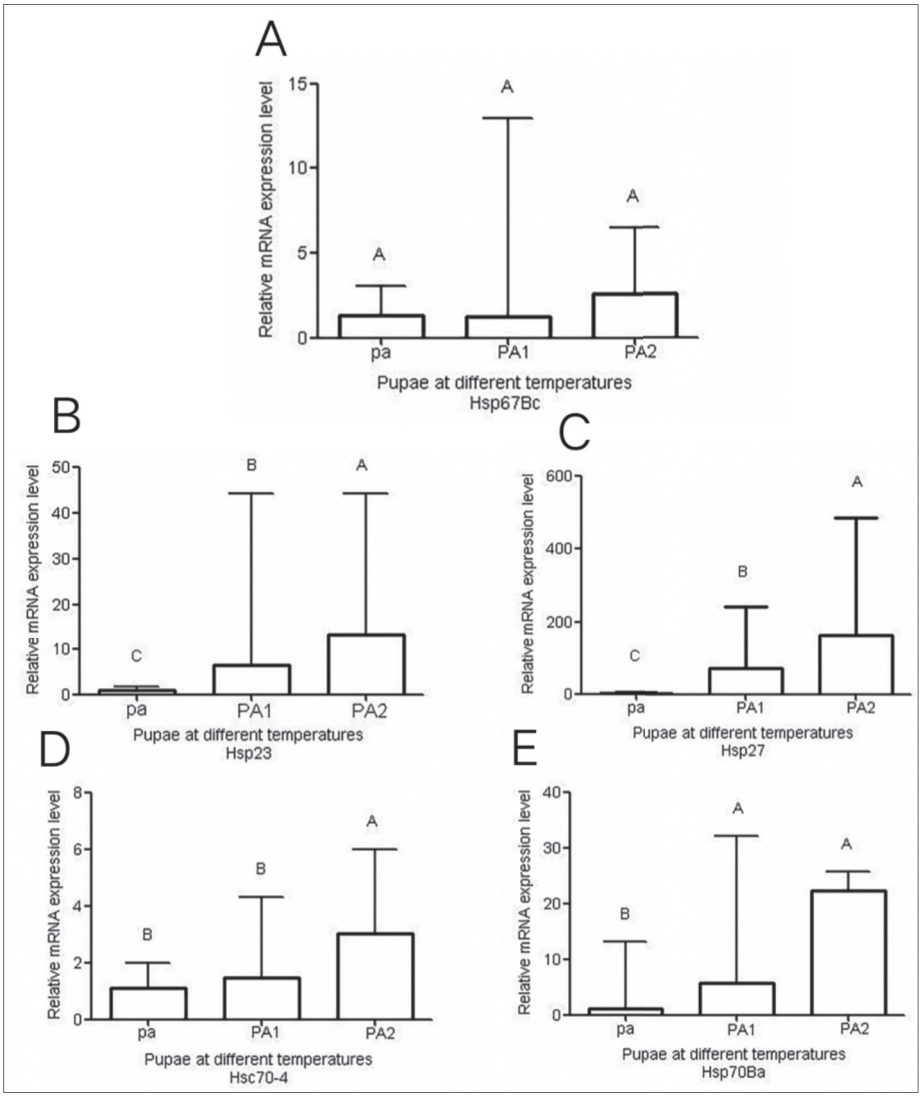


Fig 12. Transcription sequencing expression of differen unigenes at different ambient temperature. **A-** Transcription sequencing expression of HSP67Bc at different ambient temperature, **B-** Transcription sequencing expression of HSP23 at different ambient temperature, **C-** Transcription sequencing expression of HSP27 at different ambient temperature, **D-** Transcription sequencing expression of HSC70-4 at different ambient temperature, **E-** Transcription sequencing expression of HSP70Ba at different ambient temperature

under low temperature; The expression of HSC70-4 was significantly different at -10°C ($P=0.026<0.05$), while in the -5°C treated group, its expression was higher than that of the room temperature group, but it was not significantly different; Though the expression of the HSP67Bc in the cryogenic treatment pupae was higher than that of the room temperature pupae, but the difference was not significant ($P=0.271>0.05$); The expression of the HSP70Ba in pupae under low temperature was significantly different ($P=0.039<0.05$).

The results showed that the expression of HSP23, HSP27, HSP70Ba, HSP67Bc and HSC70-4 in pupae were different at different temperatures. The results of Q-PCR showed that the analysis results of HSP23, HSP27, HSP70Ba and HSC70-4 were in accordance with the data of transcriptome analysis, but the Q-PCR results of HSP67Bc were significantly different from the results of transcriptome analysis, due to the deviation may be caused by intraspecific repeatability. Among them, the expression of HSP27 in the pupa at -5°C and -10°C were significantly higher than that of the room temperature, the expression of HSP23 and HSP70Ba in the pupae at -5°C and -10°C were significantly higher than that of the room temperature. It was found that HSP27, HSP23 and HSP70Ba were related to the overwintering cold-tolerance of *Wohlfahrtia magnifica*. The expression of HSC70-4 in the pupae at -10°C were significantly higher than that of the -5°C and room temperature group, while in the -5°C treated group, which was slightly higher than that of the room temperature group, the difference was not significant. It was shown that unigenes in *Wohlfahrtia magnifica* play an important role in the cope with cold.

Discussion

The results showed that the overwintering behavior of *Wohlfahrtia magnifica* was in the form of pupa, in which the HSPs played an important role.

Through transcriptome sequencing and comparative database analysis of low temperature stressed pupae, it was found that the pupae at -5°C and -10°C had 9519 and 7319 different transcripts compare with the pupae at room temperature, respectively. Through comparison

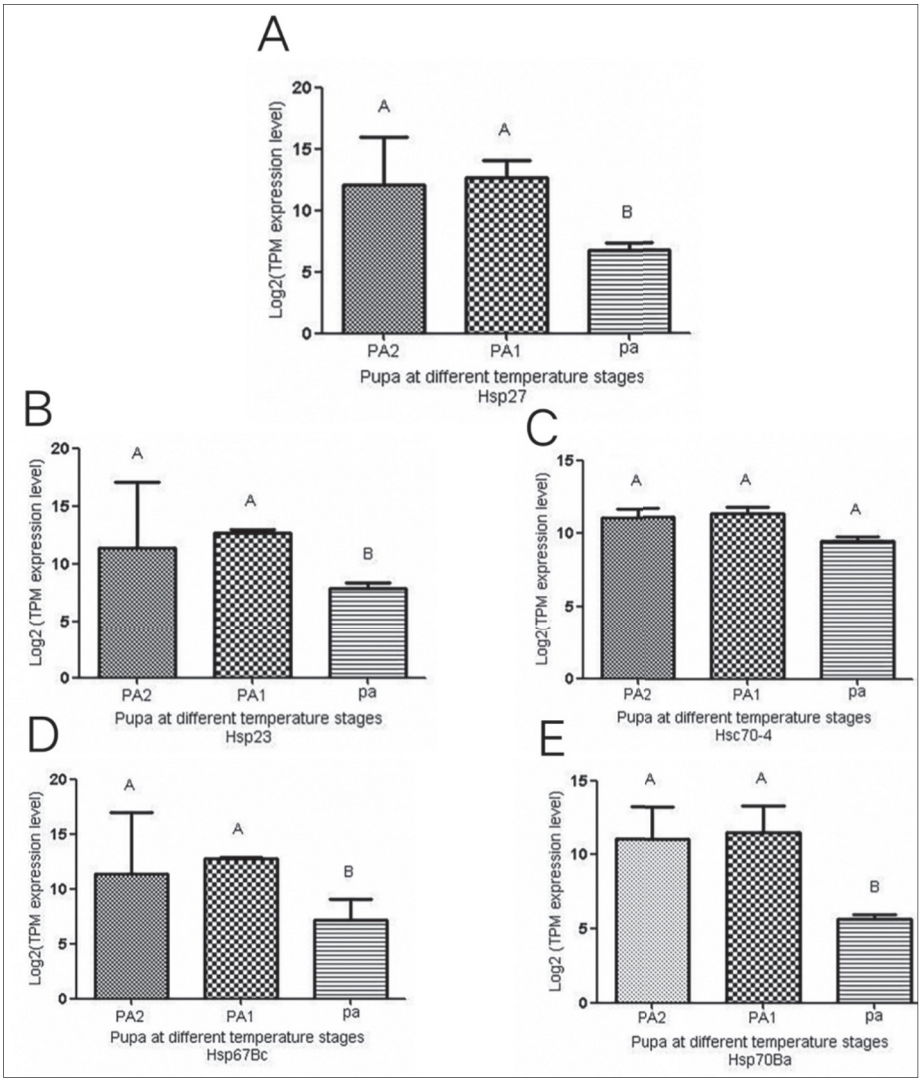


Fig 13. The expression levels of different unigenes in *Wohlfahatia magnifica* pupae at different ambient temperature. **A-** The expression levels of HSP27 in *Wohlfahatia magnifica* pupae at different ambient temperature, **B-** The expression levels of HSP23 in *Wohlfahatia magnifica* pupae at different ambient temperature, **C-** The expression levels of HSC70-4 in *Wohlfahatia magnifica* pupae at different ambient temperature, **D-** The expression levels of HSP67Bc in *Wohlfahatia magnifica* pupae at different ambient temperature, **E-** The expression levels of HSP70Ba in *Wohlfahatia magnifica* pupae at different ambient temperature

with major databases, we found many homologous unigenes were similar to that in other insects (such as *Lucilia cuprina* [29]), and among them there were many kinds of differential unigenes, indicating that the transcriptome data for the pupae of *Wohlfahrtia magnifica* under low temperature stress is very important to study its cold-tolerance related unigenes.

In this study, RNA-seq and Q-PCR technology were used to explore the molecular mechanism of the pupae survival under low temperature. Through GO enrichment analysis, HSP67Bc, HSP23, HSP27, HSC70-4 and HSP70Ba were found to be enriched in proteins folding and cold stress that related to cold-tolerance in pupae under -5°C and -10°C stress, but no AFPs were found. These results showed that HSPs play an important role in the cold-resistant environment of *Wohlfahrtia magnifica* pupa.

Q-PCR results showed that HSP23, HSP27, HSC70-4 and HSP70Ba were significantly differential expressed. It was reported that the cold-tolerance of *Drosophila melanogaster* was decreased after the HSP23 was knockouted [30], indicating that this unigene plays an important role in the cold-tolerance in *Drosophila melanogaster*. Results showed the pupae under low temperature stress, the expression of HSP23 was significantly different, indicating that the HSP23 is very important in the overwintering of *Wohlfahrtia magnifica* pupa. Studies on the cold-tolerance of HSP27 in flies have not been reported at present, but when the *Drosophila mediterranean* are heat stressed, this unigene will maintain cell stability [31], speculating that the HSP27 plays an important role in maintaining cell stability of *Wohlfahrtia magnifica* pupa during the overwinter. HSP67Bc is similar to the earliest found human HSPB8, it enhances the dissolution of denatured protein and amyloid protein [32]. Although the expression of HSP67bc was not significantly different in this experiment, but its expression level increasing with the decreasing temperature means that HSP67Bc plays a certain role in the overwintering process of *Wohlfahrtia magnifica* pupa. The expression of HSP70 and HSC70 in the *Drosophila melanogaster* were increased after it was cold-shocked at -10°C [33]. Results showing that the expression of HSC70-4 and HSP70Ba were significantly different in the cold stressed pupae,

inferring that they are very important to the overwintering process of *Wohlfahrtia magnifica* pupa.

When insects are stimulated by cold stress, AFPs can reduce the freezing point of aqueous solution to produce freeze-tolerance and promote the survival of insects in cold conditions [34]. It is reported that true AFPs with high thermal hysteresis are found in freeze-avoiding animals (those that must prevent freezing, as they die if frozen), especially marine fish, insects and other terrestrial arthropods act as preventing freezing below the temperature at which the organism normally experiences [35], such as *Dorcus hopei binodulosus (Dhb)* synthesizes at least six hyperactive AFPs (Dhb AFP) to enhance its cold-tolerance [36]. Some insects respond to cold stress by using HSPs, because HSPs play an important role in the anti-freezing ability of insects, which improves the survival and adaptability of insects in low temperature environment [37], for example, the expression of HSPs increased in larvae of *Eurosta solidaginis* before and during winter diapause [38]. Insects cold-tolerance are also related to the up-regulation of proline [39], such as no antifreeze protein was found in *Drosophila melanogaster* [40], while high concentration of proline made *Drosophila melanogaster* have cold-tolerance [23]. In addition, pyrroline-5-carboxylate reductase and hyaluronoglucosaminidase precursor in *Culex piniens* were up-regulated during its overwintering diapause [39].

This study showed that HSPs play an important role in the overwintering process of *Wohlfahrtia magnifica*, that laid a foundation for the research of molecular mechanism of *Wohlfahrtia magnifica* overwintering.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author and can be provided on your request.

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Competing Interests

The authors declared that there is no competing interests.

Authors' Contributions

H.B.L. performed the experiment research in detail; summarized and analyzed the experiment results. J.Q.X. consulted a large number of literature and completed the writing of the paper. D.E. planned the experimental research program and implementation process; gave guidance to the writing of the paper. B.X.H. put forward valuable suggestions on the revision and improvement of the paper.

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RESEARCH ARTICLE

Effect of Tocilizumab on *Acinetobacter baumannii* Lung Infection in an Immunosuppressed Rat Model

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Abstract

Our study aimed to investigate effect of tocilizumab on the lung tissue in the presence of *Acinetobacter baumannii* infection in immunosuppressed rats. A forty-eight female Wistar albino rats were divided equally into eight groups: Group 1: Healthy (H), Group 2: Immunosuppressed (IM), Group 3: Healthy rats given *A. baumannii* bacteria (H+BAC), Group 4: Immunosuppressed rats given *A. baumannii* bacteria (IM+BAC), Group 5: Healthy rats given tocilizumab (H+TCZ), Group 6: Immunosuppressed rats given tocilizumab (IM+TCZ), Group 7: Healthy rats given *A. baumannii* bacteria and tocilizumab (H+BAC+TCZ), Group 8: Immunosuppressed rats given tocilizumab and *A. baumannii* bacteria (IM+BAC+TCZ). Fourteen days after the immunosuppression of group 2, 4, 6 and 8 with hydrocortisone, group 3, 4, 7 and 8 were *A. baumannii* was dropped into the trachea. One hour after *A. baumannii* application, TCZ was administered to Groups 5, 6, 7 and 8. NF-κB, IL-6 and NLRP3 mRNA expressions were decreased in the IM group compared to the healthy group (P<0.05). Although NF-κB, IL-6 and NLRP3 mRNA expression decreased in the IM+TCZ group compared to the healthy group (P<0.05) NF-κB, IL-6 and NLRP3 mRNA expression increased in the H+TCZ group (P<0.05). Despite decreasing cytokines, *A. baumannii* has been shown to increase infection-related lung injury. This suggests that in patients currently or recently using steroids, tocilizumab may increase organ damage due to opportunistic infection.

Keywords: *Acinetobacter baumannii, Tocilizumab, Immunosuppressed rat*

Tocilizumab'ın İmmünsüprese Rat Modelinde *Acinetobacter baumannii*'nin Akciğer Enfeksiyonu Üzerindeki Etkisi

Öz

Çalışmamızda, immünsüpresyon oluşturulmuş ratlarda *Acinetobacter baumannii* enfeksiyonu varlığında tocilizumabın akciğer dokusundaki etkisini araştırmayı amaçladık. Toplam kırk sekiz dişi Wistar albino rat sekiz eşit gruba ayrıldı: Grup 1: Sağlıklı (H), Grup 2: İmmünsüprese ratlar (IM), Grup 3: *A. baumannii* bakterisi verilen sağlıklı ratlar (H+BAC), Grup 4: *A. baumannii* bakterisi verilen immünsüprese ratlar, Grup 5: Tocilizumab verilen sağlıklı ratlar (H+TCZ), Grup 6: Tocilizumab verilen immünsüprese ratlar (IM+TCZ), Grup 7: *A. baumannii* bakterisi ve tocilizumab verilen sağlıklı ratlar (H+BAC+TCZ), Grup8: Tocilizumab ve *A. baumannii* bakterisi verilen immünsüprese sıçanlar (IM+BAC+TCZ). Grup 2, 4, 6 ve 8'in hidrokortizon ile immünosupresyonundan 14 gün sonra, grup 3, 4, 7 ve 8'e *A. baumannii* suşu trakeaya transtrakeal yolla enjekte edildi. Grup 5, 6, 7 ve 8'e *A. baumannii* uygulamasından bir saat sonra TCZ verildi. İmmünsüprese grupta (IM) sağlıklı gruba (H) göre NF-κB, IL-6 ve NLRP3 mRNA ekspresyonları azaldı (P<0.05). Her ne kadar TCZ verilen IM grubunda (IM+TCZ) NF-κB, IL-6 ve NLRP3 mRNA ekspresyonu sağlıklı grup (H) ile karşılaştırıldığında azalmış olsa da (P<0.05) NF-κB, IL-6 ve NLRP3 mRNA ekspresyonu sadece tocilizumab uygulanan grupta (H+TCZ) arttı (P<0.05). *A. baumannii* enfeksiyonunda tocilizumab kullanımı, steroidlerle immünsüprese edilmiş ratlarda inflamatuar sitokinleri önemli ölçüde azalttı. Azalan sitokinlere rağmen, *A. baumannii*'nin enfeksiyona bağlı akciğer hasarını arttırdığı gösterilmiştir. Bu, steroid kullanan veya yakın zamanda steroid kullanmış hastalarda tocilizumabın fırsatçı enfeksiyon nedeniyle organ hasarını artırabileceğini düşündürmektedir.

Anahtar sözcükler: *Acinetobacter baumannii, Tocilizumab, İmmünsüprese rat*

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Introduction

Acinetobacter baumannii is an important pathogen belonging to the family Moraxellaceae, is a gram-negative, non-fermentative, aerobic, oxidase-negative, catalase-positive coccobacillus [1,2]. Despite being harmless in healthy individuals, these bacteria can cause various diseases in immunocompromised individuals [3-5]. Due to increased antibiotic resistance in recent years, *Acinetobacter* infections have become the most common cause of community- and hospital-acquired pneumonia in Asian countries, Latin America and European hospitals [6-8]. *A. baumannii* is also the etiological agent of bacteremia, meningitis, endocarditis, and urinary system, skin and soft tissue infections. The capsule structure, adhesion strength, and biofilm formation and secretion systems are among the virulence factors of *A. baumannii*, which affect its role in clinical conditions [9-15]. These bacteria can adhere well to medical devices and cause pneumonia in patients on mechanical ventilators [12,16,17]. Clinically, immunocompromised patients are particularly susceptible to *A. baumannii* infection, although the cause of this susceptibility has not been yet determined. Therefore, very different strategies should be determined in the treatment of immunocompromised patients with *A. baumannii* infection.

In terms of bacterial resistance and difficulties in treatment, *A. baumannii* is one of the most important infectious agents that has been a major problem in the last decade. Due to its capacity to develop resistance to most antibiotics, *A. baumannii* has recently been classified as a 'red alert' human pathogen [18]. Another issue that needs to be discussed in terms of the current situation is the presence of other drugs used by patients in intensive care units. Although there are many different drugs used in intensive care units, steroids have very strong anti-inflammatory effect which can stop the cytokine storm [19]. Glucocorticoids are widely used in human and veterinary medicine due to their potent non-specific immunosuppressive properties. Due to these properties, it is one of the most suitable drug groups for making immunosuppression models in rats. Steroid use can be considered to cause immune suppression through NF-*κB* and AP-1 or neutrophil phagocytic dysfunction. This can increase the risk of nosocomial infections. However, previous studies have reported conflicting results. It has been determined that the downregulation of inflammation due to glucocorticoid therapy reduces the growth factors of bacteria, which decreases the risk of nosocomial infection [20]. A question that requires furthers investigation is how the risk of nosocomial infection development or progression occurs, especially in intensive care patients using steroids. More important is the question of how to treat *A. baumannii* infection in patients who are given steroids or who are immunosuppressed for any reason. In many studies, susceptibility to and severity of *A. baumannii* infection was significantly higher in immunocompromised mice than in non-imminocompromised mice [21]. *A. baumannii* infection activates the host innate immune responses which leads to the production of proinflammatory cytokines such as IL-6 and IL-1 [22]. Immunomodulators that stimulate host innate immunity have potential as stand-alone treatment or as immune supportive for *A. baumannii* infection [22].

Tocilizumab (TCZ) is an immunomodulator that prevents IL-6 from binding to IL-6R. It has been shown that the use of IL-1 and IL-6 antagonists provides significant benefits in hyperimmune diseases such as SARS-CoV [23]. TCZ was previously indicated for use in autoimmune diseases, and Food and Drud Agency extended its use to cytokine release syndrome in 2017 [24]. However, considering the available studies, it seems very important to empirically show how the use of immunosuppressive drugs such as TCZ for cytokine storm would affect the development and progression of secondary nosocomial infections in patients using that are also using steroids. NF-κB and IL-6 are mediators involved in host defense against *A. baumannii* infections. IL-6 is a multifunctional cytokine with both pro-inflammatory and anti-inflammatory properties. While IL-6 released during infection protects the host against the agent, its irregular and continuous release can cause serious complications and death.

The current study aimed to investigate the effects of TCZ use on the lung tissue in the presence of *A. baumannii* infection in immunosuppressed rats at histo-pathological and molecular levels.

Material and Methods

Ethical Statement

The experiments were conducted according to the ethical norms approved by the Atatürk University Ethics Committee of the Experimental Animal Teaching and Research Center (No: 2017/88).

Animals

The rats were obtained from the Medicinal and Experimental Application and Research Center, and kept in standard laboratory conditions under a natural cycle of light and dark. Forty-eight female Wistar Albino rats weighing 200-220 g were used in the study. During the experiments, the animals were supplied enough water *(ad libitum)* and pellet feed. Animals were housed in groups in typical plastic cages in a well-ventilated room at 22±1°C under specific light conditions (14/10 h light/dark cycle) prior to the experiment.

Experiment Groups

The rats were randomly divided into eight groups with six rats in each group. Group 1, 3, 5 and 7 were comprised of rats with normal immunity. Group 2, 4, 6 and 8 were immunosuppressed groups via hydrocortisone.

Group 1: Healthy (H)

Group 2: Immunosuppressed with hydrocortisone (IM)

Group 3: Healthy rats given *A. baumannii* bacteria (H+BAC)

Group 4: Immunosuppressed rats given *A. baumannii* bacteria (IM+BAC)

Group 5: Healthy rats given tocilizumab (H+TCZ)

Group 6: Immunosuppressed rats given tocilizumab (IM+TCZ)

Group 7: Healthy rats given *A. baumannii* bacteria and tocilizumab (H+BAC+TCZ)

Group 8: Immunosuppressed rats given tocilizumab and *A. baumannii* bacteria (IM+BAC+TCZ)

Acinetobacter baumannii Strain

This bacterial strain was isolated by the the Atatürk University Clinical Microbiology Laboratory and placed in refrigerator at -70°C until the experiment. On the day of the experiment, *A. baumannii* was dissolved using the conventional method and standardized to 1 x 10^8 CFU/mL with sterile physiological saline.

Drug Administrations

- Hydrocortisone Administration

First step of the experiment is that to create immunosuppression. Hydrocort-Liyo® 100 mg IM/IV ampoule (Koçak Farma Pharmaceuticals and Chemical Industry Inc. Türkiye) was dissolved in saline. The rats in immunosuppressed groups (Groups 2, 4, 6 and 8) were subcutaneously given hydrocortisone at a dose of 20 mg/kg for 14 days and hydrocortisone administration continued until the experiment was terminated to the 21st day of experiment [25].

- Tocilizumab Administration

One hour after the *A. baumannii* application, tocilizumab (Actemra 400 mg/20 mL IV Concentrate Vial® The Roche Group) was dissolved in saline, diluted and intraperitoneally injected to groups 5, 6, 7 and 8 at a dose of 2 mg/kg to each rat for seven days (14th day of the experiment to 21st day) [26]. At the end of the seventh day (21st day of experiment), experiment was terminated.

Rat Model of Acinetobacter baumannii Infection

Fourteen days after the immunosuppression of group 2, 4, 6 and 8 with hydrocortisone; Group 3, 4, 7 and 8 were anesthetized using an intraperitoneal injection of 10% chloral hydrate (4 mL/kg). After the rats were placed in the supine position, the skin was cut with a cervical midline incision. In order to minimize tissue damage, fascia, the sternocleidomastoid muscle, and parathyroid glands were removed with cotton swabs; and the trachea was surgically exposed. An insulin syringe with a sterile 26 G needle was positioned intratracheally through the tracheal cartilages. In order to confirm that the needle was positioned correctly in the trachea, the syringe plunger was withdrawn to make sure that only air was observed inside the syringe barrel. 100 µL of 1 x 10^8 CFU/mL suspension of *A. baumannii* was dropped to the trachea. After tracheal instillation, the animals were kept upright for 5 min, and then left until they became conscious [27].

Lung Tissue Collection and Term of Experiment

The mental state, breathing, food and water consumption, exercise, temperature, and hair of the rats were observed daily, and the survival rate was recorded. On the 21st day of the experiment, the rats were sacrificed with diethyl ether, and left lung tissue samples were taken and the experiment was terminated.

Real-Time PCR

- Total RNA Extraction and cDNA Synthesis

The tissues (20 mg) were stabilized in RNA Stabilization Reagent (RNAlater, Qiagen), and then disrupted using TissueLyser II (Qiagen). The total RNA was purified using the RNeasy Mini Kit (Qiagen) in a QIAcube (Qiagen) device according to the manufacturer's instructions [28]. The RNA samples were then reverse-transcribed into complementary DNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA concentration and quality were assessed and quantified using the Epoch Spectrophotometer System and Take3 Plate (BioTek).

- Relative Quantification of Gene Expression

The relative NLRP3 (Rn04244622_m1), IL-6 (Rn01410330_m1) and NF-*κB* (Rn01399565_m1) mRNA expression analyses were performed with StepOnePlus Real Time PCR System technology (Applied Biosystems) using cDNA synthesized from rat lung RNA. The real-time reverse transcriptase-polymerase chain reaction (qPCR) was run using the Primer Perfect Probe mix and the TaqMan Probe-based technology (Primer Design Ltd., Southampton, UK), and the results were expressed as the relative-fold compared to the control animals. The gene expression levels were normalized by β-actin (Rn00667869_m1) as a housekeeping gene. For each tissue, triplicate determinations were performed in a 96-well optical plate for both targets using 9 mL of cDNA (100 ng), 1 mL of Primer Perfect Probe mix, and 10 mL of QuantiTect Probe PCR Master mix (Qiagen, Hilden, Germany) in each 20 mL reaction. The plates were heated for 2 min at 50°C and 10 min at 95°C, and then 40 cycles of 15 s at 94°C and 60 s at 60°C were applied. All data were expressed as fold-changes in expression compared to the expression in other animal groups, using the 2-ΔΔCt method [29,30].

Molecular Statistical Analysis

All data were expressed as mean ± SD in each group. All data were subjected to one-way analysis of variance using

IBM SPSS Statistics 20. All parametric data were analyzed with one-way analyses of variance, Tukey's test. P<0.05 was accepted as significant when compared to healthy group.

Histopathologic Examination

Tissue samples taken for the histopathological evaluation as a result of necropsy were fixed in 10% formalin solution for 48 h. Following tissue processing, the samples were embedded in paraffin blocks. 4-µm thick sections were taken from each block. The preparations prepared for the histopathological examination were stained with hematoxylin-eosin (HE) and examined with a light microscope [31]. In the sections examined, the severity of histopathological findings was evaluated as absent (-), very mild (+), mild (++), moderate (+++), severe (++++), and very severe (+++++) *(Table 1, 2, 3, 4)*.

Statistical Analysis

The Kruskal-Wallis test, one of the non-parametric tests, was used for the analysis of the differences between the groups for the data obtained semi-quantitatively in the histopathological examination, and the Mann-Whitney U test was used for the comparison of paired groups. SPSS 13.0 package program was used for all statistical analyses.

Results

Molecular Analysis

It was found that NF-κB mRNA expressions decreased in the steroid immunosuppressed group (IM) compared to the healthy group (H) (P<0.05). NF-κB mRNA expression decreased in the group of immunosuppressed rats given bacteria (IM+BAC) compared to the healthy rats given bacteria (H+BAC) (P<0.05). It was found that NF-κB mRNA expressions decreased in the immunosuppressed group given tocilizumab (IM+TCZ) compared to the healthy group given tocilizumab (H+TCZ) (P<0.05). NF-κB mRNA expressions decreased in the immunosuppression group given tocilizumab and bacteria together (IM+BAC+TCZ) compared to the healthy group given tocilizumab and bacteria together (H+BAC+TCZ) (P<0.05). NF-κB mRNA expressions considerably increased in the healthy group given tocilizumab and bacteria together (H+BAC+TCZ) and in the healthy group given bacteria (H+BAC) compared to the healthy group (H) (P<0.05). NF-κB mRNA expressions considerably increased in the immunosuppressed group given bacteria (IM+BAC) and in the immunosuppressed group given bacteria and tocilizumab together

Table 1. *Scoring according to the thickening of interstitial tissue in lung tissue sections*

Degree of Positivity	Presence of Interstitial Pneumonia
Absent (-)	No finding of interstitial pneumonia
Very mild (+)	Mild interstitial pneumonia in a very small focus in the lung section
Mild (++)	Mild interstitial pneumonia in multiple very small foci in the lung section
Moderate (+++)	Interstitial pneumonia in the form of a few foci in the lung section
Severe (++++)	Interstitial pneumonia in the form of many foci in the lung section
Very severe (+++++)	Diffuse interstitial pneumonia in the lung section

Table 2. *Scoring according to peribronchiolar cell infiltration in lung tissue sections*

Degree of Positivity	Presence of Peribronchiolar Cell Infiltration
Absent (-)	No mononuclear infiltration
Very mild (+)	1-4 mononuclear infiltrations around one bronchus/bronchiole in the lung section
Mild (++)	5-10 mononuclear infiltrations around one bronchus/bronchiole in the lung section
Moderate (+++)	11-20 mononuclear infiltrations around one bronchus/bronchiole in the lung section
Severe (++++)	20-30 mononuclear infiltrations around one bronchus/bronchiole in the lung section
Very severe (+++++)	More than 30 mononuclear infiltrations around one bronchus/bronchiole in the lung section

Table 3. *Scoring according to the presence of granuloma in lung tissue section*

Degree of Positivity	Presence of Granuloma
Absent (-)	No granuloma
Very mild (+)	1-2 granulomas in the lung section
Mild (++)	1-2 granulomas in the lung section
Moderate (+++)	3-4 granulomas in the lung section
Severe (++++)	More than 5 granulomas in the lung section

Table 4. *Scoring of histopathological findings in the lung tissues of rats treated for seven days*

Groups	Interstitial Pneumonia	Degeneration of Bronchial Epithelia	Peribronchiolar Cell Infiltration	Granuloma
Healthy	-	-	-	-
Immunosuppressed	-	-	-	-
H+BAC	++	++	++	-
IM+BAC	+++	+++	+++	-
H+TCZ	-	-	-	-
IM+TCZ	+	-	-	-
H+BAC+TCZ	++++	+++	++++	++
IM+BAC+TCZ	+++++	++++	+++++	+++

BAC: *A. baumannii;* ***TCZ:*** *Tocilizumab;* ***H:*** *Healthy;* ***IM:*** *Immunosuppressed*

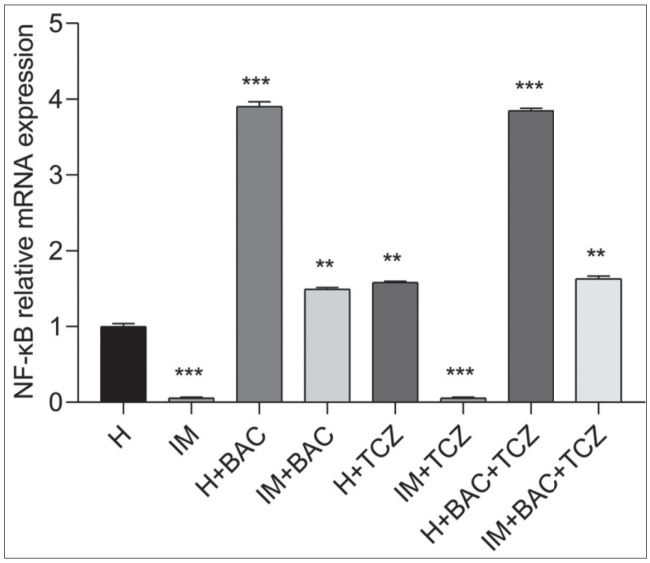

Fig 1. NF-κB relative mRNA expression. NF-*κB* mRNA expression levels in the lung tissues of all the experimental groups. The relative expression levels were calculated by the 2−ΔΔCt method. Each value is mean ± S.D. for six samples in each group. All data were expressed as mean ± SD in each group. All data were subjected to one-way analysis of variance using IBM SPSS Statistics 20. All parametric data were analyzed with one way analyses of variance, Tukey's test. P<0.05 was accepted as significant when compared to healthy group

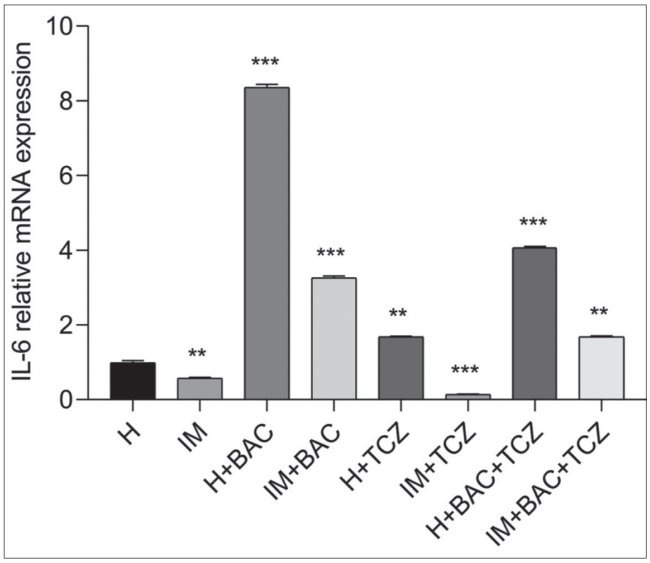

Fig 2. IL-6 relative mRNA expression. IL-6 mRNA expression levels in the lung tissues of all the experimental groups. The relative expression levels were calculated by the 2−ΔΔCt method. Each value is mean ± S.D. for six samples in each group. All data were expressed as mean ± SD in each group. All data were subjected to one-way analysis of variance using IBM SPSS Statistics 20. All parametric data were analyzed with one way analyses of variance, Tukey's test. P<0.05 was accepted as significant when compared to healthy group

(IM+BAC+TCZ) compared to the immunosuppressed group (IM) (P<0.05) *(Fig. 1)*.

It was found that IL-6 mRNA expressions decreased in the steroid immunosuppressed group (IM) compared to the healthy group (H) (P<0.05). IL-6 mRNA expression decreased in the group of immunosuppressed rats given bacteria (IM+BAC) compared to the healthy rats given bacteria (H+BAC) (P<0.05). It was observed that IL-6 mRNA expressions decreased in the immunosuppressed group given tocilizumab (IM+TCZ) compared to the healthy group given tocilizumab (H+TCZ) (P<0.05). IL-6 mRNA expressions decreased in the immunosuppression group given tocilizumab and bacteria together (IM+BAC+TCZ) compared to the healthy group given tocilizumab and bacteria together (H+BAC+TCZ) (P<0.05). IL-6 mRNA expressions considerably increased in the healthy group given tocilizumab and bacteria together (H+BAC+TCZ) and in the healthy group given bacteria (H+BAC) compared to the healthy group (H) (P<0.05). IL-6 mRNA expressions increased in the immunosuppressed group given bacteria (IM+BAC) and in the immunosuppressed group given bacteria and tocilizumab together (IM+BAC+TCZ) compared to the immunosuppressed group (IM) (P<0.05) *(Fig. 2)*.

It was found that NLRP3 mRNA expressions decreased in the steroid immunosuppressed group (IM) compared to the healthy group (H) (P<0.05). NLRP3 mRNA expression decreased in the group of immunosuppressed rats given bacteria (IM+BAC) compared to the healthy rats given bacteria (H+BAC) (P<0.05). It was observed that NLRP3 mRNA expressions decreased in the immunosuppressed

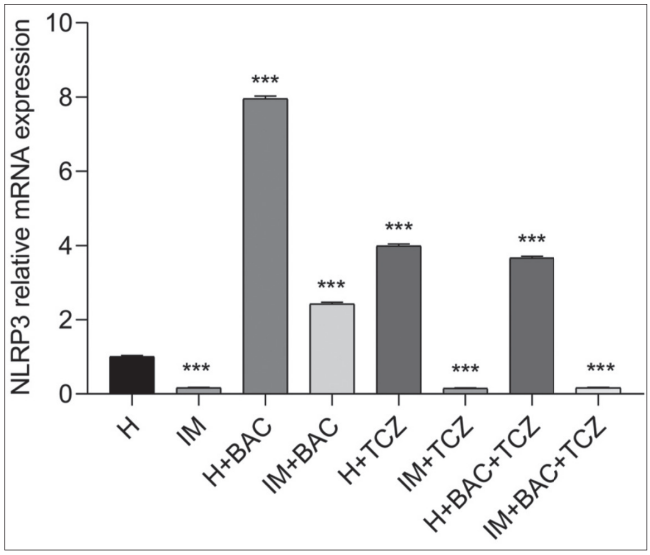

Fig 3. NLRP3 relative mRNA expression. NLRP3 mRNA expression levels in the lung tissues of all the experimental groups. The relative expression levels were calculated by the 2−ΔΔCt method. Each value is mean ± S.D. for six samples in each group. All data were expressed as mean ± SD in each group. All data were subjected to one-way analysis of variance using IBM SPSS Statistics 20. All parametric data were analyzed with one way analyses of variance, Tukey's test. P<0.05 was accepted as significant when compared to healthy group

in the healthy group given bacteria (H+BAC) and in the healthy group given tocilizumab (H+TCZ) compared to the healthy group (H)(P<0.05). NLRP3 mRNA expressions increased in the immunosuppressed group given bacteria (IM+BAC) compared to immunosuppressed group (IM) (P<0.05). About NLRP3 mRNA expressions, there was no statistical difference among the groups of immunosuppressed group given tocilizumab (IM+TCZ), immunosuppressed group given bacteria and tocilizumab together and immunosuppressed group (IM) *(Fig. 3)*.

Histopathological Analysis of Lung Tissues

When the lungs were examined histopathologically, it was determined that healthy group (H) and immunosuppressed group (IM) had a normal histological appearance *(Fig. 4-A,B)*. It was determined that the group of healthy rats given bacteria (H+BAC) had a mild interstitial pneumonia, desquamation of bronchiolar epithelia, lymphocytic cell infiltration surrounding bronchi-bronchioles, and vascular hyperemia *(Fig. 4-C)*. The group of immunosuppressed rats given bacteria (IM+BAC) had a moderate interstitial pneumonia, desquamation of the bronchial-bronchiolar epithelia, surrounded by mononuclear cell infiltration and vascular hyperemia *(Fig. 4-D)*. Statistically significant

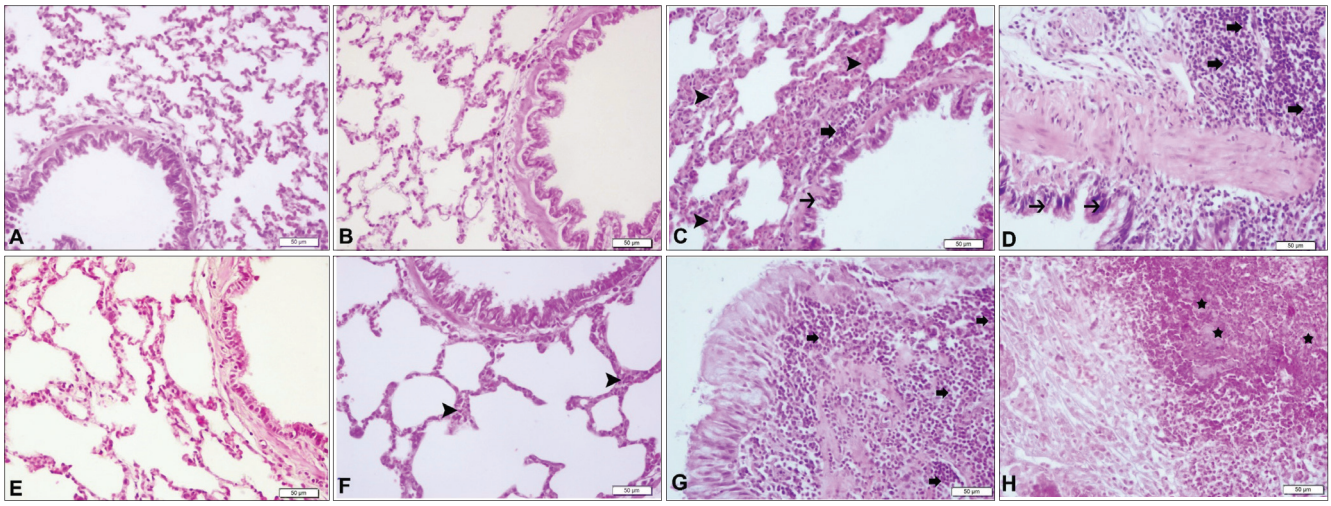

Fig 4. Lung Tissue H&E staining. Healthy group **(A)**, Immunosuppressed group **(B)**, Healthy rats given *A. baumannii* bacteria *(C)*, Immunosuppressed rats given *A. baumannii* bacteria **(D)**, Healthy rats given tocilizumab **(E)**, Immunosuppressed rats given tocilizumab **(F)**, Healthy rats given *A. baumannii* bacteria and tocilizumab **(G)**, Immunosuppressed rats given tocilizumab and *A. baumannii* bacteria **(H)**. Interstitial pneumonia (arrowheads), degeneration of bronchial epithelia *(thin arrows)*, peribronchiolar cell infiltration *(thick arrows)*, granuloma *(star)*, H&E, Bar: 50 µm. The Kruskal-Wallis test, one of the non-parametric tests, was used for the analysis of the differences between the groups for the data obtained semi-quantitatively in the histopathological examination, and the Mann-Whitney U Statistical analysis test was used for the comparison of paired groups. SPSS 13.0 package program was used for all statistical analyses

group given tocilizumab (IM+TCZ) compared to the healthy group given tocilizumab (H+TCZ) (P<0.05). NLRP3 mRNA expressions decreased in the immunosuppression group given tocilizumab and bacteria together (IM+BAC+TCZ) compared to the healthy group given tocilizumab and bacteria together (H+BAC+TCZ) (P<0.05). NLRP3 mRNA expressions considerably increased in the healthy group given tocilizumab and bacteria together (H+BAC+TCZ), difference (P<0.05) was detected when compared to group H.

It was determined that the group of healthy rats given tocilizumab (H+TCZ) had a normal histological appearance of the bronchi-bronchioles and alveoli *(Fig. 4-E)*. The group of immunosuppressed rats given tocilizumab (IM+TCZ) had a very mild interstitial pneumonia *(Fig. 4-F)*. When the

healthy group given tocilizumab and bacteria together (H+BAC+TCZ) were evaluated it was found that the lungs had interstitial pneumonia, lymphoid hyperplasia, degeneration of the bronchial-bronchiol epithelium, mild granuloma, and hyperemia in the vessels *(Fig. 4-G)*. Statistically significant difference ($P<0.05$) was detected when compared to healthy group (H). When the immunosuppressed group given tocilizumab and bacteria together (IM+BAC+TCZ) were evaluated it was found that the lungs had a very severe interstitial pneumonia, moderate granuloma, dense bacterial clusters in the middle of the granulomas, severe degeneration of the bronchiole epithelium *(Fig. 4-H)*. Statistically significant difference ($P<0.05$) was detected when compared to healthy group (H).

The histopathological results of the groups are summarized in *Table 4*. The presence of Interstitial Pneumonia, Peribronchiolar Cell Infiltration and Granuloma in the lung tissues are given in *Table 1*, *Table 2* and *Table 3*, respectively.

Discussion

In this study, we investigated the effect of IL-6 receptor antagonist on the lungs and systemic inflammatory response in healthy and immunosuppressed rats in which pneumonia was induced by *A. baumannii*. When we examined our results, we found that NF-*κB*, NLRP3 and IL-6 mRNA expressions significantly decreased in rats that were given steroids (group 2, 4, 6, 8) compared to the healthy rats (group 1, 3, 5, 7). NF-*κB*, NLRP3 and IL-6 mRNA expressions significantly increased in the groups in which *A. baumannii* pneumonia was induced without immunosuppression while this increase was significantly reduced in the immunosuppressed rats infected with *A. baumannii*. Our results indicate that treatment with IL-6 receptor antagonists caused a significant decrease in systemic inflammatory response in rats with *A. baumannii* pneumonia in the presence of steroid immunosuppression compared to the rats with *A. baumannii* pneumonia without steroid immunosuppression. Considering only the histopathological analyses, the results were reserved. The use of IL-6 receptor antagonist in *A. baumannii* infection in immunosuppressed rats caused significant lung damage compared to the rats that were not given the drug.

Lung injury due to *A. baumannii* infection is a clinical condition that causes serious morbidity and mortality. Pneumonia infections caused by *Acinetobacter* have been reported to increase the mortality rate to 42% in immunocompromised patients treated in the intensive care unit [32]. The increased release of immune modulatory mediators is required to stop this process and achieve recovery. It is important to understand the basics of host-bacterial interactions, especially the host immune response, for the development of effective treatments against *A. baumannii*, which has recently become the leading cause of pneumonia [33]. Different innate immune cells such as monocytes, macrophages, dendritic cells and natural killer cells have been identified as important factors in the body's defense against *A. baumannii*, and among them, neutrophils represent an important immune cell indispensable for the control of infection [33]. With the activation of alveolar macrophages in *Acinetobacter* infections, the production of mediators such as IL-6, tumor necrosis factor alpha (TNF-α) and NF-*κB* increase and play the main protective role for the host against the causative agent [34]. IL-6 is released from infected or lesioned cells and recognized by the pathogen recognition receptors of immune cells. These receptors consist of toll-like receptors, retinoic acid-inducible gene-1-like receptors, nucleotide-binding oligomerization domain-like receptors, DNA receptors, and NOD-like receptors [35]. In addition, IL-6 released during infection plays a supporting role for the immune system by regulating NF-κB synthesis and increasing TNF-α, and IL-1 β mRNA transcription [36]. Steroids with a very strong anti-inflammatory effect can stop the cytokine storm [19]. However, the use of steroids can be considered to lead to immunosuppression through NF-κB and AP-1 and by causing neutrophil phagocytic dysfunction. This can increase the risk of nosocomial infections. However, studies in the literature report conflicting results. It has been determined that the down-regulation of inflammation due to glucocorticoid therapy reduces the growth factors of bacteria, which decreases the risk of nosocomial infection [37]. In an *in vitro* study, glucocorticoid use downregulated inflammation, and LPS-activated monocytes were shown to reduce the gene transcription of TNF-α, IL-1β, and IL-6 [38]. Similar results have been obtained from clinical studies [39]. Studies have shown that inflammation caused by infections has a bidirectional effect. It is stated that cytokines that increase with inflammation can also be a growth factor for bacteria [37]. In addition to treatment with many steroids in immunosuppressed rat models, among the most commonly used methods are those involving the use of hydrocortisone [40]. In our study, the immunosuppression model was successfully created with the hydrocortisone administration. The use of hydrocortisone reduced NLRP3 expression and IL-6 and NF-κB levels, with the expected lung damage remaining lower.

In this study, we showed that especially in *A. baumannii* infections, the reduction of immunosuppression; i.e., increased cytokine storm could reduce the prevalence of infection and the damage it can cause. However, this was not the only focus of our study. In fact, this work was an experimental demonstration of problems that exist in many intensive care patients and always present a difficult time for the clinician. The balance between pro-inflammatory and anti-inflammatory mediators regulates the inflammatory process, which includes adhesion, chemotaxis, phagocytosis of invading bacteria, bacteria killing, and phagocytosis of debris from injured tissue. However, if this proinflammatory and anti-inflammatory mediator

balance is not disturbed, homeostasis can be restored by eliminating bacterial invasion. Otherwise, it progresses to sepsis, shock, and multiorgan insufficiency due to the direct effect of the microorganism, as well as the effect of proinflammatory cytokines [31]. Therefore, a well-planned anti-cytokine therapy and anti-inflammatory therapy is very important. The question was what we would clinically face if an IL-6 receptor antagonist was required in a patient developed *A. baumannii* pneumonia when on steroids. We demonstrated, both at molecular and histopathological levels, that the use of steroids could prevent the development of damage due to *A. baumannii* pneumonia, contrary to expectations.

NOD-like group inflammasomes are complexes of the natural immune response and they are multiproteins released at the time of tissue damage, inflammation, and infection. It has recently been stated that inflammasomes which are complexes located in the cytoplasm of multiple proteins, are responsible for the maturation of pro-inflammatory cytokines such as IL-1β and IL-18, and the initiation of pyroptosis, a highly inflammatory form of programmed cell death [30]. Inflammasomes sense either microbial stimuli or danger and send protective signals to the host. This signaling and regulation are achieved through proinflammatory cytokine release or proptosis induction. NLRP3 is one of the strongest inflammatory signal proteins among inflammasomes. It is one of the most important response elements in inflammatory response to acute respiratory distress syndrome, sepsis, and many bacterial, parasitic, viral, and fungal infections [41]. In individuals without immune system disorders, these cytokines are regulated, while in immunosuppressive cases, the host becomes susceptible to infection with the lack or absence of these cytokines. Thus, immunomodulatory therapies are seen as a strategy in the combat against *A. baumannii* infections in patients with a weak immune system [42]. IL-6, being rapidly produced through pathogen-linked molecular patterns or molecules that recognize damage-dependent molecular patterns, triggers innate immune responses in a manner that is controlled by transcriptional and posttranscriptional mechanisms; however, the irregular continued release of IL-6 has pathological effects leading to chronic inflammation and autoimmunity [43].

Damage-related inflammatory response is specific to infection, and IL-6 release increases in severe injuries and sepsis. Although IL-6 plays a role in protecting the host as a proinflammatory response, serious complications and even death can be seen as a result of its continuous and irregular release. TCZ is a humanized anti-human antibody of the immunoglobulin G1 class that prevents IL-6 from binding to IL-6R. It has been shown that the use of IL1 and IL6 antagonists provide significant benefits in diseases presenting hyperinflammation, such as SARS-CoV [23]. Although TCZ was previously only indicated for use in autoimmune diseases, the FDA extended its use to cytokine release syndrome in 2017 [24]. In a clinical study, it was shown that TCZ was used in 38% of intensive care unit-acquired bloodstream infections. This shows that this drug, which can play an active role in cytokine storm, can also contribute to many infections [44]. However, in the same study, it was emphasized that this increased risk could further increase with steroid use. There is no information about clinical progression in these patients. Therefore, the current study was the first to provide an insight into the potential results of *A. baumannii* reproduction in patients using both steroids and TCZ. Our results showed that *A. baumannii* pneumonia induced in rats that were given steroids could lead to a worse state with TCZ use. With these results, we showed that more attention should be paid to the immune status and risk of infection in the transition to TCZ use in patients using steroids. Our findings also indicated that while the use of TCZ with steroids suppressed cytokine production very strongly, this substantially reduced cytokine level led to the inability to stop *A. baumannii* infection and exacerbated lung damage.

In this study, the use of TCZ in the presence of *A. baumannii* infection significantly decreased inflammatory cytokines in rats immunosuppressed with steroids. However, despite the decreasing amounts of cytokines, *A. baumannii* was shown to increase lung damage due to infection. This suggests that in patients with a current or recent use of steroids, tocilizumab can increase organ damage due to opportunistic infection. Therefore, more care should be taken in patients using tocilizumab together with steroids.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (D. Çelebi) on reasonable request.

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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Author Contributions

DC, ZH and OC conceived and supervised the study. DC, ZH and IC carried out animal experiments. KTC and IC made laboratory measurements. NA, IH and HK collected and analyzed data. SY applied the histopathological examination

of the study. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Comparison Between Four Laboratory Tests for Routine Diagnosis of Enzootic Bovine Leukosis

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Abstract

The aim of this study was to compare the diagnostic capabilities of the agar gel immunodiffusion test (AGID) and two types of PCR, nested PCR (with nucleic acid extraction from blood) and direct blood nested PCR (db nested PCR) - without extraction, vs. enzyme-linked immunosorbent assay (ELISA) as the gold standard in the routine diagnostics of this disease. A total of 409 blood samples were obtained from cattle 18 mo. to 5 yrs. of age, and all the samples were analyzed using the four assays. Following the initial testing, the samples were stored at -20°C and re-tested using all the four techniques after a month of freeze storage to determine the reproducibility of the results. ELISA detected 57 animals as positive (13.9%) versus 33 ones using AGID and 56 using the two types of nested PCR. AGID showed low sensitivity of 57.9% and moderate agreement compared to ELISA. In addition, AGID did not show consistency in the results from the two independent measurements. The two types of nested PCR showed nearly full agreement with ELISA with a kappa value of 0.99. Since AGID showed lower sensitivity and lack of reproducibility in the results for 22 samples as compared to the other techniques used in this study, we suggest that the future application of this test for the diagnosis of Enzootic bovine leucosis in blood samples should be reconsidered. On the other hand, db nested PCR demonstrated very good sensitivity and reproducibility of results, it also requires less sample processing. All this makes it potentially suitable for routine diagnostics.

Keywords: *AGID, db nested PCR, ELISA, Enzootic bovine leukosis, Nested PCR*

Enzootik Sığır Lökozunun Rutin Teşhisi İçin Kullanılan Dört Laboratuvar Testinin Karşılaştırılması

Öz

Bu çalışmanın amacı, agar jel immünodifüzyon testi (AGID) ve iki tip PCR'nin, nested PCR (kandan nükleik asit ekstraksiyonu ile birlikte) ve kanda direkt nested PCR (db nested PCR) - ekstraksiyon olmaksızın, tanısal yeteneklerinin bu hastalığın rutin teşhisinde altın standart olarak kullanılan Enzyme-Linked Immunosorbent Assay (ELISA) ile karşılaştırılmasıydı. Çalışmada, 18 ay ile 5 yaş arası sığırlardan toplam 409 kan örneği toplandı ve tüm örnekler bu dört yöntem ile analiz edildi. İlk analizleri takiben, örnekler -20°C'de saklandı ve sonuçların tekrarlanabilirliği açısından bir aylık dondurularak depolamanın ardından dört yöntem ile yeniden analiz edildi. ELISA ile 57 (%13.9) hayvan pozitif saptanırken, AGID ile 33 ve her iki tip nested PCR ile 56 hayvan pozitif saptandı. ELISA'ya kıyasla AGID, %57.9'luk düşük sensitivite ve orta düzeyde uyum gösterdi. Ayrıca AGID, iki bağımsız ölçümden elde edilen sonuçlarla da tutarlılık göstermedi. İki tip nested PCR yöntemi de 0.99 kappa değeriyle ELISA ile neredeyse tam uyum gösterdi. Bu çalışmada kullanılan diğer tekniklere kıyasla AGID, 22 örnekte daha düşük sensitivite gösterdiği için ve tekrarlanabilirliği olmadığı için kan örneklerinde Enzootik sığır lökozunun teşhisinde bu testin gelecekte uygulanmasının yeniden düşünülmesi gerektiğini öneriyoruz. Diğer taraftan, db nested PCR çok iyi bir sensitivite ve tekrarlanabilirlik gösterdi, ayrıca daha az örnek işlenmesine ihtiyaç duydu. Bütün bunlar, db nested PCR'yi rutin teşhis için potansiyel olarak uygun hale getirmektedir.

Anahtar sözcükler: *AGID, db nested PCR, ELISA, Enzootik sığır lökozu, Nested PCR*

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Introduction

Enzootic bovine leukosis (EBL) is a well-known infectious disease in cattle worldwide. Natural infection has been shown in buffalo, zebu and capybara in some regions [1,2]. EBL is caused by an oncovirus, bovine leukemia virus (BLV), which belongs to the *Deltaretrovirus* genus of the *Retroviridae* family [3]. BLV has a strong affinity for B-lymphocytes, but also infects other cells involved in the immune defenses. Owing to the oncogenic properties of the causal agent and the immune response imbalance, animals develop tumours of various clinical manifestation depending on their location [4]. Reportedly, lymphosarcoma develops in up to 10% of infected cattle, whereas persistent lymphocytosis, in 30 to 70% of animals [5]. In most cases, the integration of viral RNA in the form of proviral DNA in the host cell genome results in asymptomatic infection which facilitates the spread of the pathogen among sensitive populations [1]. The modes of BLV transmission are well known. The current understanding is that the major mode of transmission is horizontal and that the sources of infection in herds are animals with a proven high viral load [6,7].

Besides the above-mentioned natural hosts, some other animal species are susceptible to experimental infection, sheep being most sensitive and developing tumors at a younger age than cattle [8]. Studies have investigated the association between BLV and mammary cancer in human. Giovanna et al.[9] and Buehring et al.[10] found that the presence of proviral DNA in breast tissue was associated with a neoplastic process, whereas Zhang et al.[11] observed no association between BLV and breast cancer in Chinese women. Another study from Iran reported a possible association between BLV and development of some types of lymphoma in humans [12].

Enzootic bovine leukosis causes serious direct and indirect losses to farmers in countries with developed cattle breeding [13,14]. Early detection of infected animals using modern diagnostic approaches is essential for the management of EBL in cattle farms. Agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) have been approved by the OIE as techniques for detection of anti-BLV antibodies in serum or milk [15]. In cases when antibody titers are very low or absent, e.g. in young calves with colostrum antibodies, different types of polymerase chain reaction (PCR) assays have been developed to detect proviral DNA in samples from virus-infected animals, including tumor tissue [16-19]. One of the innovations in EBL diagnosis is the use of PCR without prior DNA extraction [20]. This prompted us to adapt the nested PCR recommended by the OIE [15], as well as db nested PCR by omitting the proviral DNA extraction step from whole blood.

The introduction of state disease control programs based on diagnostic methods together with culling of positive animals has led to limitation or complete elimination of EBL in a lot of Western European countries, Scandinavia and Oceania [21]. There have been reports of high seroprevalence in North and South America, Eastern Europe and Asia [7,22]. The seroprevalence in Bulgaria varied from 0.00% to 63.85% in different regions in 2012, according to Sandev et al.[23].

The aim of this study was to perform comparative analysis of the diagnostic capabilities of laboratory tests, agar gel immunodiffusion test (AGID), nested PCR and db nested PCR vs. enzyme-linked immunosorbent assay (ELISA) as the gold standard in the routine diagnostics of EBL.

Material and Methods

A total of 409 blood samples were collected from cattle 18 months to 5 years. of age. The animals were reared in farms in the South-Central Region of Bulgaria. The blood samples were tested using four laboratory assays. All samples were taken aseptically by jugular vein puncture and were collected in Vacutainer™ collection tubes with EDTA K3 anticoagulant (Wenzhou Gaode Medical Instrument, China) for proviral DNA extraction and in sterile Serum Blood collection tubes, 5 mL (Wenzhou Gaode Medical Instrument, China) for the serological assays.

Agar Gel Immunodiffusion

The Bovine Leukosis POURQUIER AGID test (Institut Pourquier, France) was used for detection of anti-gp51 antibodies, according to the manufacturer's instructions. Petri dishes loaded with the components: antigen, positive controls and test samples, were incubated at 22°C in a humid camera and were inspected for lines of precipitation every 24 h over 3 days. The sera were re-assayed independently following storage at -20°C for 1 month.

Enzyme Linked Immunosorbent Assay (ELISA)

A competitive ELISA kit was used for the detection of antibodies against the gp51 envelope protein of BLV (IDVet, France). According to the manufacturer's instructions, we followed the brief procedure, with initial incubation at 21°C±5°C for 45 min ± 4 min. The optical density (OD) values were read at 450 nm using a microplate photometer (Biosan, Latvia). The results were validated and interpreted according to the following criteria and equations in the manufacturer's instructions:

Validation: mean value of negative controls (2 wells C1 and D1) higher than 0.7 (OD_{NC}>0.7); mean value of positive controls (OD_{PC}) (2 wells A1 and B1) at least 30% that of the negative control (OD_{NC}), or OD_{PC}/OD_{NC} <0.3;

Interpretation: Competition % = (OD_{SAMPLE}/OD_{NC}) x 100 *(Table 1)*.

Table 1. Interpretation of competitive ELISA results	
Result	**Status**
% Competition ≤ 50%	Positive
50 < % Competition < 60%	Doubtful
% Competition ≥ 60%	Negative

The sera were re-assayed using ELISA independently following storage at -20°C for 1 month.

Nested PCR Assay

Proviral DNA was obtained from whole blood (100 µL) using the ISOLATE II Genomic DNA Kit (Bioline, UK). PCR was performed in 25 µL reaction volume: 12.5 µL MyTaq red PCR mix (Bioline, UK), 3.0 µL DNA, 1 µL primers (produced by Jena Bioscience, Germany) each in a working concentration of 10 pmol/µL and 7.5 µL molecular biology grade water (Bioline, UK). The reactions were run in a Quanta Biotech Termal Cycler (Quanta Biotech, UK) with the following temperature profile for the first-round PCR: denaturation at 95°C for 3 min; 35 cycles of 30 s at 95°C, 45 s at 59.9°C and 60 s at 72°C; followed by 7 min at 72°C. The primer sequences, their positions and the PCR product sizes [15] are shown in *Table 2*.

Since the GenBank NCBI database is being constantly updated with new data, we checked the specificity of the primers recommended by the OIE for BLV diagnosis to make sure they are up to date. The primer verification and update was done using the Basic Local Alignment Search Tool (BLASTn) available at the National Center for Biotechnology Information (NCBI; Bethesda, MD). The multiple alignments of the nucleotide sequences and primers were performed with MUSCLE (Edgar, 2004) by MEGA5 software.

To optimize the nested-PCR, we ran consecutive gradient reactions. The annealing temperature range for the first primer pair (BLV-env-1 and 2) was 55.1°C - 66.2°C, and for the second one (BLV-env-3 and 4), 50.1°C - 66.3°C. The amplifications were run both with initial DNA template and with PCR products. The nested PCR temperature profile was as follows: Denaturation at 95°C for 3 min, 30 cycles, denaturation at 95°C for 30 s, annealing at 59.9°C or 61.1°C for 30 s, extension at 72°C for 50 s, final extension at 72°C for 7 min, storage at 4°C.

db Nested PCR

PCR was also performed without DNA extraction, directly on blood using a MyTaq Blood - PCR-Kit (Bioline, UK). The primers were the first primer pair that we used in the nested-PCR. The PCR program was optimized in terms of the step durations: denaturation for 15 s, annealing for 30 s, extension for 45 s, according to some requirements of the kit. The same PCR program was used with the internal primer pair. The amplification was run with 1, 2 and 3 µL of blood. The samples were re-assayed independently following storage at -20°C for 1 month.

The quality and quantity of the obtained DNA/PCR products were determined by DNA/RNA calculator GeneQuant II (Pharmacia LKB, Biochrom, UK) and by 2% agarose gel electrophoresis (Gene Shun Biotech, China) with a 100 bp DNA ladder (Bioline, UK). The electrophoresis conditions were: 120 V, 45 mA, 30 min.

Sequencing

To confirm the specificity of the products obtained after the first- and second-round PCR, 5 samples each were sequenced using a DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Giles, UK) in both directions with a forward and reverse primer. The obtained sequencing products were analyzed in a capillary MegaBACE™ 1000 automatic sequencer (Amersham Biosciences). Two controls, M13mp18DNA and MegaBACE 4 Colour Standart, of the kit used for the sequencing reaction and reading were included, respectively. The obtained sequences were analyzed by BLASTn (NCBI; Bethesda, MD).

Statistical Analysis

The agreement between the assays was evaluated using Cohen's kappa statistic according to McHugh [24], and the sensitivity and specificity of the assays were calculated according to Fenner et al.[25], ELISA was used as the gold standard [19].

Results

ELISA identified antibodies against BLV in 57 out of the 409 tested blood sera (13.9%). Of these, 27 showed doubtful/ inconclusive results in the first assay but proved negative following the confirmatory procedure in the ELISA kit.

Table 2. Primer sequences, position and product size used in the nested PCR for BLV detection			
Primers for *env* gp51 Gene	**Sequence (5'-3')**	**Position**	**Product Size, bp**
BLV-env-1	TS'TGTGCCAR'GTCTCCCAGATA	5032–5053	598
BLV-env-2	AACAACAACCTCTGGGAAGGG	5629–5608	-
BLV-env-3	CCCACAAGGGCGGCGCCGGTTT	5099–5121	444
BLV-env-4	GCGAGGCCGGGTCCAGAGCTGG	5542–5521	-
'updated positions			

Table 3. *Comparison between AGID and ELISA in BLV detection assays*

Tests Results	ELISA Positive	ELISA Negative	Total
AGID Positive	33	9	42
AGID Negative	24	343	367
Total	57	352	409

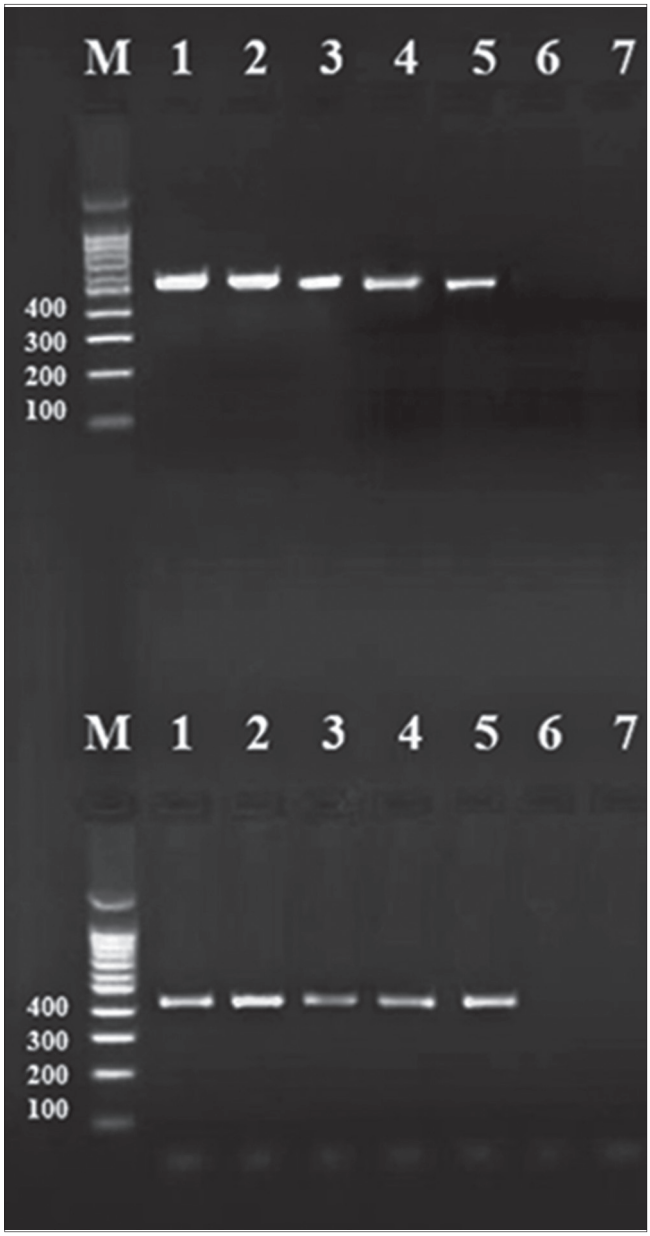

Fig 1. Results from nested and db nested PCR using two primer pairs, BLV-env-1 and 2 (top); BLV-env-3 and 4 (bottom); M - DNA Ladder 100 bp. Top, BLV-env-1 and 2: 1 - sample x; 2 - sample y; 3 - amplification of sample y directly from 1 µL blood; 4 - amplification of sample y directly from 2 µL blood; 5 - amplification of sample y directly from 3 µL blood; Bottom: 1 - sample x; 2 - sample y; 3 - amplification of sample y directly from 1 µL blood; 4 - amplification of sample y directly from 2 µL blood; 5 - amplification of sample y directly from 3 µL blood; 6 - negative sample by ELISA and AGID; 7 - negative control PCR

Table 4. *Comparison between nested PCR/db nested PCR and ELISA in BLV detection assays*

Tests Results	ELISA Positive	ELISA Negative	Total
Nested/db Nested PCR Positive	56	0	56
Nested/db Nested PCR Negative	1	352	353
Total	57	352	409

Table 5. *Data of kappa statistics showing agreement between AGID and ELISA and between nested PCR and ELISA*

Parameters	AGID/ELISA	Nested and db Nested PCR/ELISA
% Agreement	91.93	99.76
Kappa value	0.62	0.99
SE of kappa	0.06	0.01
95% CI	0.505 - 0.739	0.97 - 1.000

SE - standard error of kappa; CI - confidence interval

The results from the comparative analysis between AGID and ELISA are shown in *Table 3*. AGID identified 33/57 of the sera as positive, 9 of which as false positive. In addition, the results were inconsistent for a total of 22 sera in the first and the second testing. The sensitivity and specificity of the test were 57.9% (95% CI - 44.08 - 70.86) and 97.44% (95% CI - 95.20 - 98.82), respectively.

Gradient PCR on extracted DNA using the first primer pair had an optimum annealing temperature range of 59.7-62.6°C for amplification of a 598-bp product, and 57.0-64.3°C when a PCR product served as the template. The optimum annealing temperature range for the second primer pair was 58.9-61.1°C and 54.8-63.5°C, respectively, with a product size of 444 bp. The amplicons were best visualized using annealing temperature of 59.9°C for the BLV-env-1 and 2 primers, and 61.1°C for the BLV-env-3 and 4 primers, which we used in the subsequent analyses. The results from the nested and db nested PCR are shown in *Fig. 1*. Specific amplification was obtained both from extracted DNA and from whole blood, as well as using the three tested volumes of blood.

There was a second fragment about 100 bp in size in four of the positive samples. The sequencing procedure produced high background electropherograms, which did not allow BLASTn analysis to determine the origin of this fragment.

The sequencing results of the products from the first- and second-round PCR showed that products of about 538 bp and 397 bp, respectively, were suitable for analysis. BLASTn (NCBI) analysis confirmed that they belonged to BLV.

Table 4 presents the results from nested PCR and db nested PCR as compared to ELISA. There were no differences between the two PCR assays. As seen from *Table 4*, there

was inconsistency between nested PCR and db nested PCR vs. ELISA in only 1 sample, which tested negative by nested PCR/db nested PCR but positive by ELISA. The sensitivity of the nested PCR and db nested PCR was 98.25% (95% CI - 90.61 - 99.96%) and the specificity, 100.00% (95% CI - 98.96% - 100.00%).

The agreement between AGID and ELISA was 91.93%, with a kappa value of 0.62. The agreement of nested PCR and db nested PCR with ELISA was 99.76% and 0.99, respectively *(Table 5)*.

There were no differences between the results from the two independent tests run one month apart using either ELISA, or nested PCR and db nested PCR.

Discussion

The OIE Manual [15] recommends polymerase chain reaction, enzyme-linked immunosorbent assay and agar gel immunodiffusion test as suitable assays for the diagnosis of enzootic bovine leukosis. PCR detects sequences of the BLV *env* gene, whereas ELISA and AGID can detect antibodies against gp51, which is encoded by the *env* gene, and against the internal protein p24, which is encoded by the *gag* gene [26]. Antibodies against these proteins form shortly after infection onset and are detectable throughout the life of cattle [27]. In this study, we used four assays to test blood samples twice, the two tests being one month apart, to compare their effectiveness in the routine diagnosis of BLV.

A study has shown that AGID does not detect anti-gp51 antibodies in sera with low antibody titers associated with some physiological conditions in animals, such as advanced gestation or in the first days after calving, as well as antibodies in milk serum [28]. In our study, 42% of infected animals gave false negative results for the presence of antibodies in the AGID test. This was probably due to some of the above-mentioned factors in some of the animals. On the other hand, persistent co-infection with other viruses, such as bovine viral diarrhea virus, could lead to suppressed antibody formation against BLV [29]. Such cattle are an important source of infection from an epidemiological point of view. A drawback of AGID is the subjective factor in reading the results [30]. In our study, the results were interpreted independently by three experienced researchers, with no inconsistencies in the scoring. The second AGID testing produced inconsistent results in 22 sera: positive in the first test but negative in the second test following the freeze-thaw cycle. These are cases of weakly positive samples with faint lines of precipitation that remain undetectable possibly owing to partial antibody degradation in the second testing despite the low-temperature stability [31]. In addition, considering the type (size) of the antigen participating in the AGID reaction, which is a precipitation reaction in nature, the result will depend on the ratio between the antibody and antigen concentrations, forming a precipitation curve [32]. Besides, Rivers and Jones [33], who studied the titer of four types of IgG after 12 freeze–thaw cycles, reported that the titer of three types of IgG decreased two times after the first cycle. Based on these considerations, when a standard antigen concentration is used and the antibody concentration decreases as a result of a single freeze-thaw cycle of low-titer serum, consequently, the line of precipitation will shift to the left and visible complexes will not form. Such scenario could most likely explain the discrepancy between the results in the two AGID tests. This, along with the low sensitivity make AGID unsuitable in cases when there is irregular funding for EBL testing and samples need to be kept frozen until delivery of reagents.

Nine samples produced a false positive result, 4 of which in both tests, which was another surprising observation in this study. It could possibly be attributed to cross-reactive immune response in a natural infection with a genetically closely related retrovirus such as human T cell leukemia virus type I [34]. In addition, the k-statistic showed moderate agreement with ELISA with a kappa value of 0.62. Lojkić et al.[35] tested 12 AGID-negative sera and detected three positive samples using ELISA. Of 225 AGID-negative sera, Gonzalez et al.[36] found 69 ELISA-positive ones. Higher sensitivity of AGID than the 57.9% observed in our study was reported by Trono et al.[37]: 79.7% since AGID scored 36 out of 178 false-negative samples vs. PCR and southern blot analysis. In many countries, the costs of diagnostic tests for monitoring and elimination of EBL from farms are covered by the owners (incl. in the case of import and export of animals). Bulgaria is no exception. Thus, it is important to apply assays with high sensitivity and specificity to reduce the economic costs of farms [38] in the long run.

The high sensitivity of ELISA observed in this and other studies [26], the automated execution and interpretation of results and the use of minimum amounts of reagents make ELISA a very convenient screening method in the study of blood and milk sera to control the disease [39]. When compared to AGID, ELISA can detect anti-BLV antibodies earlier, from 3 to 12 weeks from infection onset [40], which is an advantage. Regardless of the ELISA kit used for antibody detection, Kuczewski et al.[41] reported that 5 kits produced by different companies showed high agreement between assays with kappa values of k=0.91 and k=1.

The procedures that we used for BLV detection by nested PCR and adapted db nested PCR generated amplicons of the expected size. The results showed that besides nested PCR, db nested PCR can also be used successfully as a key test in routine EBL diagnostics and as a confirmatory test for serological assays. In four of the samples, there was an additional amplified fragment which we were unable to identify after sequencing. Nevertheless, we could speculate that it might be attributed to activation of an endogenous retrovirus as a result of BLV proviral DNA integration, DNA

breakage during extraction [42] or a fragment resulting from *env* gene transcription. We could exclude the possibility of it being a fragment resulting from PCR because the same band appeared with different DNA concentrations.

In our study, there was just one sample that tested negative by nested PCR and db nested PCR but positive by ELISA. Similar results have been reported by Gregory et al.[43], who tested blood samples from cattle and interpreted 36/40 samples as positive using nested PCR, whereas 37/40 ones using ELISA. In another study, Villalobos-Cortés et al.[44] also observed ELISA to be more sensitive than nested PCR by 13%. Such discrepancy could possibly be explained by low virus titer in the infected animal, which remains below the detection threshold of nested PCR. The presence of proviral DNA and the low percentage of virus-infected cells, however, lead to constant stimulation of the immune system, which responds by producing antibodies. These antibodies - albeit present in a low amount - are detectable by ELISA [30,35]. Another reason could be a strong cytotoxic and humoral response in the first 1-8 weeks of viral infection [45], together with subsequent clearance and transition into latency via proviral integration. During latency, just 1:50 000 peripheral blood cells contain enough viral transcripts for them to be detected by *in situ* hybridization, a method of comparable sensitivity to PCR [46]. On the other hand, it is possible for not all peripheral blood mononuclear cells to be infected with the virus [27,47]. The samples that produced doubtful/inconclusive results in ELISA proved negative in the subsequent confirmatory assay, which entailed additional costs and time consumption, and was fully in agreement with the results from nested/db nested PCR. The diagnostic abilities of the three assays in our study were good, as evidenced by the kappa value of 0.99, which is interpreted as nearly perfect agreement according to McHugh [24]. A study reported 100% agreement between PCR and ELISA results [48]. Conventional PCR and INTA-ELISA showed over 90% agreement [37]; and direct filter PCR and ELISA showed 97.6%, strong agreement [19] with a kappa value of 0.88. Other authors report higher sensitivity of PCR compared to ELISA and nested PCR [17,49], however, this concerns mainly real-time quantitative PCR analysis, which still finds limited application in the routine diagnosis of EBL. In case of doubtful/inconclusive ELISA results, and following the kit's confirmatory procedure, PCR is recommended as an arbitration technique for detection of BLV proviral DNA [15].

The higher sensitivity of the PCR assays in our study, as compared to other reports of lower sensitivity of PCR vs. ELISA, could possibly be attributed to the capability of detecting more virus variants following the update of the first primer pair. Regarding the diagnostic abilities of direct blood (db) PCR, without DNA extraction, Nishimori et al.[20] observed lower sensitivity compared to nested PCR, but 100% specificity and reproducibility of results in cattle blood samples. In our study, there was full agreement between the results obtained using nested PCR and db nested PCR. This could probably be due to the fact that we used the same primers. These results indicate that, regardless of whether DNA is extracted or not, these particular PCR procedures will produce equally reliable results.

In conclusion, the low AGID sensitivity of 57.9% in this study and the inconsistent results it produced for 22 samples in two independent tests suggest that the future use of this assay in the routine EBL diagnostics should be reconsidered. The choice of method depends on the testing purpose and the population size. In eradication programs, it would be inappropriate to use AGID. This analysis would also be unsuitable for screening purposes in small farms because of the high error rate. Its implementation may be justified, to a certain extent, in large farms for initial screening; however, a positive result and confirmation by ELISA would increase the costs. Although AGID has advantages in terms of speed, ease of performance and no need of specific equipment, other highly specific assays, such as PCR and ELISA, will be required for disease control and successful monitoring programs.

Availability of Data and Materials

Datasets analyzed during the present study are available in the authors on reasonable request.

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Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

NR, IS and MCh designed the study; IS and MCh performed the samples collection and laboratory assays; NR analysed the data; NR, IS and MCh wrote the paper..

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RESEARCH ARTICLE

Serum Intestinal Fatty Acid-Binding Protein and Calprotectin Concentrations to Assess Clinical Severity and Prognosis of Canine Parvovirus Enteritis [1]

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Abstract

This study was conducted to assess the usefulness of serum intestinal fatty acid-binding protein (IFABP) and calprotectin (CALP) concentrations in comparison with other biomarkers [total leucocyte counts (TLC), C- reactive protein (CRP) and procalcitonin (PCT)] in predicting the clinical severity and prognosis of Canine Parvoviral (CPV) enteritis. Ten healthy dogs (CON group) and 40 dogs with natural CPV enteritis (INF group) were used. The INF group was also divided into survivor and non-survivor. Blood samples were collected twice in the INF group and once in the CON group. The clinical health score (CHS) was calculated for each patient by scoring certain clinical findings. Serum CRP and IFABP, and plasma PCT concentrations of the INF group at hospital admission (0 h) were significantly higher than in the CON group. Compared to the survivor subgroup, mean serum PCT and IFABP concentrations in the non-survivor subgroup were significantly higher at both 0 h and after initiation of treatment (24 h), while the mean TLC was significantly lower at 24 h. The correlation between CHS and serum IFABP (r=0.501; P=0.000) was stronger than other biomarkers evaluated. Based on the sensitivity and specificity from the Receiver Operating Characteristic curve analysis, TLC (24 h) and serum IFABP (0 h) serve as the most valuable biomarkers among the parameters in this study to predict the prognosis of CPV enteritis.

Keywords: *Canine parvovirus enteritis, Biomarker, Intestinal fatty acid-binding protein, Calprotectin*

Kanin Parvoviral Enterit'in Klinik Şiddeti ve Prognozunu Değerlendirmede Serum İntestinal Yağ Asidi Bağlayıcı Protein ve Kalprotektin Konsantrasyonları

Öz

Bu çalışma, köpeklerde Parvoviral (CPV) enteritin klinik şiddetini ve prognozunu öngörmede serum intestinal yağ asidi bağlayıcı protein (IFABP) ve kalprotektin (CALP) konsantrasyonlarının kullanılabilirliğinin, diğer belirteçlerle [toplam lökosit sayıları (TLC), C-reaktif protein (CRP) ve prokalsitonin (PCT)] karşılaştırmalı olarak değerlendirmek için gerçekleştirildi. On sağlıklı köpek (CON grubu) ve 40 doğal CPV enteritisli (INF grubu) köpek kullanıldı. INF grubu da hayatta kalan (survivor) ve hayatta kalmayan (non-survivor) olarak ikiye ayrıldı. INF grubundan iki ve CON grubundan bir kez kan örnekleri alındı. Belirli klinik bulgular puanlanarak her hasta için klinik sağlık skoru (CHS) hesaplandı. Hastaneye yatışta (0. saat) INF grubunun serum CRP, IFABP ve plazma PCT konsantrasyonları CON grubundan anlamlı derecede yüksekti. Survivor altgrubu ile karşılaştırıldığında, non-survivor altgrubunun ortalama serum PCT ve IFABP konsantrasyonları, hem 0. saatte hem de tedavi başlangıcından 24 saat sonra (24. saat) önemli ölçüde yüksekti, ortalama TLC ise 24. saatte önemli ölçüde düşüktü. CHS ile serum IFABP (r=0.501; P=0.000) arasındaki korelasyon, değerlendirilen diğer biyobelirteçlerden daha güçlüydü. Alıcı İşletim Karakteristiği eğrisi analizinden elde edilen duyarlılık ve özgüllüğe dayalı olarak, TLC (24. saat) ve serum IFABP (0. saat), CPV enteritinin prognozunu tahmin etmek için bu çalışmanın parametreleri arasında en değerli biyobelirteçler olarak hizmet ederler.

Anahtar sözcükler: *Kanin parvoviral enterit, Biyobelirteç, Intestinal yağ asidi bağlayıcı protein, Kalprotektin*

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Introduction

Canine parvovirus (CPV) enteritis is a common acute viral disease that mainly affects dogs younger than six months [1]. In infected puppies, the tissues most affected by CPV are intestinal epithelium, lymphoid tissue, and bone marrow [2,3]. The virus reaches the intestinal mucosa through the bloodstream, replicating in the germinal epithelium of the intestinal crypts [2]. It induces severe and extensive necrosis of the epithelial cells, loss of intestinal glands, and villous atrophy or collapse [4,5]. All of these contribute to disrupting the gastrointestinal mucosal barrier, which allows Gram-negative and anaerobic bacteria translocation from the intestinal lumen [6]. Thus, intestinal injuries caused by CPV result in hemorrhagic enteritis, systemic inflammatory response syndrome (SIRS), sepsis and endotoxemia [2,6]. Furthermore, the CPV leads to the destruction of leukocyte precursors in the bone marrow. Along with the destruction or collapse of the thymic cortex, this condition results in a significant decrease in total leukocyte count (TLC) in infected animals [1,7]. Insufficient immunity, combined with SIRS and sepsis, puts affected animals at high risk of developing septic shock, multiple organ failure, and even death if left untreated [7]. In this context, the mortality rate can reach 20-48% in puppies despite the aggressive treatment protocol [2,8]. Considering the high morbidity (up to 100%) and mortality rates of CPV enteritis in puppies, it is crucial to determine the prognosis of the disease. So, there is a clear need for biomarkers that are compatible with the complex pathophysiology of CPV and reflect the disorders of the affected patient.

In CPV enteritis, one of the factors affecting the clinical severity and prognosis of the disease in the patient is systemic inflammation [9,10]. Therefore, the biomarkers of inflammation are of clinical interest. C- reactive protein (CRP) is considered as a positive major acute-phase protein in dogs and one of the most widely used biomarkers for inflammation [10]. Several researchers [9,11] have revealed a significantly higher serum CRP concentration in dogs with CPV in comparison to healthy dogs. It was also demonstrated that CRP is a potent predictor of mortality in dogs with CPV enteritis [9]. On the other hand, it did not prove to be a good predictor of the outcome when used alone [10].

Procalcitonin (PCT), the precursor of calcitonin, is another valuable biomarker of sepsis [12,13]. In both human and veterinary medicine, PCT is released in response to microbial toxins and specific pro-inflammatory mediators, and its concentrations in serum rise early after exposure to an infectious stimulus [12-15]. Although the condition of serum PCT concentration in CPV enteritis was evaluated in one study [11], to the best of our knowledge, no study assessed the prognostic role of PCT in this disease.

Calprotectin (CALP) belongs to calgranulin protein family, calcium-binding cytosolic proteins mainly present in neutrophils, monocytes, macrophages, and epithelial and endothelial cells [16,17]. Concentrations of CALP rise at the site of local inflammation following its release from activated and dying cells [18,19]. As a relatively low molecular-weight protein (36.5 kDa), it diffuses freely from inflamed tissues into circulation and can be measured in serum/plasma [18,19]. Evidence for these mechanisms has prompted studies dealing with CALP in inflammatory disease in both human and veterinary medicine [16,17]. Significant increases in serum CALP concentration were described in dogs with inflammatory diseases such as inflammatory bowel disease, systemic inflammatory response syndrome, and sepsis [19,20]. The releasing mechanism of CALP and promising results in some studies [17,19,20] indicate that CALP is a potential marker of inflammation in dogs as well as in humans.

Plasma/serum CRP, PCT, and CALP concentrations are inflammatory biomarkers, and these parameters are not tissue/organ-specific [9,10,12,13,18]. It is known that the most radical method used in determining intestinal damage is a histopathological examination from tissue biopsy taken endoscopically. However, endoscopic examination is not considered practical, especially in cases like CPV enteritis that have an acute onset and require urgent intervention. In the last years, Intestinal Fatty Acid Binding Protein (IFABP) is considered as a non-invasive biomarker to evaluate the loss of intestinal wall integrity and dysfunction of the intestinal barrier in human medicine [21-23]. IFABP is an intracellular protein specifically secreted in abundance in the epithelial cells of the mucosal layer of intestinal tissue [21-23]. The presence of IFABP, especially in the mature epithelium of intestinal villi, facilitates leakage from enterocytes into the circulation when intestinal mucosal damage occurs [21-23]. Recently, two promising studies [8,24] on serum IFABP concentrations for prognostic value in dogs with CPV enteritis have been published.

The current study aims to assess the usefulness of serum IFABP and CALP concentrations in comparison with other inflammatory markers (TLC, CRP and PCT) in predicting the clinical severity and prognosis of the disease in dogs with CPV enteritis.

Material and Methods

Ethical Statement

All study procedures were reviewed and approved by the Animal Research Ethics Committee of the Aydin Adnan Menderes University, under protocol number 64583101/2018/133.

Animals

In this prospective study, a total of 50 dogs, including the healthy control (CON) group (n=10) and the CPV infected (INF) group (n=40), were evaluated. Dogs in

the INF group have been subdivided into two groups as survivors and non-survivors within 7 days of initiation of treatment *(Fig. 1)*. The INF group dogs were presented to the Aydin Adnan Menderes University Veterinary Teaching Hospital between January 2019 to February 2020 with the complaint of prominent clinical symptoms of CPV enteritis such as weakness, reduced appetite, vomiting, diarrhea/hemorrhagic diarrhea, dehydration. The dogs in the INF group were of various breeds (21 mixed, 6 Golden Retrievers, 5 German Shepherd, 3 Rottweiler, 2 English Cocker Spaniel, 2 Yorkshire Terrier, and 1 Pug), sex (14 males and 26 females), and ages (between two and six months). The dogs in this group were not vaccinated against CPV. The dogs in the CON group were selected similarly to the INF group in terms of breed (6 mixed, 2 Golden Retrievers, 1 German Shepherd, and 1 Yorkshire Terriers), sex (4 males and 6 females), age (between 2 and 6 months) and vaccination status (unvaccinated). The dogs in this group were found to be healthy according to the clinical, haematological and faecal examinations (CPV, *Isospora* spp. and *Giardia* spp.). The diagnosis in CPV enteritis suspected dogs was confirmed by determining the CPV antigen in faeces with a rapid test kit (Catalog No: E-AD-C023; Canine Parvovirus Antigen Lateral Flow Assay Kit, Elabscience Biotechnology Inc, USA). The sensitivity of 98.8%, specificity of 98.5%, and accuracy values of 98.65% for the detection of CPV antigen in this test kit are specified by the manufacturer. The faecal samples of study dogs were also examined microscopically twice, at admission to the hospital (0 h) and the 24 h of the treatment, for *Isospora* oocysts by hyperosmolar sugar flotation method and *Giardia* trophozoites by zinc sulphate flotation method. The dogs whose faecal samples were found negative in both parasitological examinations were included in the study.

Clinical Examinations

A clinical examination was performed on each dog in the INF group at admission and then daily until they heal or death. Certain clinical (body temperature and heart and respiratory rates) and haematological findings were used to evaluate the general health status of the INF dogs and to determine the presence of SIRS in the patients. In this way, dogs were evaluated according to the SIRS criteria reported by Hauptman et al.[25] on admission and were defined as SIRS positive (+) or SIRS negative (-). Furthermore, appetite, and severity and character of diarrhea, vomiting, depression, dehydration, exhaustion, and the physical aspect of faeces in the INF group were noted. Dogs in the INF group were examined once a day at 0 h (pre-treatment) and 24 h initiation of treatment. Each of the above-mentioned symptoms was scored from 0 to 3 or 4, the maximum score being 20 for death [26]. Thus, clinical health scores (CHS) for each patient were calculated at 0 and 24 h using the score assignment scheme for clinical symptoms reported by Martin et al.[26].

Sample Collection and Measurements

Faecal samples were collected from the rectum to plastic containers for parasitological examinations in the INF group at 0 and 24 h and once in the CON group. For clinical examination, blood sampling and laboratory measurements in the INF group were performed twice at hospital admission (0 h), at the 24 h initiation of treatment, and once for dogs in the CON group.

Blood samples were taken from *V. cephalica antebrachii* into tubes with lithium heparin (Vacutainer, Beckton, Dickenson) and serum separation tubes (Vacutainer, Beckton, Dickenson). Complete blood cell count has been performed with the automated blood cell counter (Abacus Junior Vet 5; Diatron MI Zrt., Hungary) using samples with lithium heparin within 30 min after blood collection. The remaining blood samples with lithium heparin were centrifuged at 2000 x g for 10 min to obtain plasma, and these samples were stored at -20°C until PCT concentrations were measured. Blood samples in serum separation tubes were centrifuged after clot retraction at 2000 x g for 10 min to obtain sera. CRP measurement from serum samples was performed with a point of care device (EUROLyser, Salzburg, Austria) using solo cCRP tests, according to the instructions provided by the manufacturer. Subsequently, serum samples were stored at -20°C until IFABP and CALP measurements. The plasma PCT (#MBS7606532, MyBioSource, Inc., USA) and, serum IFABP (#MBS2605533, MyBioSource, Inc., USA) and CALP (#CSB-EQ013485DO, Cusabio Biotech Co., China) concentrations were measured by using commercially

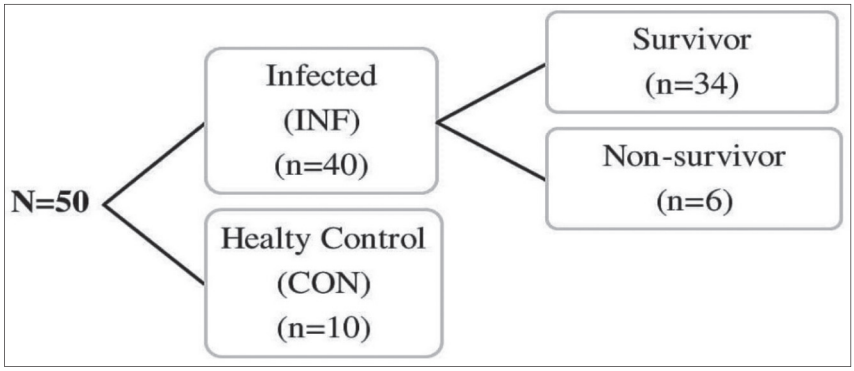

Fig 1. Grouping of dogs evaluated in this study and the number of dogs in each group

available canine-specific enzyme-linked immunosorbent assay kits following the manufacturer's instructions.

Treatment Protocol

Dogs infected with CPV were hospitalized for at least 24 h in separate cages, although hospitalization times varied depending on the severity of the disease. The discharged dogs have been admitted to our hospital for examinations and treatments twice a day until they recovered.

In general, the treatment protocol consisted of fluid therapy, antibiotic therapy, gastrointestinal support, nutritional support, supportive care, and the efficacy of the treatment was monitored. The treatment protocol used in this study was adapted from Prittie [2], Goddard and Leisewitz [27], and Judge [28]. The degree of dehydration, the presence of hypovolemia or hypovolemic shock were evaluated with a comprehensive clinical examination. The dose and rate of initial therapy varied with the patient. The first goal of fluid therapy was to correct the intravascular volume deficit. For patients displaying shock or hemodynamic compromise symptoms, initial fluid resuscitation was begun with rapid intravenous administration (7-12 mL/kg IV over 10 min) of a Lactated Ringers Solution. This procedure was repeated until signs of hemodynamic compromise were no longer present. When significant hemodynamic improvement is not achieved within 30 min of fluid therapy, the bolus of synthetic colloid hydroxyethyl starch was administered at a dose of 3-5 mL/kg, given intravenously over 10 min to prolong the effectiveness of crystalloid therapy. Lactated ringer and potassium chloride (20 mEq/L) added 5% dextrose solutions were used in the dehydrated dogs but not in shock and the maintenance fluid treatment of dogs in shock. The hydration deficit was calculated according to the degree of dehydration. This fluid volume is administered to the patient over 8-24 h and the patients' daily fluid requirement (60 mL/kg/24 h). The β-lactamase resistant penicillin (amoxicillin-clavulanate, 12.5 mg/kg SC every 12 h, at least 5 d) was used as an antimicrobial. In addition, metronidazole (10 mg/kg IV every 12 h, at least 3 d) was combined with this antibiotic in SIRS+ dogs. Butorphanol (0.1 mg/kg IV) was administered in dogs in need of pain relief. Maropitant citrate at a concentration of 1 mg/kg (SC every 24 h) was administered for 3-5 days until vomiting had ceased. Nil per os was ordered for an initial 6-h period following admission to the hospital, especially for dogs with severe vomiting and diarrhea. After suppressed vomiting, it was gradually switched to food with an easily digestible carbohydrate and a lean protein source.

The clinical examinations and treatment plans of the dogs with CPV were made by the same clinician (CDA) in order to prevent any changes that may occur due to the discretion of the clinician.

Statistical Analysis

Statistical analyses were performed using SPSS 19.0 (IBM Corporation, Armonk, USA) and MedCalc 19.1.3 (MedCalc Software bvba, Ostend, Belgium). The distributions of all parameters were checked with the Shapiro–Wilk test. Except CALP, all parameters showed normal distribution, and CALP was not distributed normally despite log transformation. Means, standard error of means (SEM), medians, and interquartile ranges (IQR) for each evaluated parameter were calculated using descriptive statistics. Parameters were analyzed using parametric (TLC, CRP, PCT and IFABP) and non-parametric (CALP) tests under consideration of their distributions.

Firstly, the above-mentioned parameters in the CON group were compared separately with 0 h and 24 h of the INF group by the independent sample t-test or the Mann-Whitney U test. Then, the INF group was divided into two subgroups as survivor and non-survivor. For the parameters evaluated, the intergroup differences between these three groups (CON, survivor and non-survivor) were assessed for each sampling time using one-way analysis of variance with post-hoc Tukey or Kruskal-Wallis tests. Additionally, dependent samples t-test or Wilcoxon Signed Rank test was used to evaluate the differences between 0 and 24 h for the INF group and each subgroup (survivor and non-survivor). The Pearson correlation coefficients *(r)* were calculated for the correlations between CHS and TLC, CRP, PCT, and IFABP. The Spearman correlation coefficient (rho) was calculated for the correlation between CHS and CALP. The strength of the linear relationship was assessed using the coefficient of determination reported by Chan [29].

The prognostic cut-off values, the area under the curve (AUC), P-value, standard error, sensitivity (%), specificity (%) for the best differentiation between survivors and non-survivors were analyzed by receiver operating characteristic (ROC) curve analysis for each parameter in both sampling times. A P-value <0.05 was considered statistically significant for all analyses.

The sample size was estimated for ROC analysis by MedCalc 19.1.3, with an AUC value of 0.8 or 0.85, an α error of 0.05, a power of 0.8 and a prevalence of non-survivors of 20%.

Results

The most common clinical findings of dogs in the INF group were inappetence-anorexia (97.5%), haemorrhagic diarrhea (72.5%), non-haemorrhagic diarrhea (25%), and vomiting (70%). Also, 65% of dogs (26 cases) in the INF group were evaluated according to the SIRS criteria by Hauptman et al.[25] were found to be SIRS positive (+). Of the 40 dogs in the INF group, 34 recovered (survivor group), and 6 died (non-survivor group) within 7 days of starting treatment. Thus, the mortality of dogs infected with CPV in this study was noted as 15%. The mean survival time of the non-survivor dogs was 3.33±0.61 days from the initiation of the treatment. In addition, 5 of the 6

Table 1. *Description of parameters in the healthy CON group (once) and the survivor and non-survivor groups (0 h and 24 h)*

Marker	Groups	0 h	24 h	P [2]
CHS	CON	-		
	survivor	10.60±0.83[a]	8.48±0.72[a]	0.000
	non-survivor	17.33±0.71[b]	15.83±1.32[b]	0.151
	P[1]	0.000	0.000	
TLC (10^9 cells/L)	CON	12.88±0.75[ab]		
	survivor	17.07±1.74	16.95±2.50[a]	0.941
	non-survivor	12.28±3.58	4.57±1.79[b]	0.022
	P[1]	0.213	0.034	
CRP (mg/L)	CON	13.95±1.68[a]		
	survivor	74.32±7.66[b]	60.68±2.69[b]	0.076
	non-survivor	75.69±3.48[b]	65.05±4.83[b]	0.104
	P[1]	0.000	0.000	
PCT (pg/mL)	CON	29.54±4.01[a]		
	survivor	59.30±8.69[a]	45.10±7.38[a]	0.006
	non-survivor	141±50.87[b]	113.39±37.63[b]	0.323
	P[1]	0.002	0.002	
CALP (mg/L)	CON	1.94(1.23-3.02)		
	survivor	2.87(1.25-4.01)	2.21 (1.47-4.35)	0.216
	non-survivor	6.3(1.81-9.56)	5.82 (1.80-16.56)	0.753
	P[1]	0.135	0.255	
IFABP (ng/mL)	CON	2.93±0.29[a]		
	survivor	5.44±0.35[b]	4.15±0.22[b]	0.000
	non-survivor	7.41±0.86[c]	5.50±0.76[c]	0.017
	P[1]	0.000	0.001	

The values have been expressed as mean ± standard error of means for CHS, TLC, CRP, PCT and IFABP, and median (interquartile ranges) for CALP. ***P[1]*** *refers to the significance at the same time points between groups. The different letters [(a, b, c)] indicate the statistical significances (P< 0.05) among the groups.* ***P[2]*** *expresses the significance of the change in time within the same group.* ***CON:*** *healthy control group;* ***CHS:*** *clinical health score;* ***TLC:*** *total leucocyte count;* ***CRP:*** *C- reactive protein;* ***PCT:*** *procalcitonin;* ***CALP:*** *calprotectin;* ***IFABP:*** *intestinal fatty acid-binding protein*

dogs in the non-survivor subgroup were determined to be SIRS + dogs.

The mean CHS of the non-survivor group was significantly (P=0.000) higher than the survivor group both at 0 h and 24 h *(Table 1)*. The mean CHS of survivors was decreased significantly (P=0.000) at 24 h after initiation treatment whereas the values between 0 h and 24 h did not differ significantly in the non-survivor group.

There was no statistically significant difference in TLC in dogs with CPV enteritis at 0 h and 24 h compared to the healthy control dogs *(Fig. 2-A)*. However, the mean TLC of the non-survivor group at 24 h (4.57±1.79) decreased dramatically compared to 0 h (12.28±3.58). Thus, the mean TLC of the non-survivor group was found to be significantly (P=0.011) lower than the survivor group at the 24 h of treatment *(Table 1)*.

Serum CRP concentrations were significantly higher (P=0.000) in dogs with CPV enteritis at 0 h and 24 h than in the healthy CON dogs *(Fig. 2-B)*. However, there was no significant difference in CRP concentrations between survivor and non-survivor groups at both sampling times *(Table 1)*.

While the mean plasma PCT concentration of the INF group at 0 h was found to be statistically higher than the CON group, no significant difference between the two groups in the mean PCT concentrations at 24 h *(Fig. 2-C)*. In addition, the plasma PCT concentrations of the non-survivor group were statistically higher than both the survivor and CON groups at both sampling times. Furthermore, PCT concentrations of the survivor subgroup decreased at 24 h of the treatment compared to the 0 h (pre-treatment). In contrast, the PCT concentrations did not change over time in the non-survivor group *(Table 1)*. In terms of serum CALP concentrations, both the 0 and 24 h values in the INF group were not statistically different from the CON group *(Fig. 2-D)*. Although the CALP concentrations of the non-survivor group were numerically higher than those of the survivor and CON groups at both 0 and 24 h, these elevations did not reach statistical significance *(Table 1)*.

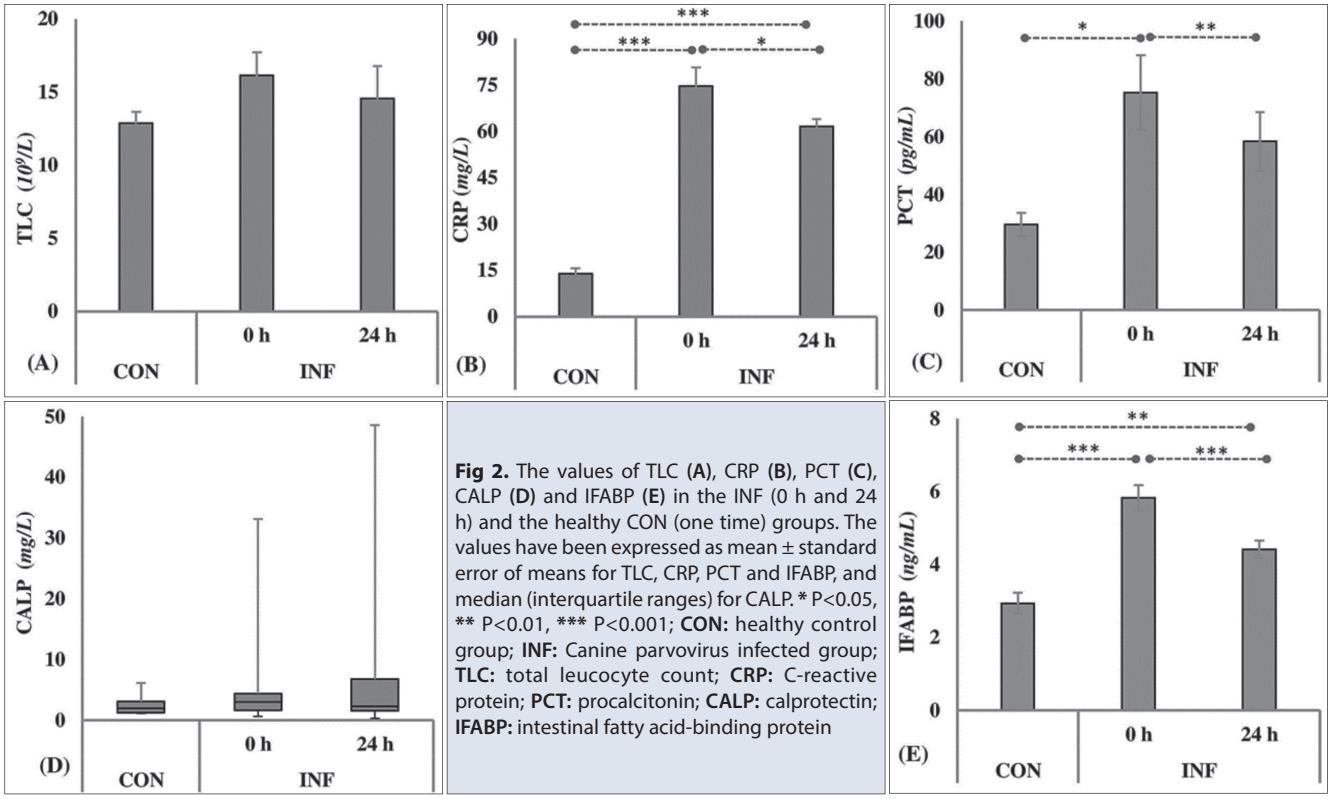

Fig 2. The values of TLC **(A)**, CRP **(B)**, PCT **(C)**, CALP **(D)** and IFABP **(E)** in the INF (0 h and 24 h) and the healthy CON (one time) groups. The values have been expressed as mean ± standard error of means for TLC, CRP, PCT and IFABP, and median (interquartile ranges) for CALP. * P<0.05, ** P<0.01, *** P<0.001; **CON:** healthy control group; **INF:** Canine parvovirus infected group; **TLC:** total leucocyte count; **CRP:** C-reactive protein; **PCT:** procalcitonin; **CALP:** calprotectin; **IFABP:** intestinal fatty acid-binding protein

Table 2. *Correlation coefficients (r/rho) and significance (P) levels between CHS and biomarkers in CPV-infected dogs*

Parameters		Correlation Coefficients	P-Value
CHS	TLC	*r* = -0.195	P = 0.130
	CRP	*r* = 0.375	P = 0.003
	PCT	*r* = 0.271	P = 0.033
	CALP	*rho* = 0.389	P = 0.002
	IFABP	*r* = 0.501	P = 0.000

CHS: *clinical health score;* ***TLC:*** *total leucocyte count;* ***CRP:*** *C- reactive protein;* ***PCT:*** *procalcitonin;* ***CALP:*** *calprotectin;* ***IFABP:*** *intestinal fatty acid-binding protein*

Serum IFABP concentrations of the INF group on both sampling days were significantly higher than that of the CON group *(Fig. 2-E)*. The mean IFABP concentrations of the non-survivor, survivor, and CON groups were significantly different from each other at both the 0 and 24 h of the study. Also, IFABP concentrations of the non-survivor group were significantly higher than the survivor and CON groups on both days. Besides, time-dependent changes of mean serum IFABP concentration within the group were found to be significant for both the survivor (P=0.000) and non-survivor (P=0.017) groups *(Table 1)*.

To reveal the relationship between the clinical severity of the disease and measured parameters, correlation analyses between the CHS and these biomarkers were performed considering both 0 and 24 h values of all canine parvovirus infected dogs. As seen in *Table 2*, while the relationship between CHS and TLC was not significant (*r*=-0.195; P=0.130), the positive correlations were found significant between CHS and CRP (*r*=0.375; P=0.003), PCT (*r*=0.271; P=0.033), CALP (*rho*=0.389; P=0.002) and IFABP (*r*=0.501; P=0.000).

The ROC curves *(Fig. 3)* were drawn using survivor and non-survivor dogs at 0 and 24 h to determine the prognostic roles of measured parameters in dogs with CPV enteritis. Data from ROC analysis were offered in *Table 3*. In this context, the ROC analysis on 0 h for the utility of CRP and IFABP in differentiating in the INF group between the survivor and non-survivor dogs estimate an AUC of 0.733 (cut-off value of >69.05 mg/L with 83.33% sensitivity and 64% specificity) and 0.80 (cut-off value of >6.39 ng/mL with 83.3% sensitivity and 80% specificity), respectively. The ROC curve analysis also indicated that TLC and PCT were effective in distinguishing survivors from non-survivors at 24 h, with the AUCs of 0.873 (cut-off value of ≤5.04 10^9/L with 83.33% sensitivity and 94.3% specificity) and 0.78 (cut-off value of >47.86 pg/mL with 66.7% sensitivity and 85.7% specificity), respectively. On the other hand, the CALP was not effective enough to predict mortality at both 0 h and 24 h.

Discussion

Canine parvovirus enteritis is among the most common causes of gastrointestinal emergencies in puppies [30]. Considering both its' prevalence and the poor outcomes, predicting the severity and prognosis of the disease

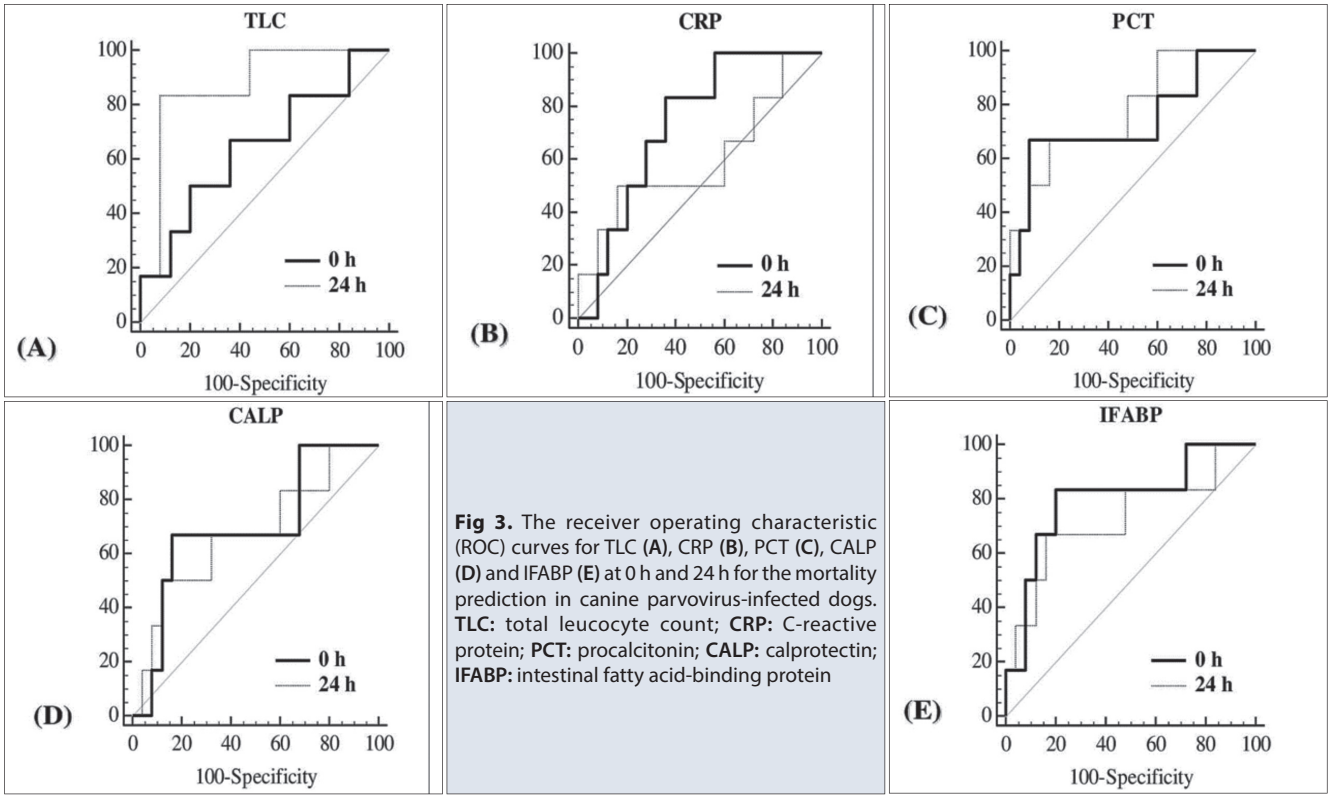

Fig 3. The receiver operating characteristic (ROC) curves for TLC **(A)**, CRP **(B)**, PCT **(C)**, CALP **(D)** and IFABP **(E)** at 0 h and 24 h for the mortality prediction in canine parvovirus-infected dogs. **TLC:** total leucocyte count; **CRP:** C-reactive protein; **PCT:** procalcitonin; **CALP:** calprotectin; **IFABP:** intestinal fatty acid-binding protein

Table 3. *The receiver operating characteristic (ROC) curve analysis of biomarkers at 0 h and 24 h for the mortality prediction in canine parvovirus-infected dogs*

Parameters	Hours	AUC	Standard Error	P Value	95% Confidence Interval	Sensitivity (%)	Specificity (%)	Cut off Value
TLC	0 h	0.647	0.140	0.293	0.455-0.809	66.67	64.0	≤13.27 (10^9/L)
	24 h	0.873	0.078	< 0.001	0.705-0.965	83.3	94.3	≤ 5.04 (10^9/L)
CRP	0 h	0.733	0.098	0.017	0.545-0.875	83.33	64	>69.05 (mg/L)
	24 h	0.600	0.156	0.521	0.409-0.771	50	84	>69.65 (mg/L)
PCT	0 h	0.740	0.141	0.088	0.552-0.880	66.67	92	>99.97 (pg/mL)
	24 h	0.780	0.114	0.014	0.595-0.908	66.7	85.7	>47.86 (pg/mL)
CALP	0 h	0.693	0.132	0.143	0.503-0.845	66.67	84	>4.103 (mg/L)
	24 h	0.673	0.137	0.207	0.482-0.830	80	88	>8.05 (mg/L)
IFABP	0 h	0.80	0.116	0.01	0.618-0.921	83.3	80.0	>6.39 (ng/mL)
	24 h	0.727	0.140	0.104	0.538-0.870	66.67	84	>5.09 (ng/mL)

AUC: *area under the curve;* ***TLC:*** *total leucocyte count;* ***CRP:*** *C- reactive protein;* ***PCT:*** *procalcitonin;* ***CALP:*** *calprotectin;* ***IFABP:*** *intestinal fatty acid-binding protein*

are essential in guiding the treatment protocol and monitorization of the patient. Therefore, the need for biomarkers compatible with the pathophysiological mechanism of the disease is obvious. Additionally, considering the outcomes of CPV enteritis, we believe that evaluating TLC, the inflammatory biomarkers and intestinal damage marker together will shed light on the pathophysiology of the disease. The findings of this

research that investigated the associations of selected markers with the severity and prognosis of CPV enteritis mainly include: (1) IFABP showed a moderate correlation (*r*=0.501; P=0.000) with the clinical severity of the disease, which was superior to other evaluated markers in determining the clinical severity of the CPV enteritis, (2) CRP and IFABP values at 0 h (hospital admission) and TLC and PCT values at 24 h (24 h of treatment) were significant for predicting poor outcomes, (3) serum CALP was not sufficiently successful in predicting neither the clinical severity nor the prognosis of CPV enteritis.

The decrease in TLC during CPV enteritis is generally the most consistent haematological finding [31,32]. This finding is widely accepted to be attributable to the destruction of hematopoietic progenitor cells of the various leukocyte types in lymphoproliferative organs, mainly in the bone marrow. Potential sepsis and endotoxemia that can cause to margination of neutrophils and pronounced loss of neutrophils through the inflamed intestinal wall are also are thought to contribute to the reduction in TLC [1,32]. The prognostic significance of total or differential leukocyte counts on admission or overtime in dogs with CPV enteritis has previously been assessed [5,31]. In this study, although there was no statistical difference in TLC between survivor and non-survivor dogs at hospital admission, TLC at the 24 h of treatment was significant in predicting the prognosis *(Fig 3-A; Table 3)*. Similarly, Goddart et al.[31] and Eregowda et al.[8] emphasize the role of TLC in determining prognosis at 24 h and 72 h of treatment, respectively. In this context, persistent leukopenia is one of the most successful findings for predicting outcomes in dogs with CPV enteritis among the evaluated parameters in the current study.

Multiple factors such as gut inflammation, cellular destruction and disruption of the gastrointestinal mucosal barrier contribute to the development of SIRS and sepsis in CPV enteritis [2,6,11]. The fact that 65% of the dogs in the INF group and, 5 of the 6 dogs who died in our study were SIRS positive (+) confirms that systemic inflammation and possible sepsis are important in the pathogenesis of this disease. Thus, CRP, PCT and CALP were evaluated in this study within the scope of inflammatory markers.

The status of CRP, a major acute-phase protein of dogs in CPV enteritis, has been addressed previously. Our results on serum CRP *(Fig. 2-B)* are similar to previous studies [9-11] which proved significant increases in serum CRP concentration in dogs with CPV enteritis. To differentiate survivors from non-survivors, a serum CRP concentration of >92.4 mg/L at the time of admission had a sensitivity and specificity of 91% and 61%, respectively [9]. A cut-off of 97.3 mg/L in CRP at 24 h after admission appeared to have greater sensitivity and specificity (86.7% and 78.7%, respectively) than did values at 0 h [10]. In this study, a sensitivity of 83.33% and a specificity of 64 % for a cut-off of 69.05 mg/L at 0 h *(Fig. 3-B)* are comparable with the values previously reported. The differences in the sensitivity and specificity of CRP between the studies may be explained that infected dogs are first evaluated at different times during their disease (some are examined soon after the onset of clinical signs, while others may be ill for a day or longer), as McClure et al.[10] pointed out.

Procalcitonin is one of the promising inflammatory markers which is relatively more recent than CRP in veterinary medicine [13-15]. In humans, increased blood PCT concentrations are used to differentiate bacterial sepsis from non-infectious systemic inflammation [12]. Similarly, the plasma PCT concentrations of dogs with sepsis were found higher than those of healthy dogs [13,14]. In this context, PCT concentrations at admission to the hospital in dogs with sepsis may predict organ dysfunction and septic shock, and serial measurement of serum PCT can provide prognostic information in dogs with sepsis [14]. Kubesy et al.[11] reported that dogs with CPV enteritis had a higher serum PCT concentration compared to healthy controls, but this did not reach statistical significance. In the present study, although the plasma PCT concentrations of dogs with CPV enteritis were higher than those of healthy CON dogs, its concentrations in survivor dogs of the INF group were not statistically different from the healthy CON group *(Table 1)*. This suggests that the clinical severity of the disease and its complications, such as sepsis, may affect plasma PCT concentration. Additionally, the plasma PCT concentrations of the survivor dogs decreased statistically at 24 h of the treatment whereas it did not significantly reduce despite the treatment in the non-survivor group *(Table 1)*. This finding can be related to the short kinetics (12-24 h) of plasma PCT. Although blood culture for determining sepsis in our study was not performed, one reason for no significant decrease in the plasma PCT despite treatment in the non-survivor group may be associated with possible sepsis caused by CPV enteritis. Moreover, this study demonstrated that plasma PCT concentrations at 24 h of dogs with CPV enteritis were moderately effective (AUC=0.78; P=0.014; *Table 3*) in predicting poor outcomes and this result is not comparable because of no previous present study.

Calprotectin is released into the circulation after the activation of neutrophil granulocytes, regulates the adhesion of leukocytes to the endothelium and extracellular matrix during the inflammatory process and protects cells against microorganisms [33]. Therefore, the utility of serum CALP concentration in evaluating systemic inflammatory conditions and sepsis in human medicine [33,34] and for detecting inflammation in dogs with inflammatory bowel disease [19] has been demonstrated. In a study conducted on dogs with sepsis and non-septic SIRS [20], serum CALP concentrations were significantly higher than that of dogs in the healthy control group. In this study, serum CALP concentrations in non-survivor subgroup of INF group were only numerically higher than those of the survivor and CON groups on both sampling days *(Table 1)*. The

result in our study is in agreement with a previous study by Thames et al.[20] in which no significant difference in serum CALP levels between survivor and non-survivor dogs with sepsis and non-septic SIRS has been proved. In addition, a weak positive correlation *(rho*=0.389; P<0.033) between serum CALP concentration and CHS indicated that serum CALP appeared insufficient for predicting clinical severity and prognosis in dogs with CPV enteritis. Since the information on serum CALP concentrations (e.g. serum CALP kinetics, the relation-ship between serum CALP and local-circulating neutrophil count) in dogs is quite scant, it is not easy to make a firm judgment on the results of this study. This situation may also be originated from the relatively small sample size in this study, apart from factors related to the patho-physiology of CPV enteritis and the biology of serum CALP.

It is known that IFABP is found in the cytoplasm of the enterocytes and enters the bloodstream when the integrity of the intestinal mucosa is disrupted [21-23]. Thus, its use in various diseases has been investigated to detect intestinal damage especially in humans [21-23]. On the contrary, studies on the condition of serum IFABP in various diseases are scant in veterinary medicine. Two recent studies [8,24] demonstrated that IFABP is a valuable biomarker in dogs with CPV. Eregowda et al.[8] reported that serum IFABP concentration at 72 h of treatment in CPV enteritis could serve as a reliable predictor of prognosis (AUC=0.888; P<0.003). Gulersoy et al.[24] also revealed that serum IFABP concentration could be used to predict mortality (AUC=0.787; P=0.043) at admission to the hospital (0 h) in dogs with CPV enteritis. In accordance with the finding of the above-mentioned previous studies, IFABP values at 0 h (pre-treatment; hospital admission) in this study can be considered as a reliable biomarker with high sensitivity (83.3%) and specificity (80%) in predicting mortality. Apart from the results of previous studies, serum IFABP concentration and the CHS in dogs with CPV enteritis were *moderately positively correlated* (*r*=0.501; P=0.000) and thus the importance of serum IFABP among the other parameters examined for determining the clinical severity of the disease was proved *(Table 2)*. A strong positive correlation between clinical signs and the extent of intestinal epithelial necrosis in CPV enteritis has been described [4]. Given the biology of IFABP, this suggests that IFABP may be a direct indicator of CPV-induced intestinal damage. However, there is a clear need for studies investigating the relationship between the histopathological grade of intestinal damage and serum IFABP concentration in dogs with CPV enteritis.

This study has some limitation factors for the results and these especially include the inability to uniformize environmental and stress factors due to its clinical nature, and the absence to evaluate Coronavirus and other possible concurrent agents that may occur between 2 to 6 months of age in dogs. Furthermore, the polymerase chain reaction analysis was not used for the diagnosis of CPV in this study. However, the diagnosis of CPV in the INF group was made by evaluating both clinical findings and the rapid test kit result, which has high sensitivity and specificity. Despite these limitations, this study provides valid information for the assessment of clinical severity and prognosis in dogs naturally infected with CPV. Additionally, a future study with a larger sample size could increase the statistical power.

In conclusion, TLC in whole blood and PCT concentration in plasma at 24 h after initiation of treatment and, serum CRP and IFABP concentrations at hospital admission (0 h) in CPV-infected dogs could be used as prognostic indicators in predicting disease outcomes. However, based on ROC curve analysis results, TLC (24 h) and serum IFABP (0 h) concentration serve as the most valuable biomarkers among the parameters in this study. Compared with other parameters evaluated, serum IFABP concentration correlated strongly with the clinical severity of the disease.

Competing Interests

The authors declared that there is no conflict of interest.

Availability of Data and Materials

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' Contributions

Design of the study: CDA, HV and BU. Preparation of the study, management of the patients and data collection: CDA, GET, and GSEA. Performing the laboratory analysis: CDA and GSEA. Article writing, data analysis and editing: CDA, GET, HV, and BU. All authors reviewed and approved the final manuscript.

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RESEARCH ARTICLE

Effect of Imidocarb on DNA Damage in Sheep with Babesiosis

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Abstract

In this study, it was aimed to determine the DNA damage using the comet assay, which specifically shows DNA damage in naturally *Babesia* spp.-infected sheep and to evaluate the damage before and after imidocarb application. Blood samples obtained from 10 infected sheep with positive clinical signs and symptoms of babesiosis and whose diagnosis was confirmed by Giemsa staining and PCR methods, and blood samples from 10 healthy sheep were used as study material. DNA damage was examined by the comet assay from the blood samples of the infected patient group and the control group obtained during the disease and after the treatment, and the results were compared with statistical methods. When DNA damage was examined in sick animals diagnosed with babesiosis, the tail length and the tail moment values were found to be statistically significantly higher than the control group ($P<0.001$). According to the results obtained after imidocarb application, it was determined that DNA damage and tail moment decreased statistically with imidocarb, and the difference was statistically significant, and the values were higher than the control group ($P<0.001$). As a result, *Babesia* infection can cause DNA damage, has been confirmed by the determination of direct DNA damage using the comet assay, and imidocarb given for treatment was successful and reduced the damage.

Keywords: *Babesiosis, DNA damage, Imidocarb, Sheep*

Babeziozisli Koyunlarda İmidokarb Uygulamasının DNA Hasarına Etkisi

Öz

Bu çalışmada, doğal olarak *Babesia* spp. ile enfekte koyunlarda spesifik olarak DNA hasarını gösteren comet testi kullanılarak DNA hasarının belirlenmesi ve imidokarb uygulaması öncesi ve sonrası hasarın değerlendirilmesi amaçlanmıştır. Çalışma materyali olarak babeziozis klinik belirti ve semptomları pozitif olan ve Giemsa boyama ve PCR yöntemleri ile tanısı doğrulanan 10 enfekte koyundan alınan kan örnekleri ve 10 sağlıklı koyundan alınan kan örnekleri kullanıldı. Enfekte hasta grubu ve kontrol grubundan hastalık sırasında ve tedavi sonrasında alınan kan örneklerinden comet testi ile DNA hasarı incelendi ve sonuçlar istatistiksel yöntemlerle karşılaştırıldı. Babeziozis tanısı konulan hasta hayvanlarda DNA hasarı incelendiğinde kuyruk uzunluğu ve kuyruk momenti değerleri kontrol grubuna göre istatistiksel olarak anlamlı derecede yüksek bulundu ($P<0.001$). İmidokarb uygulaması sonrası elde edilen sonuçlara göre DNA hasarı ve kuyruk momentinin imidokarb ile istatistiksel olarak azaldığı ve aradaki farkın istatistiksel olarak anlamlı olduğu ve değerlerin kontrol grubuna göre daha yüksek olduğu belirlendi ($P<0.001$). Sonuç olarak *Babesia* enfeksiyonunun DNA hasarına neden olabileceği, comet testi kullanılarak direkt DNA hasarının belirlenmesi ile doğrulanmış ve tedavi için verilen imidocarb başarılı olmuş ve hasarı azaltmıştır.

Anahtar sözcükler: *Babeziozis, DNA hasarı, İmidokarb, Koyun*

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Introduction

Babesiosis is a tick-borne haemoparasitic disease that causes high morbidity and mortality and high economic losses in tropical and subtropical regions of the world, and it is the most critical blood-borne parasitic disease of small ruminants [1,2]. Six species of *Babesia (B. ovis, B. motasi, Babesia (Lintan), B. crassa, B. foliata*, and *B. taylori)* have been described, of which *B. ovis* and *B. motasi* have been reported to be pathogenic *Babesia* species [2-4]. Babesiosis is a disease that causes high economic losses in the livestock industry worldwide. Clinically, symptoms such as fever, anemia, jaundice, and hemoglobinuria are observed in babesiosis. Babesiosis can be seen as a "protozoan sepsis" in different animals and is expressed to be clinically similar to septic conditions characterized by systemic inflammatory response syndrome (SIRS) and multiple organ failure syndrome (MODS) [5].

Microscopic examination of samples stained with Giemsa stain is the most commonly used method in the diagnosis of babesiosis, but this method is not specific. Since there are false negative results in low-density parasitemia and some *Babesia* species cannot be distinguished from Theileria, serological methods and molecular-based tests such as PCR have frequently been used in epidemiological studies in recent years [6]. Molecular-based tests are more sensitive and allow identification of species by using appropriate primers [7-9]. Early diagnosis and successful treatment of babesiosis reduces the mortality rates. Clinicians use imidocarb in the treatment of babesiosis. Imidocarb is a carbanilide derivative and is usually available in the form of the dipropionate salt. For the treatment of babesiosis in sheep, intramuscular (IM) administration of 1.2 mg/kg once is recommended. However, a second dose may be given 10 to 14 days later for disease control. It is stated that it can be used at a dose of 2.4 mg/kg for prophylaxis of the disease in sheep [10].

DNA is a sensitive molecule and DNA damage can occur for various reasons. DNA damage leads to necrosis or cellular mutation when damage is high or repair systems are insufficient and this plays an important role in mutagenesis, carcinogenesis and aging [11-13]. Free radicals can attack any macromolecule, including DNA, and can cause lipid peroxidation, protein oxidation and DNA damage [14-17]. DNA damage is characterized by structural damage such as disruption of chromatin structure, oxidation of DNA bases, mismatch and suppression of tubulin polymerization, chemical modification of bases, chromatin abnormalities, strand breakage, DNA-DNA and DNA-protein crossovers [18-20]. Parasitic infections cause activation of inflammatory cells that play an important role in host defense. In addition, parasites increase the amount of free radicals in the tissues, organs and cells they inhabit and cause lipid peroxidation, which causes tissue and cell damage in the host. It has been reported that erythrocyte membrane fragility occurs as a result of increased lipid peroxidation and decreased antioxidant defense in the erythrocytes of animals with piroplasmosis [21-23]. Increased activation of inflammatory cells and therefore, increased oxidant-producing enzymes, have been reported in sheep infected with *Babesia* spp., but the extent of DNA damage has not been determined by specific methods [24].

The Comet assay [single-cell gel electrophoresis (SCGE)], which is among the methods used for detection of DNA damage, is a fast, simple, sensitive and widely used technique. The comet assay method is based on the principle that DNA molecules with different molecular weights and different electrical charges migrate differently in the electrical field at alkaline pH. While healthy DNAs do not form comets during transport, damaged DNAs move at different speeds in the electrical field, forming a tail-shaped image. DNA degradation, antioxidant status (resistance to H_2O_2 degradation) and DNA repair levels in lymphocytes can also be measured with the comet assay [25,26].

Babesiosis is common worldwide, especially among small livestock, and causes serious economic losses. It has been reported that oxidative stress occurs in *Babesia* spp. infections, but studies showing precise DNA damage are insufficient in number [27]. In this study, it was aimed to determine DNA damage in sheep naturally infected with *Babesia* spp. using the comet assay, which specifically shows DNA damage, and to evaluate the damage before and after imidocarb application.

Material and Methods

Sample Collection and Identification of Babesia

In this study, 10 mature Akkaraman sheep with a weight of 25-40 kg, aged 3-5 years, showing clinical babesiosis symptoms (40-42°C fever, anemia, hemoglobinuria, jaundice, etc.), located in a farm in the Özalp District of Van, Türkiye in July 2021 were included as the patient group. The control group consisted of 10 healthy sheep, which were subject to the same region and rearing conditions, had no disease history and clinical findings specific to babesiosis and other diseases, and were found to be negative for *Anaplasma* spp. and *Theileria* spp. with microscopic examination (5% Giemsa stain) and blood samples were obtained from these sheep for analysis. Before and after the treatment (Day 10), blood samples were taken from the sheep diagnosed with the disease for laboratory analysis. All tracked animals were kept in their natural habitat for the duration of the study. The study was performed with the Van YYU Animal Experiments Local Ethics Committee (VAN YUHADYEK) decision (It was decided that ethics committee approval was not required) (Approval no: 2020/12-08, date: 31/12/2020).

Microscopic Diagnosis of Babesia spp.

Blood smear staining was performed with 5% Giemsa stain

by taking blood samples from the Vena jugularis of the animals. Piroplasma forms were found in erythrocytes with microscopic examination.

Molecular Diagnosis of Babesia spp.

- DNA Extraction

PCR test was performed by isolating DNA from all samples suspicious for *Babesia* by microscopic examination using the Invitrogen PureLink™ Genomic DNA Mini Kit (USA, K182002), according to the manufacturer's protocol.

- PCR Reaction

Orunç Kılınç et al.[28] performed amplification of the 18S rRNA gene region, using BJ 5'-GTCTTGTAATTGGAATGATGG-3' and BN2 5'-TAGTTTATGGTTAGGACTACG-3' primers [29]. A 5 pmol forward and reverse primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1U Taq Polymerase and 10X PCR buffer (500 mM Tris-HCl, pH 8.8, 160 mM $(NH_4)SO_4$ and 0.1% Tween®20), Nuclease Free Water and 2 µL of DNA were used in 25 µL master mix for one sample. At the end of the microscopic examination were PCR tested with positive animals, negative animals, in addition to 1 positive and 1 negative control. The reaction was followed by pre-denaturation at 95°C for 15 min, with each cycle consisting of denaturation (30 sec at 95°C), bonding (30 sec at 55°C) and elongation (40 sec at 72°C) steps, in 40 cycles and a final extension of 10 min at 72°C. The obtained PCR products were stained with Safe-T-Stain and images were obtained on 2% agarose gel.

- DNA Damage Analysis

The Comet analysis method was used to determine DNA damage. It was applied on gel-coated slides according to the Comet protocol and spread was achieved. Prepared slides were run by the electrophoresis method [30]. Three times the sample volume LMA was added and mixed with Whole Blood with EDTA. It was added to slides that had been applied with NMA. 3 samples were studied from each group. The slides were scanned with a fluorescence microscope, and visual damage levels were counted (Oxion Microscopy for Fluorescence, The Netherlands). DNA damage levels were calculated based on the genetic damage index (GDI) formula. The genetic damage index reflects the number of Arbitrary Units [15]. The % DNA Damage and the % Tail Moment measurements from these images were calculated using the "Image J" program (a program distributed freely by the National Institute of Health of the SA *(https://imagej.nih.gov/ij/download.html)*.

Statistical Analysis

All results are reported as mean ± standard error of the mean. The data of each sampling time of all groups were evaluated with the One-Way Anova test. The significance of the difference between the groups was evaluated with the Duncan test (SPSS® v.19 Evaluation Version for Windows, IBM).

Results

In the present study, clinically high fever >40°C, hemoglobinuria, jaundice, increased heart and respiratory rate were determined in the patient group. The smears obtained from blood samples taken from animals with clinical symptoms were stained with the Giemsa staining method and examined microscopically, and piroplasms were observed in erythrocytes *(Fig.1)*. In order to confirm the results of the microscopic examination, in the PCR test performed on suspicious blood, as a result of the amplification of the 18S rRNA gene region, specific fragments specific for *Babesia* spp. were obtained with a size of approximately 447 bp in all 10 samples *(Fig. 2)*.

DNA damage in sheep with babesiosis after the comet analysis has been demonstrated in *Fig. 3*. When DNA damage *(Table 1)* and tail moment *(Table 2)* were examined

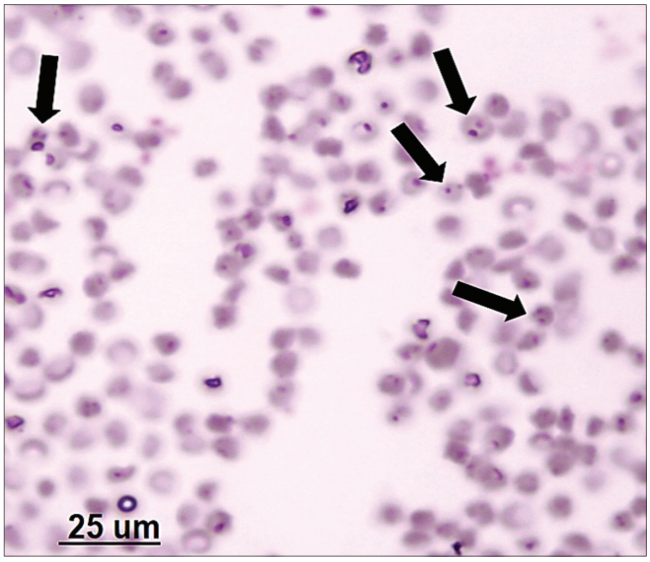

Fig 1. *Babesia* spp. (Giemsa staining)

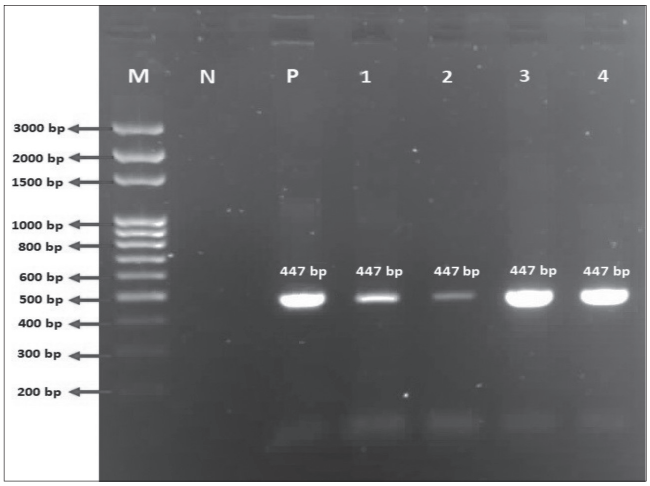

Fig 2. *Babesia* spp. agarose gel image (M: marker, P: positive control, N: negative control, 1,2,3,4: positive samples)

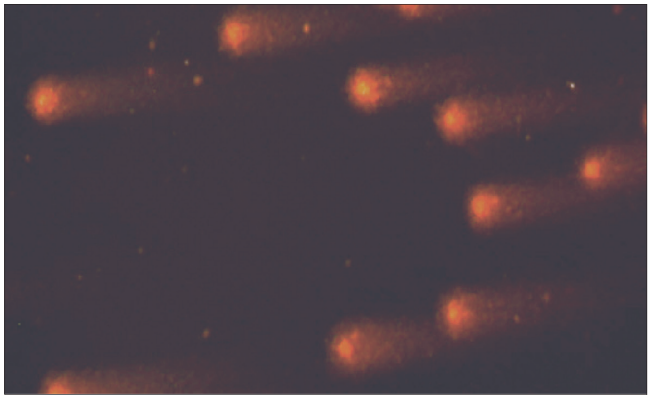

Fig 3. DNA damage in sheep with babesiosis (the comet analysis)

Table 1. *Imidocarb application DNA damage table in babesiosis treatment (n:10)*

Groups	Mean	Std. Error
Control	5.71784	0.7169242 [c]
Patient	36.2785	1.5562375 [a]
Treatment	16.58399	0.8135902 [b]

[a,b,c] *Indicates the difference between groups P<0.001*

Table 2. *Imidocarb application tail moment table in babesiosis treatment (n:10)*

Groups	Mean	Std. Error
Control	6.68563	0.4814676 [c]
Patient	39.46755	1.4941459 [a]
Treatment	16.48585	0.5343389 [b]

[a,b,c] *Indicates the difference between groups P<0.001*

in sick animals diagnosed with babesiosis, the values were found to be statistically significantly higher than that of the control group (P<0.001). After imidocarb administration, it was determined that DNA damage, which was found to be significantly different to sick animals as the treatment group, decreased, but there was a significant difference compared to the control group and its value was higher (P<0.001) *(Table 2)*.

Discussion

Free radicals react with proteins and lead to modification of amino acid residues by oxidation, nitrosation and carbonylation. In fact, protein carbonyl (PCO) derivatives are produced when enzymes and proteins are deactivated and modified by free radicals. In addition, oxidative DNA damage can cause a range of changes including mutations, replication errors, genomic instability and cell death [31]. DNA damage may be associated with hydroxyl radicals (·OH) produced in parasitic infections [32]. It has been reported that the level of 8-hydroxyguanine (8-OHG), which can react with DNA nitrogen bases and is one of the critical biomarkers of oxidative stress, increases in babesiosis and the *Babesia* spp. causes DNA damage [33,34].

Küçükkurt et al.[34] found that *Babesia* infection increased the oxidative stress markers and DNA damage and decreased (total antioxidant activity) AOA and glutathione (GSH) in goats, and that the increase in the production of free radicals generated during infection not only contributed to the host defense strategies of organisms to kill the parasite, but also induced leads to the acceleration of lipid peroxidation in other cells. As a result, they reported a DNA damage in goats with comet assay. In our study, DNA damage occurring in sheep with babesiosis was detected by the comet assay *(Fig. 3, Table 1, Table 2)*, and these results support the literature information mentioned above.

Ostling and Johanson [35] were the first to measure DNA damage in cells using a microgel electrophoresis technique known as "single-cell gel electrophoresis" or "Comet assay". However, the neutral conditions they used allowed detection of only DNA double-stranded breaks. Later, this method was adapted under alkaline conditions by Singh et al.[36]. It led to a sensitive version of the analysis that could evaluate both double- and single-stranded DNA breaks, as well as alkaline variable regions in DNA, expressed as open-strand breaks. However, this method has been modified at several stages (lysis, electrophoresis) to make it suitable for assessing various types of damage in different cells [25,26]. There are previous studies reporting that DNA damage may occur in babesiosis, but these studies investigated oxidative stress and 8-OHG markers [34-37]. Comet, on the other hand, is a method that reveals specific DNA damage, and in this study, it has been confirmed that DNA damage occurs in babesiosis *(Table 1, Table 2, Fig. 3)*.

There are many studies on babesiosis and imidocarb administration. In one study, it was shown that imidocarb dipropionate (IMD) was more effective compared to diminazene aceturate [38]. In another study, it was reported that the use of imidocarb dipropionate together with oxytetracycline produced more successful results, and it was more effective than the combined use of diminazene aceturate and oxytetracycline [39]. In another study, the combined application of imidocarb and alpha-lipoic acid (ALA) was reported to be successful in treatment in dogs experimentally infected with *Babesia canis vogeli* [40]. In our study, the presence and extent of DNA damage during infection and after treatment in sheep with babesiosis was investigated, and it was revealed that DNA damage occurred during infection and this damage decreased after treatment; hence, imidocarb application was successful *(Table 1, Table 2)*.

In this study, blood samples were obtained from 10 sick Akkaraman sheep, in which *Babesia* spp. were diagnosed microscopically and molecularly *(Fig. 1, Fig. 2)*, and DNA damage was examined using the comet method, and blood samples were obtained from 10 healthy sheep. As

a result of the study, when DNA damage *(Table 1)* and tail moment *(Table 2)* were examined in sick animals diagnosed with babesiosis, the values were found to be statistically significantly higher than the control group (P<0.001). After imidocarb administration, it was determined that DNA damage, which was found to be significantly different in sick animals, decreased, but there was a significant difference compared to the control group and its value was higher (P<0.001) *(Table 2)*.

In conclusion, with this study, it has been confirmed that *Babesia* spp. cause DNA damage. It is concluded that further molecular and biochemical studies are needed in the future to better understand the pathogenesis of this infection. This study may set an example for other babesiosis-like piroplasmoses.

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Conflict of Interest

The authors declare no conflict of interest.

Availability of Data and Materials

Datasets analyzed during the current study are available to the corresponding (A. C. Öner) author on reasonable request.

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Author Contributions

ACÖ planned, designed, and supervised the research procedure. AA, FE and ÖOK performed the parasitological analysis, AU and ACÖ performed DNA damage and comet analysis, the statistical analysis, the imaging stage, and the language editing of the final manuscript. AU, FE and ACÖ has revised the manuscript for contents, and approved the final version.

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RESEARCH ARTICLE

Antioxidant and Anti-Inflammatory Effects of Nicotinamide Adenine Dinucleotide (NAD^+) Against Acute Hepatorenal Oxidative Injury in An Experimental Sepsis Model [1]

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Abstract

The aim of this study is to determine the antioxidant and anti-inflammatory effects of Nicotinamide Adenine Dinucleotide (NAD^+) in preventing multi-organ damage caused by sepsis. Twenty-eight male Wistar-albino rats were randomly divided into four groups. The study groups comprised Sham group, sepsis group (CLP), sepsis + 100 mg/kg NAD^+ (CLP+N100) and sepsis + 300 mg/kg NAD^+ group (CLP+N300). Sepsis was induced by the cecum ligation perforation (CLP) method. NAD^+ was administered intraperitoneally for five days before cecum perforation and 6 h after operation. Serum, liver and kidney tissues were taken from the rats 24 h after the operation. MDA, GSH, CAT, TNF-α, IL-6, IL-1β, and caspase-3 parameters were measured in tissue samples with biochemical and immunohistochemical methods. In the histopathological and immunohistochemical examination, increases in TNF-α, IL-6, IL-1β, and caspase-3 expressions were observed in the liver and kidney tissues of the CLP group and severe damage was seen in tissue morphology ($P<0.001$). Hepatorenal injury was significantly decreased in the treatment groups. Sepsis increased MDA levels in all tissues, but significantly decreased GSH and CAT activities. While NAD^+ administration significantly increased GSH and CAT activity in the liver and kidney tissues, it caused a significant decrease in MDA levels. This study shows that nicotinamide may be a potent therapeutic agent for the treatment of sepsis.

Keywords: *Anti-inflammation, Hepatorenal injury, Nicotinamide, Oxidative stress, Sepsis*

Deneysel Sepsis Modelinde Nikotinamid Adenin Dinükleotidin (NAD^+) Akut Hepatorenal Oksidatif Hasara Karşı Antioksidan ve Anti-inflamatuar Etkileri

Öz

Bu çalışmada amaç, sepsisin neden olduğu çoklu organ hasarını önlemede Nikotinamid Adenin Dinükleotid'in (NAD^+) antioksidan ve anti-enflamatuvar etkilerini belirlemektir. 28 erkek Wistar-albino sıçan rast gele yedişerli dört gruba ayrıldı. Çalışma grupları; Sham grubu, sepsis grubu (CLP), sepsis + 100 mg nikotinamid uygulama grubu (CLP+N100) ve sepsis + 300 mg nikotinamid uygulama grubu (CLP+N300) şeklinde oluşturuldu. NAD^+ çekum perforasyonundan önce beş gün boyunca ve operasyondan 6 saat sonra intraperitoneal yoldan verildi. Operasyondan 24 saat sonra ratlardan serum, karaciğer ve böbrek dokuları alındı. Alınan doku örneklerinde MDA, GSH, CAT, TNF-α, IL-6, IL-1β, kaspaz-3 parametreleri biyokimyasal ve immünohistokimyasal metod kullanılarak ölçüldü. Histopatolojik ve immünhistokimyasal incelemede, CLP grubunun karaciğer ve böbrek dokularında TNF-α, IL-6, IL-1β ve kaspaz-3 ekspresyonlarında artışlar gözlendi ve doku morfolojisinde ciddi hasar görüldü ($P<0.001$). Tedavi gruplarında hepatorenal hasar anlamlı olarak azaldı. Sepsis, tüm dokularda MDA seviyelerini arttırdı, ancak GSH ve CAT aktivitelerini önemli ölçüde azalttı. NAD^+ uygulaması dokularda GSH ve CAT aktivitesini önemli ölçüde artırırken, MDA düzeylerinde anlamlı düşüşe neden oldu. Bu çalışmadan elde edilen veriler; sepsisin tedavisinde Nikotinamidin güçlü bir teropatik ajan olabileceğini göstermektedir.

Anahtar sözcükler: *Anti-inflamasyon, Hepatorenal hasar, Nikotinamid, Sepsis, Oksidatif stres*

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Introduction

Sepsis causes many changes such as increased microvascular permeability, acute lung damage, coagulation disorders, hypovolemia, decreased myocardial contractility, hypoxia, and decreased systemic vascular resistance, resulting in respiratory, renal, hepatic, cardiovascular and endocrine organ dysfunctions [1,2]. It was determined that sepsis activates the complement system through its endotoxic effect, thus basophil and mast cells are stimulated and some vasoactive mediators are secreted from these cells [3]. The increased inflammatory response in sepsis is maintained by pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β produced by monocytes in response to infection or endotoxins. Pro-inflammatory cytokines cause endothelial breakdown and microvascular damage [4,5]. Endotoxin, TNF-α, IL-1β, leukotrienes, nitric oxide (NO) and toxic oxygen radicals that are released from neutrophils and lysosomal enzymes increase endothelial permeability. Increased endothelial permeability and endothelial damage facilitates extravasation and formation of microthrombi. As a result, organ perfusion deteriorates and organ failure develops [6]. In sepsis, reactive oxygen species (ROS) directly induce cytotoxicity in organs, causing changes in cell signaling pathways. ROS products are destroyed by a complex antioxidant defense system consisting of enzymatic antioxidants such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase and various non-enzymatic antioxidants such as melatonin, albumin, bilirubin, transferrin, ceruloplasmin, and uric acid [7]. For this reason, in studies about the treatment of sepsis conducted in recent years, it was reported that the prevention of ROS formation also reduces tissue and organ damage caused by sepsis in addition to reducing cytokine release, as well as reducing the mortality rate [8].

Nicotinamide adenine dinucleotide (NAD^+) is an important cofactor for most enzymes in cells that oxidize substrates and are itself reduced to NADH. Conversely, NADPH often acts as a cofactor for enzymes that degrade substrates. NAD^+ is a hydride acceptor that, along with its reduced form NADH is vital to the redox reactions in metabolic processes. Under physiological conditions, NAD^+ production and consumption in cells is kept in a constant balance. Therefore, NAD^+ homeostasis has key roles in cellular catabolism and anabolism [9]. With its NAD^+ metabolite, nicotinamide is involved in many important biological events, including energy production, fatty acids, cholesterol and steroid synthesis, signal transduction, and preservation of genome integrity. NAD^+ is also required for DNA repair and ADP-ribosylation of proteins [10]. Immune system dysfunction of NAD^+, known for its anti-inflammatory and antioxidant properties, was reported to be a potent cytoprotectant blocking early apoptotic phosphatidylserine exposure and late nuclear DNA degradation, during disorders including diabetes and aging-related diseases [11]. Although various medical treatments were attempted for the treatment of sepsis, there is still no adequate treatment to prevent multiple organ damage. However, more effective treatment protocols can be developed by explaining the mechanisms associated with sepsis. In our study, we aimed to investigate the anti-oxidant and anti-inflammatory role of NAD^+ in sepsis induced by cecal ligation and perforation in preventing damage to serum, liver and kidney tissue with biochemical and histopathological methods.

Material and Methods

Ethical Approval

This study was conducted at Sakarya University Animal Laboratory and Research Centre (SUDATEM). The experimental protocols were carried out in accordance with international guidelines, after obtaining approval from the University Animal Care and Use Ethics Committee (protocol number: 05/08/2020-43).

Experimental Procedure and Animals

All rats were kept in wire cages under standard laboratory conditions (08.00-20.00 light/dark light cycle, warm 22±2°C humidity 50-60%). All rats were fed with standard pellet feed and tap water before the study. They were only allowed water for 12 h before and after the operation. Ketamine HCl (Ketalar®, 50 mg/mL, Pfizer, Istanbul) and xylazine HCl (Rompun® 2%, Bayer, Istanbul) were used for anesthesia and NAD^+ (Sigma Chemical East ellsworth rd. Item no; N0636, USA) was used as antioxidant agent.

Twenty-eight Wistar-Albino adult male rats with an average weight of 270±30 grams were used in the study. The rats were randomly divided into 4 groups (each group n=7).

Sham group: The rats in this group were operated under anesthesia, but CLP was not performed. One mL 0.9% NaCl was given intraperitoneally (i.p.) for 5 days before the operation and 6 h after the operation.

CLP group: Sepsis was induced using the CLP method under general anesthesia. One mL 0.9% i.p. NaCl was given for 5 days before the operation and 6 h after the operation.

CLP + N100 group: Sepsis was induced using the CLP method under general anesthesia as in the CLP group. In addition, the rats in this group were given Nicotinamide i.p. at a dose of 100 mg/kg, after dissolution in 0.9% NaCl for 5 days before the CLP procedure and 6 h after the operation [12].

CLP+N300 group: Sepsis was induced using the CLP method under general anesthesia as in the CLP group. Nicotinamide was given i.p. after dissolution in 0.9% NaCl at a dose of 300 mg/kg for 5 days before the CLP procedure and 6 h after the operation [12].

Animal Sepsis Model Induced by Cecal Ligation and Perforation

The CLP model applied in the study was performed in

accordance with the method previously reported by Cadirci et al.[13] Sepsis was induced in rats using the CLP-induced sepsis model. All rats were anesthetized intraperitoneally (i.p) with ketamine HCl (50 mg/kg) and Xylazine HCl (15 mg/kg). Anesthetized rats were placed on the operating table in supine position. The abdominal area was shaved and washed with povidone iodine. The cecum was isolated with a 2 cm incision from the abdominal region of the rats to reach the peritoneum. Ascending colon contents were pushed down to fill the cecum. After the cecum was tied under the ileocecal valve using 3/0 silk thread, it was pierced with an 18 'G needle (2 holes) and the cecum was placed inside the abdomen. The abdomen was closed using 3/0 silk thread. After the surgical procedure, 1% lidocaine solution was applied to the incision areas of rats as an analgesic to reduce pain stress. Animals were fasted after surgery, but were allowed to take water after 6 h postoperative.

All rats were sacrificed with high-dose blood collection while under general anesthesia 24 h after the operation. Liver, kidney and serum samples were obtained. For serum, the blood taken into a 5 mL sterile syringe was placed in a red-capped serum tube and centrifuged at 2500 rpm for 10 min at +4°C. Tissue and serum samples examined for biochemical parameters were stored at -20°C until laboratory analysis.

Determination of MDA, GSH Levels and CAT Activity

Liver and kidney tissues (100 mg) were washed with 0.9% NaCl solution and then centrifuged (+4°C, 3000g, 10 min). Then, 10% tissue homogenates were prepared by mixing cold 1.15% KCl with 0.01 M sodium potassium phosphate (pH=7.4) solution. Homogenates were centrifuged (10.000xg) for 20 min at +4°C. Protein was measured from the obtained supernatants using the Lowry method [14]. Later, MDA, GSH levels and CAT activity were measured in liver and kidney supernatants and serum. Determination of MDA, which is one of the final products of lipid peroxidation, was made using Buege's method [15], and results are given as nM/mg. Determination of reduced glutathione (GSH) concentration was performed using Agergaard's method [16], and results are given as µM/mg. CAT activity was determined using Hadwan's method [17], and results are given as U/mg.

Histopathological Evaluation

Liver and kidney tissues were fixed in 10% neutral buffered formaldehyde for 72 h and washed in tap water for 1 day. Then, the samples were passed through alcohol series and the dehydration process was applied. The tissues were made transparent with xylol and embedded in paraffin blocks. Sections of 4 microns were taken and stained with hematoxylin eosin (H&E). Photographs were taken during examination under a light microscope (Olympus CX31-Japan). Histopathological evaluations of liver tissue in terms of sinusoidal congestion, necrosis and vacuolization were performed using the semi-numerical modified scoring system of Suzuki [18]. The scale for kidney injury designed to semi-quantitatively evaluate kidney tissue sections (tubular necrosis, cytoplasmic vacuolization, tubular dilatation and interstitial hemorrhage) was used [19].

Immunohistochemical Analysis

After the deparaffinization of the tissues placed on a slide with "Poly-L-Lysine", 4 µm thick sections from tissues fixed in neutral formaldehyde solution were boiled in a citrate buffer for antigen retrieval for 20 min in a microwave oven. After being allowed to cool to room temperature, they were washed with phosphate buffer solution (PBS). For endogenous peroxidase inactivation, they were incubated with 3% hydrogenperoxide (H_2O_2) for 20 min after PBS bath. After cooling, TNF-α (sc-52746, Santa Cruz, USA), IL-6 (sc-32296, Santa Cruz, USA), IL-1β (sc-52012, Santa Cruz, USA), caspase-3 (sc-56053, Santa Cruz, USA) primary antibodies were used as primary antibody and the rat and rabbit specific HRP/DAB detection IHC kit was used as secondary antibody. Immunopositivity in samples was evaluated after counterstaining with hematoxylin by giving a semi-quantitative number for positive cells.

Scoring of Immunoreactivity

The immunohistochemical staining results were evaluated with the most common method used semi-quantitative H scoring in mouse models. The percentage of positive cells was graded as follows: no stained cells (Grade 0), 1-25% stained cells (Grade 1), 26-50% stained cells (Grade 2), 51-75% stained cells (Grade 3), and 76-100% stained cells (Grade 4) in the representative area. The immunostaining intensity was scored as 1+ (weak), 2+ (moderate), 3+ (intense) and 4+ (strong/intense). For this, at least 500 cells were counted. The total score was calculated using the following formula: total score = [percentage of positive cells] x [immunostaing intensity] [20]. The evaluation was made in at least five areas with an inverted microscopic lens (x400 objective). Results were expressed as a percentage.

Statistical Analysis

Statistical analyses were performed using the SPSS 24.0 package program (SPSS Inc. and Lead Tech. Inc. Chicago. USA). Numerical data are given as mean ± standard deviation (SD). The Kolmogorov-Smirnov test was used to check normal distribution of data. In comparison of more than two variables, one-way ANOVA and Kruskal Wallis test were used according to normality. In evaluating the significance within the group, TUKEY HSD was used for variables where variances were homogeneous, and Tamhane's T2 test was used for non-homogeneous variables. Histopathological and immunohistochemical parameters were evaluated semi-quantitatively and the number of positive cells were evaluated statistically. Results with $P<0.05$ were considered significant.

Results

Biochemical Analysis

Fig. 1 presents in the tissue MDA levels. Sepsis induced by CLP caused an increase in MDA levels, which is an indicator of lipid peroxidation. The level of MDA in tissues increased significantly in the CLP group compared to the Sham, and treatment groups. While serum MDA level increased significantly in the CLP group compared to the sham group (P=0.016), there was no significant difference in comparison with the treatment groups (P>0.05). Liver MDA levels were significantly lower in the CLP+N300 group compared to the Sham and CLP groups (P=0.001, P=0.023, respectively). Kidney tissue MDA levels were significantly lower in the treatment groups compared to the Sham and CLP groups (P<0.05).

GSH levels of tissues in the experimental groups are presented in *Fig. 2*. GSH levels in the tissues decreased in the CLP group, and NAD treatment increased GSH levels. However, this increase was statistically significant between groups only for the kidney. There was a statistically significant decrease in renal GSH levels in the CLP group compared to the Sham and treatment groups (P=0.001, P=0.008, P=0.012, respectively).

CAT levels decreased in serum, liver and kidney tissues with sepsis induced by CLP *(Fig. 3)*. While high-dose NAD^+ treatment significantly increased CAT levels, especially in the liver and kidney (P<0.001 for both), this effect was not significant in serum. When CAT levels in the liver and kidney tissues were compared in the CLP group and the treatment groups, a significant significance was found between the groups (liver, P=0.005, P=0.000, kidney, respectively; P=0.007, P=0.001). NAD^+ administration significantly increased CAT levels in the liver and kidney (respectively, P=0.014, P=0.003) in the treatment groups compared to the Sham group.

Histological and Immunohistochemical Evaluation

Histopathological findings for the groups are given in *Table 1*. When liver tissue sections were evaluated in terms of sinusoidal congestion, necrosis and vacuolization, all scores were significantly higher in the CLP group than Sham, CLP+N100 and CLP+N300 groups (for all P=0.001). The liver samples of the treatment groups had nearly normal parenchyma morphological structure *(Fig. 4)*. There was common sinusoidal congestion, necrosis and vacuolization observed in the CLP group. Necrosis was significantly reduced in the CLP+N100 group, and there was no necrotic area in the CLP+N300 group. In the CLP+N100 and CLP+N300 groups, more normal cell contours were observed, but there was no significant difference between the CLP+N100 and CLP+N300 group *(Fig. 4)*.

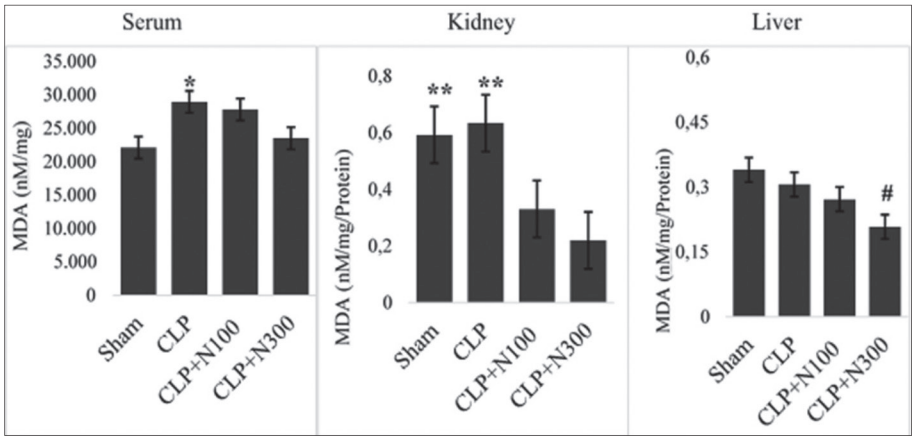
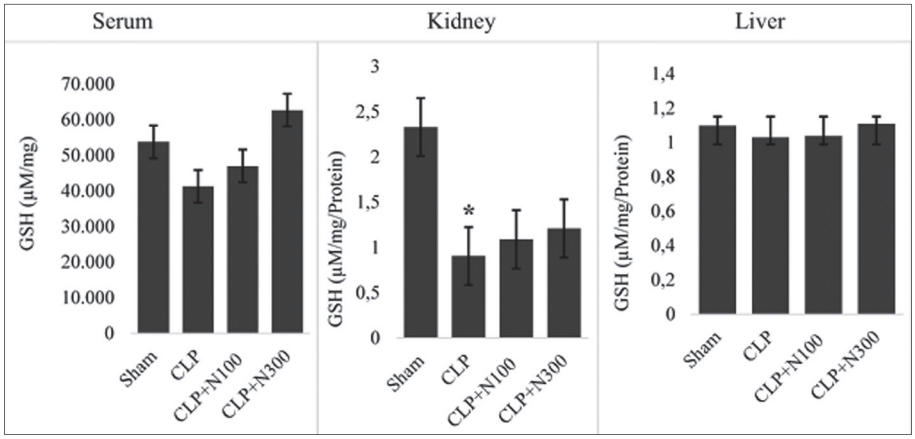


Fig 1. Tissue MDA Levels. MDA: Malondialdehyde; * P<0.05 compared to the Sham group; ** P<0.05 compared to the CLP+N100 and CLP+N300 group; # P<0.05 compared to the Sham and CLP group. The results are presented mean ± SD

Fig 2. Tissue GSH Levels. GSH: Glutathione. *P<0.05 compared to the Sham, CLP+N100 and CLP+N300 group. The results are presented mean ± SD



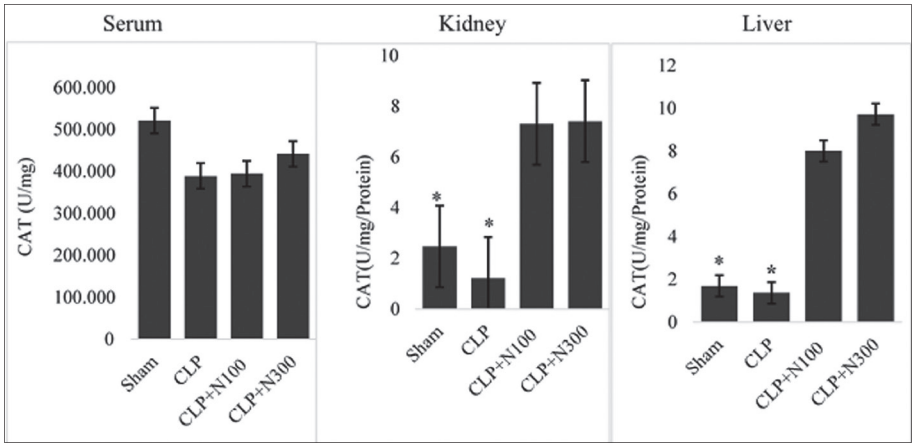


Fig 3. Tissue CAT levels. CAT: Catalase. * P<0.05 compared to the CLP+N100 and CLP+N300 group. The results are presented mean ± SD

Table 1. *Comparison of histopathological scores of experimental groups*

Groups/Parameters		Sham	CLP	CLP+N100	CLP+N300	P Value
Liver	Sinusoidal Congestion	0 (1)	2 (1)*	0 (1)	0 (1)	0.001
	Necrosis	0 (1)	2 (1)*	0 (1)	0 (0)	0.002
	Vacuolization	0 (1)	2 (1)*	0 (1)	0 (0)	0.001
Kidney	Tubular Necrosis	0 (1)	2 (1)*	1 (1)	1 (1)	0.001
	Cytoplasmic Vacuolization	0 (1)	2 (0)*	1 (1)	0 (1)	0.000
	Tubular Dilation and Interstitial Hemorrhage	0 (0)	3 (1)*	1 (1)	0 (1)	0.000

*The results are presented median (Inter Interquartile Range) (n=7). * P<0.05 as compared to the Sham and treatment groups*

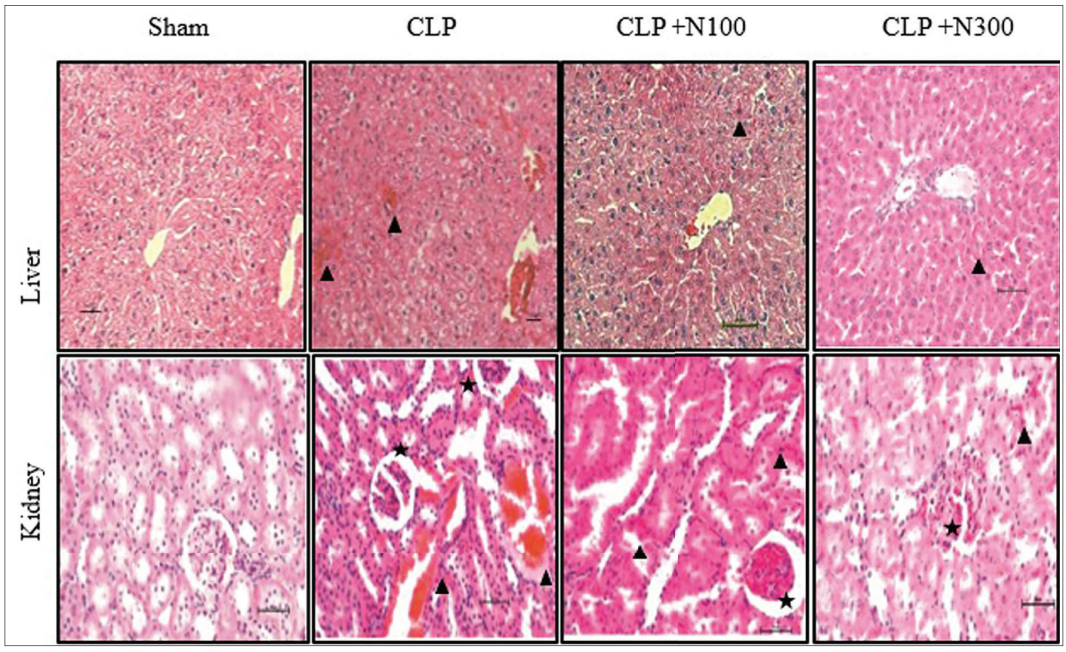


Fig 4. Microscopic images of liver and kidney samples belonging to the sham, untreated and treated sepsis groups after H&E staining. *Liver tissue:* Sham group normal liver parenchyma morphology; sinusoidal congestion and hemorrhage and damaged hepatocytes (arrowhead) are seen in the CLP group. Sinusoidal congestion and hemorrhage and a decrease in damaged hepatocytes (arrowhead) are observed in the sepsis groups receiving treatment. *Kidney tissue:* Normal kidney morphology in the sham group; in the CLP group, Bowman's space enlargement and glomerular congestion (*), vascular congestion and hemorrhage and damage to the tubule epithelium *(arrowhead)* are seen. In the treatment groups, Bowman's space enlargement and glomerular congestion (*), vascular congestion and hemorrhage, and a significant reduction in tubular epithelium damage (arrowhead) are observed. H&E, 200X, 50 scale bar

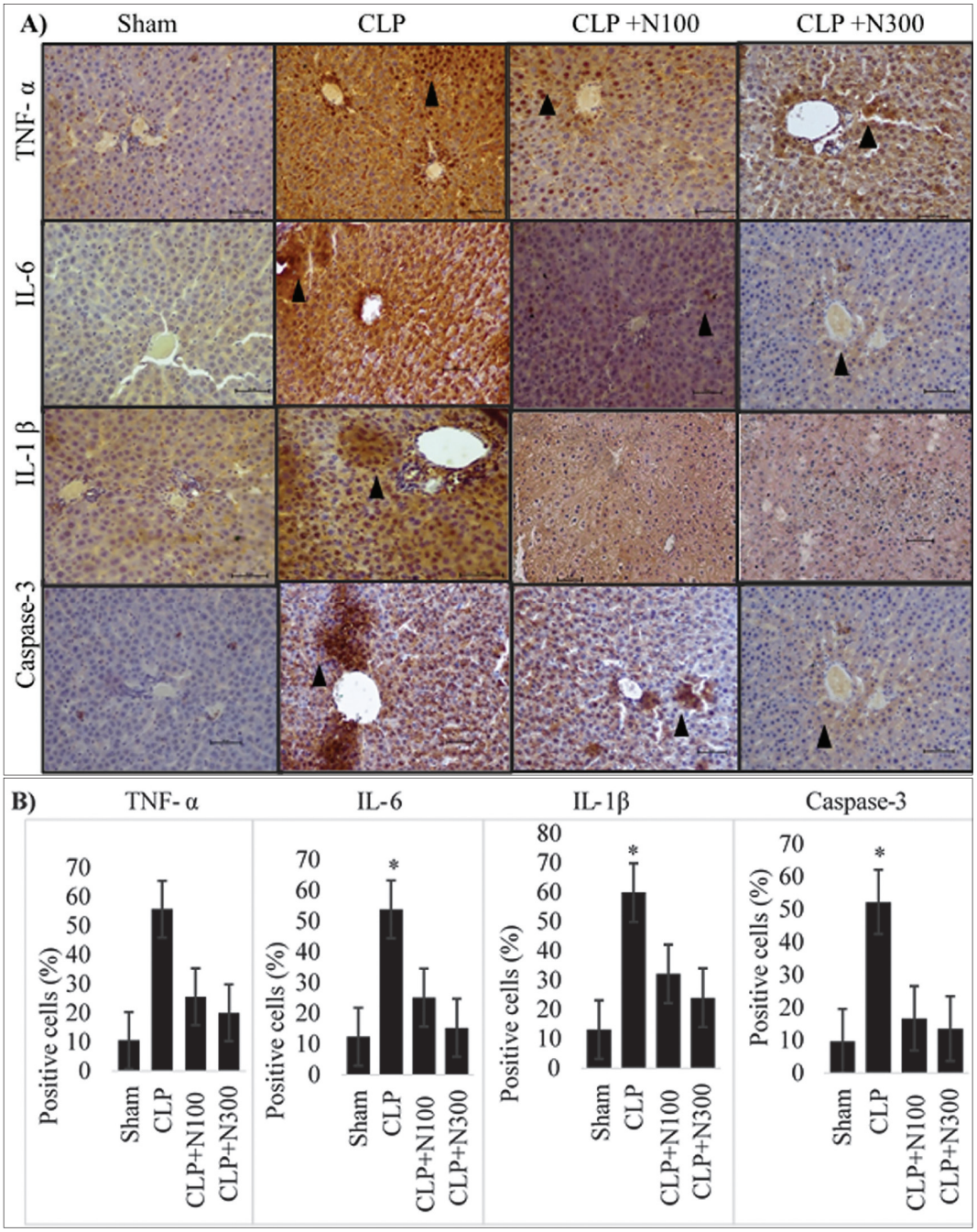


Fig 5. Liver tissue. Sham group, TNF-α, IL-6, IL-1β and caspase-3 normal percentage of positive cells and expression. In the CLP group increased TNF-α, IL-6, IL-1β, and caspase-3 expression (A) *(arrowhead)* and percentage of positive cells (B), while these reduced in the treated CLP+N100 and CLP+N300 sepsis groups *P<0.001 compared to the Sham and treatment groups. IHC, 200X, 50 scale bar

In the histopathological evaluation of the kidney samples, tubular necrosis, cytoplasmic vacuolization and tubular dilation and interstitial hemorrhage were evaluated as CLP-induced morphological kidney damage markers. Tubular necrosis, cytoplasmic vacuolization and tubular dilation and interstitial hemorrhage scores were significantly increased in the CLP group compared to the Sham and treatment groups (for all P=0.001). NAD treatment caused tubular morphology recovery *(Fig. 4)*. In the CLP+N300 group had better preserved renal morphology. In the CLP+N300 group, vacuolar degeneration in tubular epithelium were rarely observed compared to the CLP+N100 group *(Fig. 4)*. There was no significant difference between the Sham and treatment groups for all the histopathological parameters (P>0.05).

For the immunohistochemical examination of both liver and kidney tissue, normal levels of TNF-α, IL-6, caspase-3, and IL-1β expression were observed in the Sham group. TNF-α, IL-6, caspase-3, and IL-1β expression in both liver and

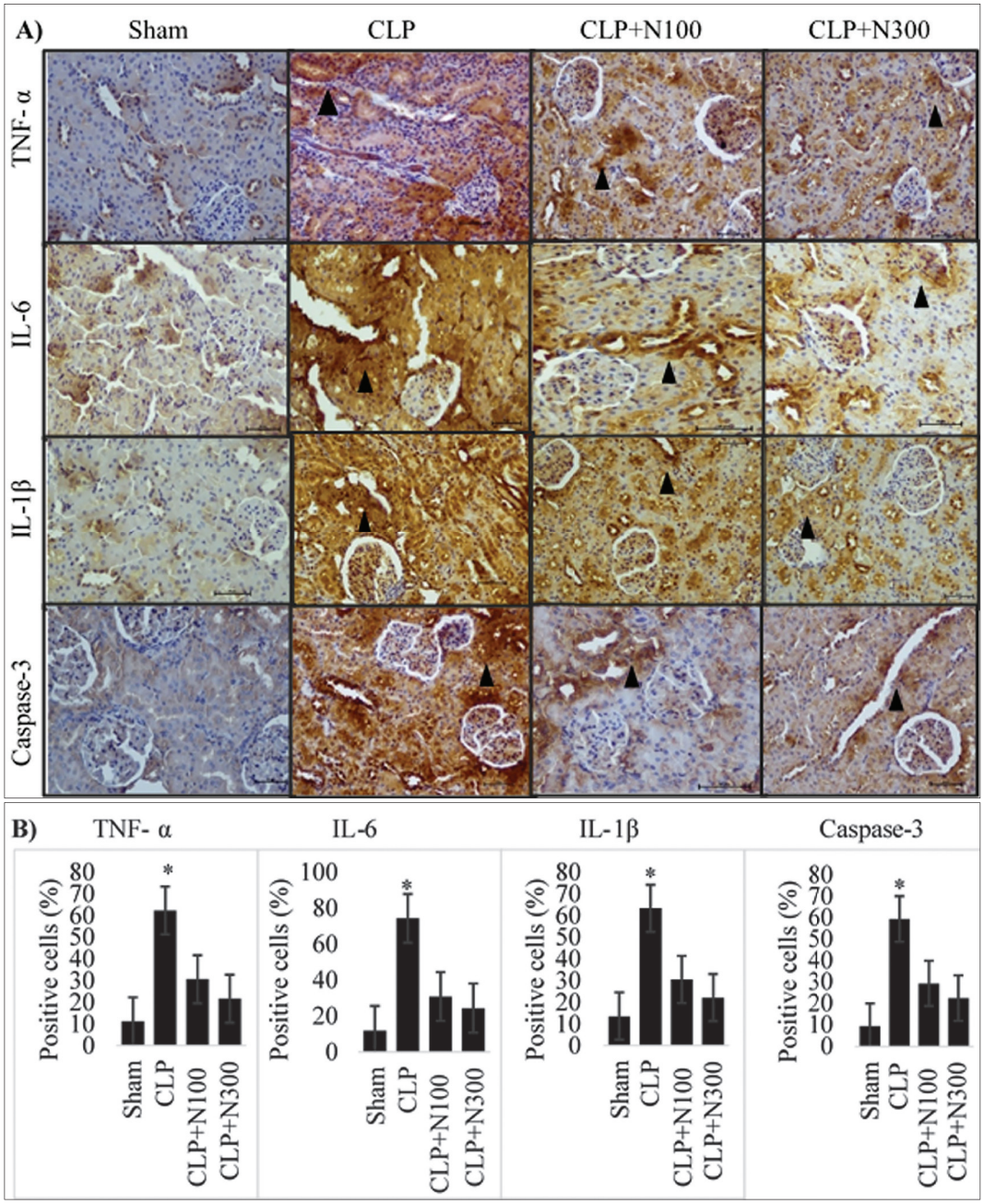


Fig 6. Kidney tissue. Sham group, TNF-α, IL-6, IL-1β and caspase-3 normal percentage of positive cells and expression. In the CLP group increased TNF-α, IL-6, IL-1β, and caspase-3 expression (A) *(arrowhead)* and percentage of positive cells (B), while these were reduced in the treated CLP+N100 and CLP+N300 sepsis groups. *P<0.001 compared to the Sham, and treatment groups. IHC, 200X, 50 scale bar

kidney tissues in the CLP group was highly immunopositive compared to the Sham and treatment groups *(Fig. 5-A,B, Fig. 6-A,B)*. The highest percentage of positive cells in the liver and kidney tissue was in the CLP group (Liver; TNF-α; 55.71%, IL-6; 53.85%, caspase-3; 52.28%, and IL-1β; 59.85% and kidney: TNF-α; 62.42%, IL-6; 74.42%, caspase-3; 59.42%, and IL-1β; 63.42%). The percentage of positive cells was found to be statistically significant (P=0.000 for all) *(Fig. 5-B, Fig. 6-B)*. In addition, immunopositivity and the number of positive cells decreased significantly in the CLP+N300 group (Liver; TNF-α; 20%, IL-6; 15.28%, caspase-3; 13.57%, and IL-1β; 24% and Kidney: TNF-α; 21.71%, IL-6; 24.57%, caspase-3; 22.42%, and IL-1β; 22.28%) compared to the CLP+N100 group (Liver; TNF-α; 25.57%, IL-6; 25.14% caspase-3; 16.71%, and IL-1β; 32.14% and Kidney: TNF-α; 30.71%, IL-6; 31%, caspase-3; 29.28%, and IL-1β; 30.57%), and this decrease was statistically significant (P=0.000) *(Fig. 5-B, Fig. 6-B)*.

Discussion

Sepsis is one of the leading causes of death in intensive care units worldwide. Multi-organ failure is the main feature of

sepsis and death is accompanied by medical complications such as multi-organ dysfunction. One of the main causes of multi-organ dysfunction is the development of oxidative stress [21]. ROS, which are released as a result of oxidative stress caused by sepsis, cause damage to structural proteins and DNA as well as phospholipids found in cellular membranes. Oxidative damage in cells and tissues causes increases in MDA and other oxidation products, and decreases in SOD, GSH levels and CAT activity. In the case of DNA damage, it causes widespread tissue damage by triggering apoptosis [22].

The current study results show that sepsis induced by CLP causes significant damage to the liver and kidneys, and this damage is significantly reduced by nicotinamide treatment. In our study, we saw that microscopic damage in liver and kidney samples in the group with untreated sepsis was associated with an increase in MDA levels, which is an oxidative stress biomarker, and a decrease in endogenous antioxidant GSH and CAT activity. However, in our study, the high degree of increase in the expression of TNF-α, IL-6 and IL-1β cytokines induced by sepsis and involved in the inflammatory process is parallel to MDA levels and this result shows that oxidative damage in tissues triggers the inflammatory process. Specifically, our results showed that nicotinamide slows the increase in the release of CLP induced cytokines. In addition, the increase in caspase-3 expression in tissues showed that apoptosis was triggered by the formation of cellular damage in the tissue.

Sepsis increases the expression and release of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β in tissues. The inflammatory process progressing in sepsis becomes stronger with ROS and turns into a cytokine storm, also known as the cytokine cascade. For this reason, studies about the treatment of sepsis reported that, in addition to reducing cytokine release, preventing ROS formation also decreases tissue and organ damage caused by sepsis and decreases the mortality rate [8]. Inflammasome is essential for the innate immune response to infection and is also important in apoptosis due to sepsis. It was shown that TNF-α, IL-6, IL-1A and IL-1β may be associated with sepsis susceptibility. IL-6 levels in septic rats were showed to increase mRNA expiration and IL-6 plays important roles in heart, liver and kidney damage in septic rats [23].

In our study, in the immunohistochemical examination of both liver and kidney tissue, TNF-α, IL-6, caspase-3, and IL-1β expression were at normal levels in the Sham group. However, TNF-α, IL-6, caspase-3, and IL-1β expression increased in both liver and kidney tissues in the CLP group. It was observed that immunopositivity decreased in the treatment groups. These results indicate that sepsis stimulates intense inflammation pathways in these tissues and causes tissue damage. In our study, it was found that NAD^+ also reduces and corrects the negative effects of sepsis in kidney and liver tissue.

Inflammatory pathogenesis and organ damage leading to death from sepsis, especially for vital organs such as the liver, are still not fully understood. Post-sepsis liver dysfunction was shown to be an independent risk factor for multiorgan dysfunction and death from sepsis. In sepsis, the liver-mediated immune response is responsible for the clearance of bacteria and toxins, but also causes inflammation, suppression of the immune system and organ damage. Mitigation of liver damage and restoration of liver function reduce morbidity and mortality rates in patients with sepsis [24]. Liu et al.[25] showed that there was significant cellular damage in liver tissue in the sepsis group and a significant increase in caspase-3 level in their studies investigating the mechanism of action of sepsis-induced liver damage in rats.

NAD^+ is an essential coenzyme that plays important roles in a variety of metabolic pathways and was approved as a valuable strategy for increasing overall content and treating a wide variety of pathophysiological conditions [26,27]. Our results showing that nicotinamide suppresses pro-inflammatory cytokines are consistent with other studies. Traister et al.[28] reported that NAD^+ reduced the expression of proinflammatory (IL-1β, TNF-α TGF-beta2 and macrophage chemotactic protein-1) and pro-fibrotic cytokines in liver cells. Mukherjee et al.[29] reported that NAD^+ increases liver regeneration, and NAD^+ metabolism can be modulated to support recovery from liver damage. Hong et al.[30] used nicotinamide riboside (NR) application, which is a precursor of NAD, and reported increased NAD^+ levels and decreased oxidative stress, inflammation and caspase-3 activity in lung and heart tissues. In another study, nicotinamide was shown to be protective against acetaminophen-induced liver toxicity, even when administered after injury [31].

In a study investigating the effects of experimental acute sepsis on kidney damage, mitochondrial dysfunction was reported in proximal tubule epithelial cells. In addition, a decrease in nicotinamide adenine dinucleotide and mitochondrial membrane potential was seen [32]. In our study, we found important results showing that NAD^+ corrected the damage caused by sepsis in kidney tissue. In our study, interstitial hemorrhage, enlargement of the Bowman capsule, glomerular congestion and dense hemorrhage areas, and degeneration in the tubular epithelium were observed in the kidney tissues of the CLP group. In the treatment groups, this deterioration in tissue decreased and improvement was almost normal. The improvement in the CLP+N300 group was greater. These findings show that NAD^+ may be a very important biological agent in preventing tissue damage in sepsis, especially in acute microbial sepsis. The pathogenesis of acute kidney injury in sepsis is associated with apoptosis. Ying et al.[33] reported that caspase 3 expression increased in the kidney tissue of septic mice.

Numerous studies showed that nicotinamide may have an important role in infection and sepsis by inhibiting

poly (ADP-ribose) polymerase (PARP) activation, pro-inflammatory mediators, and antioxidant damage by restoring mitochondrial function to restore adenosine triphosphate (ATP) levels. Thus, nicotinamide plays a role in the antioxidant system as well as energy metabolism [34,35]. In recent years, the results of clinical and experimental studies also show NAD+ homeostasis as a determinant of the ability of kidney tissue to resist various stressors [36]. Guan et al.[37] applied the NAD precursor nicotinamide mononucleotide (NMN) to young and old rats, which they created acute kidney injury (AKI). As a result of the study, they reported that NAD levels increased in both age groups. In another study, it was reported that nicotinamide administration decreased the severity of AKI by increasing renal NAD^+ [38].

As a result, increased oxidative stress due to sepsis induced by CLP, inflammation and apoptosis caused severe damage in tissues. Our findings show that the damage from sepsis is prevented by the antioxidant, anti-inflammatory and anti-apoptotic effects of nicotinamide adenine dinucleotide. It is clear that nicotinamide has great potential for multiple diseases, but the development of new therapeutic strategies needs to be elucidated with further investigations of new cellular pathways largely and closely directed by nicotinamide.

Availability of Data and Materials

The authors declare that data supporting the findings of this study are available within the article.

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This research did not receive any financial support from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest

We declare that there is no conflict of interest between the authors.

Authors Contributions

SD, planed this study design, conducted this experiment, sitatistics and biochemical analysis, and wrote this manuscript. OB conducted histopalogical and immuno-histocemical examination and evaluation, study design, contributed to literature searches, data acquisition. AS and NB contributed to write, biochemical analysis, literature searches.

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RESEARCH ARTICLE

Effect of Probiotic Mixture Supplementation to Drinking Water on the Growth Performance, Carcass Parameters and Serum Biochemical Parameters in Native Turkish Geese

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Abstract

This study aims to evaluate the effects of a probiotic mixture (PM) supplement on the growth performance and the serum biochemical parameters of native Turkish geese. A total of 60 one-day-old goslings were randomly divided into three groups, and each group was divided into four subgroups with five animals each. While no supplement was added to the drinking water of the control group, 0.25 mL and 0.50 mL of PM were supplemented in the drinking water of the PM-1 and PM-2 groups, respectively. The trial was completed in 10 weeks. The results revealed that supplementation of 0.50 mL/L PM in drinking water improved the live weight, live weight gain, feed consumption, feed/gain ratio. The supplementation of PM improved dressing and the liver weight, but it did not have an impact on the heart and gizzard weight of geese. It was also observed that 0.50 mL of PM increased the serum biochemical parameters such as glucose, total protein, albumin, calcium, phosphorus levels. At the same time, it decreased the triglycerides, total cholesterol, LDL-Cholesterol, Aspartate Aminotransferase levels. The present study showed that PM added at 0.50 mL/L in drinking water could be used as a supplement by local breeders for the growth performance of geese.

Keywords: *Biochemical parameters, Carcass, Goose, Growth performance, Probiotic*

Yerli Türk Kazlarında İçme Suyuna Probiyotik Karışımı İlavesinin Büyüme Performansı, Karkas Parametreleri ve Serum Biyokimyasal Parametreleri Üzerine Etkisi

Öz

Bu çalışmada, probiyotik karışımı (PM) ilavesinin yerli Türk kazlarının büyüme performansı ve biyokimyasal parametreleri üzerine etkilerinin değerlendirilmesi amaçlanmıştır. Toplam 60 adet 1 günlük kaz civcivi rastgele üç gruba ayrıldı ve her grup her biri beş hayvan olmak üzere dört alt gruba ayrıldı. Kontrol grubu içme suyuna katkı maddesi eklenmezken, PM-1 ve PM-2 gruplarının içme suyuna sırasıyla 0.25 mL ve 0.50 mL PM ilave edildi. Deneme 10 haftada tamamlandı. Sonuçlar içme suyuna 0.50 mL/L PM ilavesinin canlı ağırlık, canlı ağırlık artışı, yem tüketimi, yem/ağırlık artışı oranını iyileştirdiği ortaya konmuştur. PM ilavesi, karkas ve karaciğer ağırlığını iyileşmiş, ancak kazların kalp ve taşlık ağırlığı üzerine bir etkisi olmamıştır. Ayrıca %0.50 PM seviyesinin glikoz, total protein, albümin, kalsiyum ve fosfor gibi serum biyokimyasal parametrelerini artırdığı, trigliserit seviyesini düşürdüğü görülmüştür. Bu çalışma, içme suyuna 0.50 mL/L düzeyinde ilave edilen PM'nin yerel yetiştirici koşullarında beslenen kazların büyüme performansına kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Biyokimyasal parametreler, Karkas, Kaz, Büyüme performansı, Probiyotik*

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Introduction

The use of antibiotic supplements has for many years created risks of developing resistant bacteria, leaving residue in animal products, and resulting in adverse effects on human health, which, in turn, has led to concerns among the industry stakeholders [1]. Therefore, the use of antibiotics in animal production is prohibited. There is interest in seeing the effect of how probiotic, prebiotic, and herbal-originated natural alternative supplements have improved the efficiency as well as how they have helped to preserve the health of the animals [2,3].

Probiotics are defined as microorganisms that help to create a beneficial population in the gastrointestinal tract of poultry, exhibit an antagonistic effect on pathogens, and create beneficial conditions for the use of nutrients [4]. These microorganisms are composed of bacteria and yeast [2]. Various studies in the literature report the positive effect of probiotics on yield in animal feeding. However, the most important factors contributing to its effectiveness have been the probiotic strain used and the level of its use [5]. In this context, investigating new strains with good probiotic properties and effective optimization of their concentrations is quite important for the efficiency of the applications of probiotics. Strains such as *Lactobacillus, Bacillus, Bifidobacterium, Streptococcus, Enterococcus, Aspergillus, Candida,* and *Saccharomyces* have been used as probiotics for the nutrition of poultry, and they are still used today [6]. Among these strains, the species of *Lactobacillus* spp. and *Saccharomyces cerevisiae* have been the most commonly used ones [7]. According to numerous studies, *Lactobacillus* spp. and *S. cerevisiae* were found to prevent the development of harmful bacteria in the digestive system of poultry and to preserve the health of the flock [4,8]. Furthermore, *Lactobacillus* was found to be resistant to the high acidity of the stomach, shows resistance against salinity and bile, offers a high adaptation to ambient temperature [9]. Besides, It was determined that by increasing the concentrations of *Lactobacillus* species present in the digestive tract of geese, they showed higher antimicrobial activity against pathogens [10].

Geese are raised under traditional conditions in Turkey, both as an alternative to the consumption of animal protein and as important contributions to family economies. Geese are regularly fed on the pasture once they are 2-3 weeks of age until they have reached the appropriate weight for slaughter. Geese are released to pasture during the daytime and kept in houses at night. Concentrated feed is added as well as pasture in goose ration [11]. In the literature, a limited number of studies have been conducted on the improvement of the growth performance of native Turkish geese [11,12].

Probiotic mixture (PM) has been hypothesized to have beneficial impacts on growth performance. Probiotic supplementation also improves the metabolic response due to regulating serum biochemical profiles of poultry. This research was designed to evaluate the growth performance of native Turkish geese and various serum biochemical parameters by using a PM.

Material and Methods

Ethical Statement

Ethical Approval: This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAÜ - HADYEK/2019-117).

Animal, Feed and Experimental Design

The study was carried out at the Research Farm of Kafkas University. A total of 60 male one-day-old native Turkish goslings were used in the study. The animals were subjected to 70 days (14 days of adaptation and 56 days of feeding period) feeding program. After the goslings were fed the uniform basal diet (20% CP and 2900 kcal/kg ME) for an adaptation period through 1-14 days of age, they were grazed *ad libitum* on the pasture for the trial period lasting from day 15 to day 70. On day 15, based on the homogeneous average weight of geese per subgroup, all geese were weighed and assigned to three groups, with four subgroups per group and five birds per subgroup. An enclosed space of about 0.4 to 0.5 m^2 was provided per animal. The PM was added at 0 (control group), 0.25 mL and 0.50 mL to drinking water. The geese were given 450 mL/day of drinking water in individual drinkers and it was confirmed that the geese consumed the water. The PM liquid solution was administered via drinking water from day 15 to day 70 at a dose rate of 0.25 and 0.50 mL per liter of drinking water by way of a dosage pump. The drinking water was non purified well water with a pH of 7.4 and no residual chlorine at 0.5 mg/L. The geese were fed in the pasture between 08.00 and 16.00. After grazing, the geese were supplemented with as much barley meal as they could consume. The feed was provided *ad libitum*. The pasture samples were collected from five different parts of the pasture at the beginning, middle and end of the trial. The grass was mixed, and its dry matter and crude protein contents were analyzed *(Table 1)*. The nutrient content of the feed used in the study was determined following the methods reported in [13]. In the calculation of metabolized energy, the formula recommended by TSE was used [14].

The results of the nutrient analysis of feed and barley meals are presented in *Table 2* and *Table 3*. The PM, which was used in the experiment, was obtained from a commercial manufacturer (EM Premium® - EM Agriton Ltd. Sti. Izmir/Türkiye). PM (1×10^7 cfu/g; pH: 3.00-3.85) contains five bacteria and one yeast strain: *Lactobacillus fermentum, L. plantarum, L. rhamnous, L. casei, L. delbrueckii,* and *S. cerevisiae*.

Table 1. *Nutrient composition of pasture used in the experiment*

Items *	Periods		
	Begining	Middle	Finishing
Dry matter (%)	18.90	28.50	32.30
Crude protein (%)	12.20	13.15	13.65
** Analyzed values*			

Table 2. *Nutrient and chemical values of starter diet*

Ingredients	%
Maize, yellow	55.30
Soybean meal, 44% CP	29.26
Barley meal	2.85
Wheat bran	4.40
Sunflower meal, 36% CP	3.50
Vegetable oil	2.20
Limestone	0.85
Dicalcium phosphate	0.90
DL-Methionine	0.10
L-Lysine HCl	0.03
Salt	0.35
Vit-Min Mix[1]	0.25
Chemical Analysis	
Dry matter (%)	89.50
Crude protein (%)	20.00
Ca (%)	0.65
Available P (%)	0.31
Metabolic Energy (kcal/kg)	2900

[1] In each kg of diet: 7.000.000 I.U. Vit. A; 60.000 I.U. Vit. D_3; 20.000 I.U. Vit E; 2.000 mg Vit. K_3; 1.500 mg, Vit. B_6; 7 mg Vit. B_{12}; 5.000 mg, Nicotinamide; 40 mg Folic acid; 0.40 mg, Zinc sulphate; 0.50 mg, Iron sulphate; 0.04 mg, Mn sulphate; 0.15 mg, Copper sulphate

Table 3. *Chemical analysis of barley meal*

Items	Values
Dry matter (%)	87.10
Crude protein (%)	11.50
Ash (%)	2.50
Crude fiber (%)	5.00
Neutral detergent fiber (%)	20.90
Acid detergent fiber (%)	6.50
Starch (%)	58.70
Metabolic Energy (kcal/kg)	2700

Growth Performance

The LW and live weight gain (LWG) values of the geese were determined by weighing them individually each week. Also, feed consumption (FC) was determined weekly. Thus, the feed/gain ratio (F/G) was calculated by dividing FC by LWG (FC/LWG). The concentrate was considered while determining feed consumption and feed/gain ratio.

Carcass

Six animals from each group were randomly selected at the end of the experiment. After the slaughtering, the processes of plucking, separating the legs, and removing internal organs were carried out consecutively. Then, their carcass and visceral organ weights were determined. The dressing was found by calculating the ratio of cold carcass weight to the LW.

Collection and Analysis of Serum Samples

At the end of the trial, blood samples (6 geese/per group) were taken from *Vena brachialis*. Then, serum samples were obtained by centrifuging the blood samples at 4000 rpm for 10 min. Then the samples were stored at -20°C until analysis. After the serum was thawed at room temperature, the values of glucose (GL), total protein (TP), albumin (Alb), bilirubin (Bil), calcium (Ca), phosphorus (P), triglyceride (TG), Total cholesterol (TC), HDL-Cholesterol (HDL), Aspartate Aminotransferase (AST), Alanine aminotransferase (ALT) and Gama Glutamyl Transferase (GGT) were measured spectrophotometrically by using a Colorimetric Assay kit (Elabscience, UK).

Statistical Analysis

The data were evaluated using the SPSS 20 (IBM Inc., Chicago - IL) software package. The results were analyzed using the ANOVA test. The Tukey's range test was conducted to determine the mean separation among the groups. The level of statistical significance was assumed to be $P<0.05$.

Results

At the end of the experiment, significant differences were found between the groups in LW and LWG *(Table 4)*. According to the results of the experiment, it was found that the supplementation of 0.5 mL/L (PM-2) probiotic mixture in the drinking water of the geese significantly increased the LW and LWG compared to the control group ($P<0.05$). Also, the PM-2 group was found to have the highest FC value and the best F/G ($P<0.05$).

Significant differences were found between the groups in terms of dressing ($P<0.05$). Internal organ weights were affected by the supplementation of PM *(Table 5)*.

It was found that the addition of PM supplied a significant increase in biochemical parameters of GL, TP, Alb, Ca, and P levels. It caused a significant decrease in the TG level ($P<0.05$). Bil level was not influenced by PM supplementation ($P>0.05$) *(Fig. 1)*.

Table 4. *Effect of PM on performance parameters of geese*

Parameters	Groups			P
	Control	PM-1	PM-2	
Initial LW (g/bird)	551.69±2.30	546.96±2.21	548.63±2.97	0.408
Final LW (g/bird)	3612.90±21.87^{b}	3573.25±16.88^{b}	3701.05±18.67^{a}	0.001
ADG (g/bird/day)	54.66±0.41^{b}	54.04±0.29^{b}	56.29±0.34^{a}	0.001
ADFC (g/bird/day)	198.96±1.11^{b}	197.79±1.08^{b}	201.52±1.05^{a}	0.047
F/G	3.64±0.03^{a}	3.66±0.02^{a}	3.58±0.02^{b}	0.025

LW: *Live weight,* ***ADG:*** *Average daily gain,* ***ADFC:*** *Average daily feed consumption;* ***F/G:*** *Feed/Gain;* ***PM-1:*** *0.25 mL/L Probiotic mixture;* ***PM-2:*** *0.50 mL/L Probiotic mixture;* $^{a-c}$ *Values in the same row with different superscripts were significantly different (P<0.05)*

Table 5. *Effect of PM on carcass parameters and visceral organ weights of geese*

Parameters	Groups			P
	Control	PM-1	PM-2	
Dressing (%)	67.04±0.24^{b}	66.67±0.26^{b}	68.12±0.15^{a}	0.010
Heart (g)	22.80±0.97	21.80±1.66	23.13±0.80	0.317
Liver (g)	133.80±6.76^{b}	118.80±2.73^{c}	145.40±4.77^{a}	0.002
Gizzard (g)	152.40±7.87^{ab}	145.20±8.00^{b}	161.73±6.88^{a}	0.013

PM-1: *0.25 mL/L Probiotic mixture;* ***PM-2:*** *0.50 mL/L Probiotic mixture;* $^{a-c}$ *Values in the same row with different superscripts were significantly different (P<0.05)*

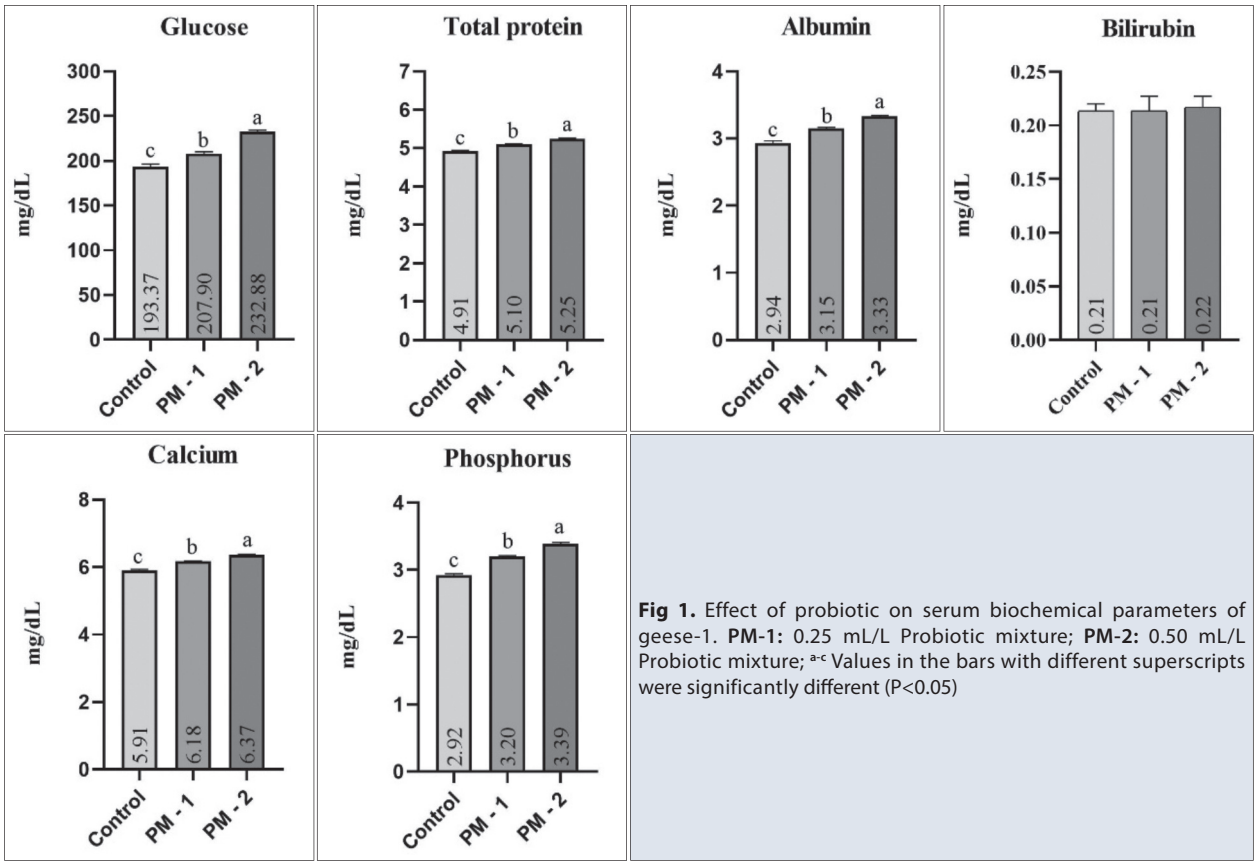

Fig 1. Effect of probiotic on serum biochemical parameters of geese-1. **PM-1:** 0.25 mL/L Probiotic mixture; **PM-2:** 0.50 mL/L Probiotic mixture; $^{a-c}$ Values in the bars with different superscripts were significantly different (P<0.05)

As presented in *Fig. 2*, levels of serum TG, TC, and LDL in PM groups decreased compared to the control group (P<0.05). HDL level was not impacted by PM supplementation (P>0.05). While dietary PM decreased significantly on activities of AST (P<0.05), it did not have any significant effect on activities of ALT and GGT (P>0.05).

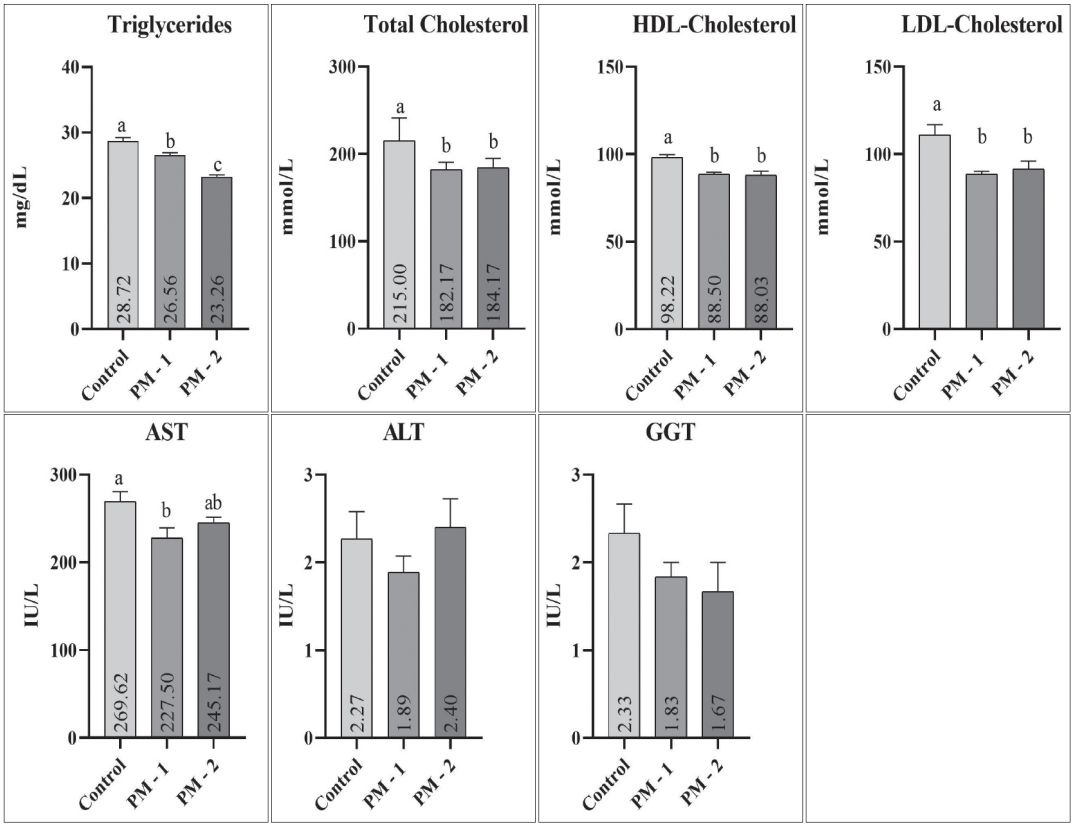

Fig 2. Effect of probiotic on serum biochemical parameters of geese-2
PM-1: 0.25 mL/L Probiotic mixture; **PM-2:** 0.50 mL/L Probiotic mixture; [a-c] Values in the bars with different superscripts were significantly different (P<0.05)

Discussion

The probiotic supplemented drinking water resulted in significant differences in native Turkish geese in terms of LW and LWG during the ten-week experiment. The present study showed similar results with some studies in the literature [15,16]. It was reported that a 0.5% and 1% probiotic supplement increased live weight [17]. Also, a 150 g/ton commercial probiotic supplement was reported to increase the LW, LWG and FC values and improve the F/G [18] in broilers. It is thought that the improvement in performance values in the cited study was the result of the probiotic supplement to the diet, which increased the digestion and absorption of nutrients in the digestive tract, reduced the toxic components, and demonstrated an antagonistic effect against pathogenic bacteria [19].

Contrary to the results of the present study, it has been reported that the probiotic supplement did not affect the performance values of the geese [20]. Yaman et al.[20] reported that a 0.20% and 0.50% probiotic supplement to the goose feeds did not affect LW, LWG, FC, and F/G. In a study using similar bacteria and yeast to the current study, it was determined that Kefir contains strains of *L. fermentum, L. plantarum, L. acidophilus, L. casei, L. delbrueckii* and *S. cerevisiae* was increased the performance parameters of geese [8]. Moreover, the results are not consistent with the results of studies reporting that the combiotic supplement (probiotic + prebiotic) or just probiotic added to quail and broiler rations did not affect the LWG, FC, and F/G [21,22]. These differences in the results may be due to viability of the probiotic mixture. The viability of the product is not continuously examined before it is applied. In addition, it is important to choose strains with maximum capacity for survival and growth rate in the digestive system. It is important to note that the efficacy of probiotics not only depends on the strains it contains but also on the animal's digestive system and diet. common probiotics like *Lactobacillus* and *S. cerevisiae* were used in the present study [15,20]. According to the results of present study, the positive results obtained from the probiotic supplemented groups in terms of performance parameters indicate that the supplement used is an alternative feed supplement that supports growth.

At the 0.5% level of PM supplementation, the dressing and the organ weight of the geese increased. A similar result was reported by Toghyani et al.[23] who found differences in the dressing and liver of broilers when probiotics were supplemented into the diet. Alam and Ferdaushi [24] stated that probiotic supplementation improved dressing and liver weight but had no effect on the weights of the heart and gizzard of broilers. On the contrary, studies have reported that the supplementation

of probiotics had no effect on dressing and weights of internal organs [25,26].

The differences in results of dressing and internal organ weights are thought to be due to the probiotic strain, administration of the probiotic and animal varieties. An increase in dressing and liver and gizzard weights could be due to their great body weight in the current study.

The serum biochemical parameters are usually used as the indicator of the physiological status of animals. The inclusion of probiotic mixture had a significant effect on serum glucose, total protein, albumin, calcium, phosphorus, and triglycerides. These effects could be explained by the increasing absorptive capability of the intestinal mucosa due to supplementation of the probiotic mixture. Therefore, animals could benefit further from the nutrients [27]. This situation may explain the change in the above-mentioned parameters with a probiotic mixture supplement.

In this study, the commercial probiotic supplement was found to increase the serum GL levels in geese. These results are similar to the studies reporting that natural and commercial probiotics significantly increased the serum GL levels in quail, broiler, and ducks [9,28]. However, several studies reported that the supplementation of probiotics did not affect the GL level [22,29]. The differences between the results of the studies are caused by the difference in the types of the probiotic supplement and the levels of use as well as the diet content.

The results of the present study are consistent with the studies reporting that the probiotic supplement increased the serum TP and Alb levels in broilers and quails [9,30]. This is because lactic acid bacteria increase the use of amino acids and proteins by preventing the breakdown of proteins into nitrogen and hindering pathogenic bacteria that reduce the efficiency of protein in the feed [31]. However, the results of the present study conflict with the results of the studies by and Abdel-Hafeez et al.[32], which reported that the probiotic supplement reduced TP levels in the broiler compared to the control group, and the study of Sahin et al.[22], which reported that the combiotic added to quail diets did not affect the serum the TP and Alb levels. While some studies showed that serum Bil level was affected by supplementation of probiotic, another research demonstrated that bil level was not affected [33,34]. Capcarova et al.[35] also reported that serum Bil level was not influenced by probiotic supplementation in the drinking water for broilers.

In the present study, the Ca and P values increased significantly due to the probiotic supplementation. Scholz-Ahrens et al.[36] reported that a probiotic might increase calcium absorption in the intestines by reducing the gastrointestinal pH of short-chain fatty acids (SCFAs) produced by certain probiotic bacteria. Siadati et al.[9] and Hosseini et al.[37] found the highest P level in the probiotic-supplemented group. Eizaguirre et al.[38] reported that the probiotic decreased pH in the intestinal tract and increased the absorption of mineral substances by increasing their solubility.

While the results of the present study were similar to the results of the studies reporting that natural and commercial feed supplements (probiotic, prebiotic) increased the P level in broilers and quails, they were not consistent with the results stating that these supplements did not the affect calcium levels [9]. Also, the results of the present study contradicted the results of the study reporting that the serum calcium level increased with the probiotic supplement but the phosphorus level did not change [39]. Furthermore, two studies reported that the serum Ca and P levels were not affected by the probiotic supplement [39,40].

The results of the present study were similar to the results of the study reporting that various levels of probiotic supplements to broiler diets decreased TG, TC and LDL levels significantly compared to control and antibiotic groups [17,18]. Several studies report that TG, TC and LDL levels in poultry were not affected by the probiotic supplement [29,41]. Studies have also been reported that HDL level was not affected by the addition of probiotics [42,43]. These results were similar to the Kalavathy et al.[44] who demonstrated that the addition of PM reduced serum LDL level and but had no influence on serum HDL level in broilers.

Geese blood biochemical indices have been reported, but activities of key enzymes of fat metabolism are rarely described. Probiotics affect digestive enzymes, amino acids, B vitamins, unknown factors that impact the animal gastrointestinal system. Probiotics can support entero-hepatic circulation and arrange bile acid synthesis to reduce cholesterol. Probiotics can also develop fat metabolism by affecting the activity of enzymes. They reduce TG synthesis and decrease TG concentrations [45]. Lactic acid bacteria lower cholesterol by absorbing cholesterol in the intestinal system, producing bile salt hydrolase, an enzyme responsible for the deconjugation of bile salts and helps to secrete more bile acids in the feces [30]. Besides, it was reported that the probiotic supplement reduced the serum TC level indirectly by limiting the activity of acetyl-CoA carboxylase [46]. In the present study, reduction in serum TG, TC and LDL were observed during the growth phase of native Turkish geese in response to probiotic supplementation in drinking water. There seemed to be some interaction between growth parameters and probiotics by resulting in advanced usage of nutrients and boosting the speed of lipid metabolism in the present study.

ALT, AST and GGT exist widely in the liver. They are also major markers of liver function and get a strong link with animal growth performance [47]. ALT and GGT levels,

exception AST, were not affected by PM addition. Similar results were noticed in some studies [35,48]. It was reported that *L. plantarum* and *B. infantis* added to the diet caused a diminished ALT level in rats [49]. It was also determined that dietary *S. cerevisiae* increased serum ALT [50]. PM by decreasing effects of stress can cause a lower enzyme activity and be a protective agent for liver against damage factors in geese. Also, these liver enzyme parameters measured in the native Turkish geese can also be used as reference values for the literature.

In conclusion, the present study revealed that a 0.5 mL/L supplementation of probiotic might have an improving effect on performance and biochemical parameters in native Turkish geese. It was also concluded that probiotic supplements added to traditional feeding methods could increase the yield performance and the effectiveness of the probiotic on the native Turkish geese should be examined in detail.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (M. Ölmez).

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Conflict of Interest

The authors declared that there is no conflict of interest.

Authors' Contributions

MÖ and TŞ designed the study. MÖ, TŞ, and SD performed the feeding period. ÖK and SD collected the data. MÖ carried out biochemical analysis. MÖ and MAY carried out the statistical analysis. The manuscript was written by MÖ, MAY and TŞ. All authors approved the final version.

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SHORT COMMUNICATION

First Detection of Tacheng Tick Virus 2 in Hard Ticks from Southeastern Kazakhstan

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Abstract

We aim to detect the presence of Tacheng tick virus 2 (TcTV-2) in ticks of southeastern Kazakhstan. A total of 205 ticks were collected and separated into four species, namely *Hyalomma scupense, Dermacentor marginatus, Hyalomma asiaticum* and *Hyalomma anatolicum*. The partial S segment of TcTV-2 was detected in individual RNA of separated ticks by reverse transcriptase polymerase chain reaction. 11.22% (23/205) of ticks were positive to the viral S segment. This is first report of presence of the TcTV-2 in *Hy. scupense* and *Hy. anatolicum* from Kazakhstan. This finding extends tick species and the geographic distribution of TcTV-2.

Keywords: *Hyalomma scupense, Hyalomma anatolicum, Kazakhstan, Tacheng tick virus 2*

Güneydoğu Kazakistan'da Sert Kenelerde Tacheng Kene Virüsü 2'nin İlk Tespiti

Öz

Güneydoğu Kazakistan'a ait kenelerde Tacheng kene virüsü 2'nin (TcTV-2) varlığının araştırılmasını amaçladık. Toplam 205 kene toplandı ve keneler *Hyalomma scupense, Dermacentor marginatus, Hyalomma asiaticum* ve *Hyalomma anatolicum* olmak üzere dört türe ayrıldı. TcTV-2'nin kısmi S segmenti, tür ayırımı yapılmış kenelerin bireysel RNA'sında reverse transkriptaz-polimeraz zincir reaksiyonu ile tespit edildi. Kenelerin %11.22'si (23/205) viral S segmenti yönünden pozitifti. Bu, Kazakistan'da *Hy. scupense* ve *Hy. anatolicum*'da TcTV-2'nin varlığına dair ilk bildirimdir. Bu bulgu, kene türleri ve TcTV-2'nin coğrafi dağılımı hakkında bilgi sunmaktadır.

Anahtar sözcükler: *Hyalomma scupense, Hyalomma anatolicum, Kazakistan, Tacheng kene virüsü 2*

INTRODUCTION

Tacheng tick virus 2 (TcTV-2) is an emerging tick-borne virus which is a member of the genus *Uukuvirus* in the family *Phenuiviridae* order to *Bunyaviruses*. In 2019, TcTV-2 was detected in human and caused fever, headache and multiple clinical symptoms in the Xinjiang Uygur Autonomous Region (XUAR, northwestern China), and had

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potential risk of person-to-person transmission. Presence of ticks in China for *Dermacentor nuttalli*, *Dermacentor silvarum*, *Dermacentor marginatus* and *Hyalomma asiaticum* were 5.46% (10/183), 16.67% (2/12), 14.81% (16/108) and 11.90% (5/42), respectively [1]. TcTV-2 was detected in *Rhipicephalus sanguineus* in Turkey and in *Dermacentor reticulatus* in Romania by high-throughput transcriptome sequencing [2,3].

Kazakhstan is located in Central Asia and borders five countries (China, the Russian Federation, Kyrgyzstan, Uzbekistan and Turkmenistan). To date, at least 12 species of ticks have been detected in southeastern Kazakhstan [4]. However, it is still unclear whether TcTV-2 can be detected in hard ticks in this region, which is adjacent to XUAR, China.

Our study aims to confirm whether TcTV-2 can be detected in hard ticks in Kazakhstan. In the present study, TcTV-2 was detected in hard ticks in three oblasts of southeastern Kazakhstan.

Material and Methods

Ethical Statement

The sampled ticks were treated and imported into Chinese lab according to the requirement of the Administration of Animal and Plant Quarantine of the People's Republic of China.

Tick Collecting and RNA Extraction

From March to May during 2018-2019, contemporaneously with the peak activities of adult ticks in Kazakhstan, a total of 6107 hard ticks were collected from Eastern and Southern Kazakhstan. According to our previous work, the morphological characteristics of ticks were taxonomically identified using a stereoscopic dissecting microscope [4,5]. Here, 205 adult ticks were sampled from their natural hosts in four oblasts including East Kazakhstan, Almaty, Jambyl and South Kazakhstan. Parasitizing ticks were collected from the entire body of each animal including cattle, horses and sheep. The geographical information was shown in *Table 1*. The total RNA of each tick was extracted by RNAprep Pure Tissue Kit (Tiangen, Biotech Co., Ltd., Beijing, China).

Nested Reverse Transcriptase Polymerase Chain Reaction (nRT-PCR) Conditions and Phylogenetic Analysis

The partial S segment of TcTV-2 was detected in individual RNA by nRT-PCR. The used primers were referred previous literature [1]. The first round PCR: the cycling conditions consisted of an initial 5 min denaturation at 94°C, followed by 35 cycles at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 8 min. The second round PCR: the cycling conditions consisted of an initial 5 min denaturation at 94°C, followed by 35 cycles at 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 8 min. A total of 205 samples were amplified in a 10 μL reaction containing 2×SYBR Premix Ex Taq II (Tiangen, Biotech Co., Ltd., Beijing, China). Each PCR assay included a negative control (distilled water instead of tick DNA template) and a positive control (with cDNA from TcTV-2-postive ticks from China). Amplicons were visualized by electrophoresis in a 1.5% agarose gel (1×TAE, pH 8.0) stained with Goldview Nucleic Acid Gel Stain (Equitech-Bio, Shanghai,China). The PCR products were sequenced and analyzed by BLASTn and Mega7 (Maxmum likelihood, Bootstrap 1000) [6].

Results

After the nRT-PCR, the S segment produces a single 252 bp fragment *(Fig. 1)*. The viral S segment was detected in 11.22% (23/205) of ticks including 10 *Hyalomma scupense*, 10 *D. marginatus*, 2 *Hy. asiaticum*, 1 *Hyalomma anatolicum*, shown in *Table 1*. Nucleotide sequences were deposited in the GenBank database (GenBank accession number were MT302558, MK286259, MK282660 and MK282666).

BLASTn analysis indicated that TcTV-2 S segment from Kazakhstan shared 99.5% (190/191) identity with those in China, and shared 96.9% (185/191) identity with those in Romania. Furthermore, phylogenetic analysis showed that TcTV-2 in Kazakhstan was closer to the Chinese virus strains in patient, Asian badger, cattle, sheep and hard ticks, and clustered with Yongjia tick virus 1 *(Fig. 2)*.

Table 1. *Geographical distribution, positive rate and tick species of ticks in southeastern Kazakhstan*

Neighboring Country	States	Districts	Number	Tick Species	Origin	Positive Rate
China-Russia	East Kazakhstan	Zaysan County	68	*Hy. scupense*	cattle	14.70% (10/68)
			26	*D. marginatus*	cattle	11.54% (3/26)
China-Kyrgyzstan	Almaty	Karablak County	14	*D. marginatus*	cattle	7.14% (1/14)
			27	*D. marginatus*	horse	11.11% (3/27)
		Ussalle County	14	*Hy. scupense*	cattle	0.00% (0/14)
		Kuksu County	14	*D. marginatus*	cattle	21.42% (3/14)
		Ustobe County	22	*Hy. asiaticum*	horse	9.09% (2/22)
Kyrgyzstan-Uzbekistan	Jambyl	Lugovoy	20	*Hy. anatolicum*	sheep	5.00% (1/20)

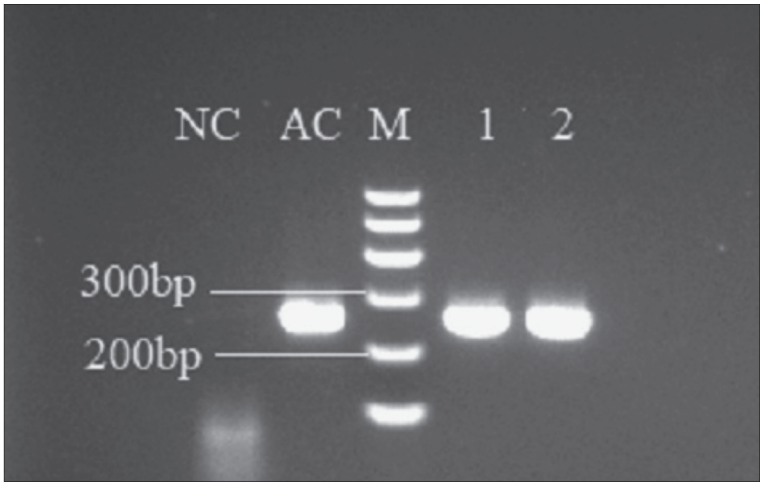

Fig 1. PCR product of TcTV-2 S segment amplification. Lane 1: Blank control; Lane 2: Positive control; Lane 3: 100 bp DNA Ladder Marker; Lane 4 and Lane 5: positive result on 252bp

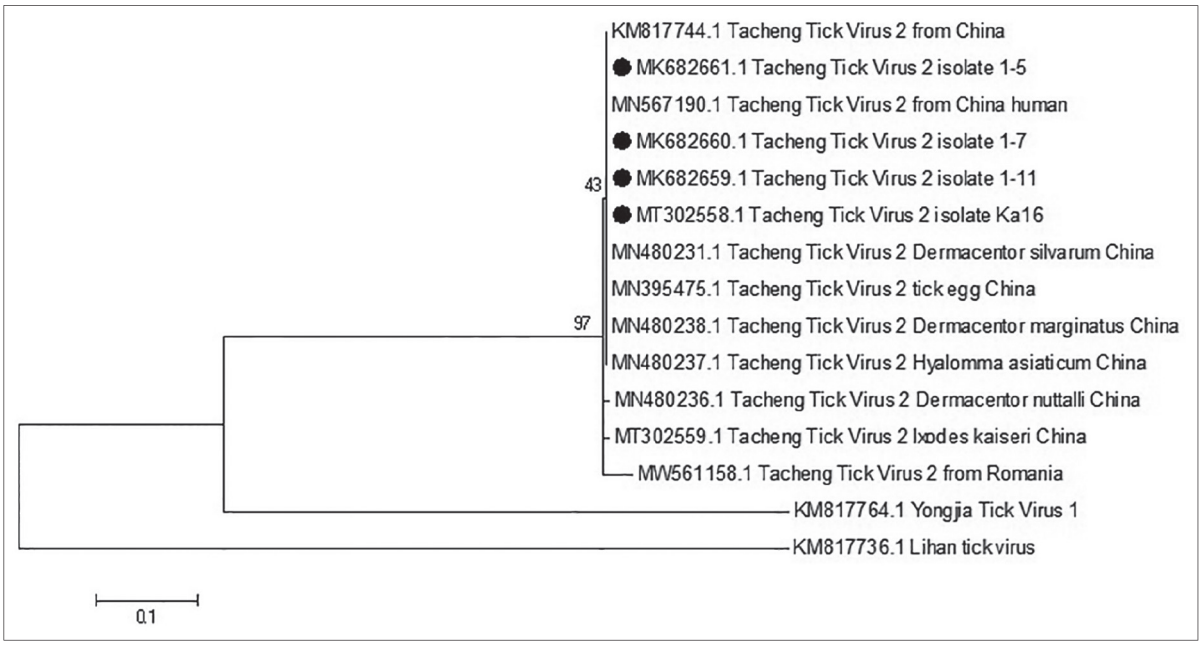

Fig 2. Phylogentic analysis of TcTV-2 partial S segment in Kazakhstan. Phylogenetic analysis was analyzed by Mega7 (Maxmum likehood, Bootstrap 1000), reference sequences were marked with black circle

Discussion

Tick-borne viral diseases have attracted much attention in recent years because of their increasing effects on human and animal health. Previously, a variety of tick-borne emerging viruses were detected in Kazakhstan, including Chim virus *(Orthonairovirus, Bunyavirales)* [7], Tacheng tick virus 1 (MK639367, *Orthonairovirus*, *Bunyavirales*), Tacheng tick virus 5 (MK656451, unclassified ssRNA negative-strand viruses) and Kemerovo virus (*Orbivirus*, *Reovirales*) [8]. In this study, TcTV-2 was detected in four tick species in Kazakhstan, and firstly documented in *Hy. scupense* and *Hy. anatolicum* ticks. To date, more than 30 species of human-biting ixodid ticks in Kazakhstan [9]. In our study, 6107 hard ticks were detected, and shown that 2935 (48.06%) were *Dermacentor*, 1592 (26.07%) were *Hyalomma* and 122 (2%) were *Rhipicephalus* [4]. This suggests *Dermacentor* and *Hyalomma* were dominant tick species in Kazakhstan. In the future, TcTV-2 should be further investigated in more tick species (especially in genera *Hyalomma*, *Dermacentor* and *Rhipicephalus*) sampled from more loci in Kazakhstan.

Previously, TcTV-2 was found in Turkey (36°N-40°N), Romania (Iasi City, 47°N) and China (Qinghe County, Wenquan County, Wusu City, Fuyun County, Gongliu County, Xinyuan County, Shawan City and Fuhai County, 43°N-47°N) [1-3]. Here, TcTV-2 was also detected in hard ticks in Almaty,

Jambyl and East Kazakhstan oblasts (25°N-47°N). This finding suggests that TcTV-2 may be detected in more regions (at least ranging from 25°N to 47°N), although it should be confirmed by more investigations.

TcTV-2, being a marked tick-borne pathogen, was previously detected in Asian badgers, cattle and sheep in China. The data came from GenBank, and their accession numbers were MW725300, MW725298 and MW725299, respectively. Here TcTV-2 was also found in 10.20% (5/49) of horse ticks (*D. marginatus* and *Hy. asiaticum*), which suggested that further investigation on TcTV-2 infection in horses should be carried out in Kazakhstan.

In summary, TcTV-2 was found first time in *Hy. scupense* and *Hy. anatolicum* ticks, and its mean positivity was 11.22% in hard ticks from southeastern Kazakhstan. This finding extends tick species and the geographic distribution of TcTV-2.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author and can be provided on your request.

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Competing Interests

The authors declared that there is no conflict of interest.

Authors' Contributions

YJ, SW and YW conceived and designed the study, and critically revised the manuscript. KG, KO and NU collected the ticks. YJ, SW, MY and ZL performed the experiments and analyzed the data. ZL and YW provided funds and contributed to writing the manuscript. All authors read and approved the final manuscript.

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SHORT COMMUNICATION

Is There an Association Between Breed, Age, and Sex with High and Low Serum Creatinine Levels in Dogs? - From the Analysis of Electronic Medical Record Data

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Abstract

Medical record data were analyzed for serum creatinine level in dogs to determine useful associations between the data. Differences in sex, age, and breed were analyzed using Fisher's exact test, and multiple factors such as sex were analyzed by contingency table analysis. Of the 3347 dogs that were tested for serum creatinine level, 243 dogs had creatinine over 1.4 mg/dL more than once. The overall rate of renal dysfunction in all breeds was 7.3%, but the rate for cavalier King Charles Spaniels was 14.1% (P<0.05), and for Shetland Sheepdogs it was also 14.1% (P<0.05), both significantly higher than the overall rate.

Keywords: *Creatinine, Dog, Electronic chart, Predisposing factor, Renal dysfunction*

Köpeklerde Yüksek ve Düşük Serum Kreatinin Düzeyleri İle Irk, Yaş ve Cinsiyet Arasında Bir İlişki Var mı? - Elektronik Tıbbi Kayıt Veri Analizi

Öz

Veriler arası ilişkilerin saptanması amacıyla köpeklerde serum kreatinin seviyeleri yönünden tıbbi kayıt verileri analiz edildi. Cinsiyet, yaş ve ırk farklılıkları Fisher'in kesinlik testi ile ve cinsiyet gibi çoklu faktörler kontenjans tablosu ile analiz edildi. Test edilen 3347 köpekten 243'ünde serum kreatinin düzeyleri birden fazla kez 1.4 mg/dL'nin üzerinde saptandı. Tüm ırklarda böbrek fonksiyon bozukluğuna ait genel oran %7.3 iken, Cavalier King Charles Spaniels köpeklerde bu oran %14.1 (P<0.05) ve Shetland Çoban Köpeklerinde %14.1 (P<0.05) saptandı ve her iki ırkta da bu oran genel ortalamaya göre önemli ölçüde daha yüksekti.

Anahtar sözcükler: *Kreatinin, Köpek, Elektronik tablo, Predispozan faktör, Böbrek fonksiyon bozukluğu*

INTRODUCTION

The use of medical data obtained by searching electronic medical records and diagnostic images is a rapidly developing field. In human medicine, this method is being used in many different areas, for example, to improve diagnostic accuracy by amassing test data from multiple hospitals [1], to investigate prognostic predictors [2], and in the development of new drugs [3]. Efforts to make use of electronic data on companion animals in clinical practice lag behind those of human medicine. The VetCompass Animal Surveillance Project was launched in the UK

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in 2007 [4], and VetCompass Australia was developed in Australia in collaboration with the UK in 2013 [5]. In both countries, the collection of the medical record data held by individual veterinarians has been contributed to the development of companion animal medicine through knowledge obtained from massive amounts of data that go far beyond individual experience. O'Neill et al.[6] analyzed the electronic medical records of more than 100.000 dogs and reported risk factors for chronic kidney disease (CKD) in dogs among their findings.

In veterinary medicine, the digitization of diagnostic imaging has contributed to the gradual expansion of the use of electronic medical records in secondary veterinary medical institutions and others, and data on matters such as the status of use of anticancer agents are beginning to be published [7]. However, the use of electronic data in most veterinary clinics remains a work in progress, and platforms for the various types of data have yet to be developed. Particularly in cities, there is a tendency for people to keep small dogs. The analysis of electronic medical records from the breeds of dogs commonly kept in cities help to verify risk factors for diseases from a new perspective. In particular, CKD in dogs is a condition commonly encountered by clinicians and obtaining information about CKD in small dog breeds that make up the majority of dogs in cities would contribute to prevent its progress. Stage classification of CKD is mainly based on the patient's serum creatinine (CRE) level [8]. In this study, several years' worth of test data aggregated from the electronic medical record system of a secondary veterinary medical institution was analyzed, and informative associations between different data was investigated, focusing particularly on serum CRE levels, which is important for the classification of CKD stages in dogs.

Material and Methods

Clinical Test Values

We obtained data on blood biochemistry test results (FUJI DRI-CHEM, 7000VZ; FUJIFILM Medical Co., Ltd., Tokyo, Japan), dog breeds, date of birth, medical record number, and testing dates linked to the Anicom Receptor customer management system (Anicom Pafe, Inc., Tokyo, Japan) used by the Animal Medical Center of Tokyo University of Agriculture and Technology, and analyzed the records of 3347 dogs for which CRE test results were available. Data that were outside the measurement range of the test device were excluded.

Observation Period

June 2008 to November 2016 (duration of 8 years and 6 months).

Age and Sex Categories

Dogs aged ≥7.5 years were defined as senior dogs [7], those aged ≥6 months but <7.5 years as adult dogs [9], and those aged <6 months as young dogs. Sex was classified into entire male, neutered male, entire female, and spayed female.

Analysis Software

Statistical Analysis System (SAS) software (SAS Institute Japan Ltd., Tokyo, Japan) was used for analysis. Differences in sex, age, and breed of dog were analyzed using Fisher's exact test, and multiple factors such as age and sex were analyzed by contingency table analysis.

Methods of Analysis

To analyze differences between breeds of dog, the numbers of dogs in each breed were tallied, and the proportions of dogs with renal dysfunction for each breed were calculated. Breeds that included more than 50 dogs were categorized as its own breed, and breeds that were represented by less than 50 dogs were grouped together as "other breeds". The proportions of dogs with renal dysfunction in each breed of dogs were compared with the analysis population excluding these breeds.

Classification of CRE Levels Used in This Study

In reference to the IRIS CKD risk staging [6,7], patients who had serum CRE ≥1.4 mg/dL, the lower limit of CKD stage 2, on ≥2 occasions were classified as the "high CRE group" and the rest were classified as the "low CRE group".

Results

Of the 3347 dogs that were tested for CRE, 243 dogs were classified into high CRE group. By sex, 16.5% were male, 29.7% neutered male, 33.8% female, and 28.2% spayed female, with no sex recorded in 1.8%, and the rate of high CRE group was highest in spayed females (P<0.01) *(Table 1)*. By age, 20.0% were young dogs, 33.0% adult dogs, and 60.6% senior dogs. There were significant differences between each age group in the rate of high CRE group, which was most common in senior dogs (P<0.0001) *(Table 2)*.

The overall rate of high CRE group in all breeds was 7.3%, but the rate for Cavalier King Charles Spaniels and Shetland Sheepdogs were both 14.1% (P<0.05), significantly higher than the overall rate. Conversely, the rate among Dachshunds was 3.4% (P<0.01) and those for Poodles (P<0.05) and Yorkshire terriers (P<0.01) were both 0.7%, all significantly lower than the overall rate *(Table 3)*.

Discussion

Examples of the use of big data in veterinary medicine include a report of mean life expectancy from cremation data of 12039 animals [10], a report of cause of death and mean life expectancy from data of 299555 dogs enrolled in voluntary health insurance [11], and another study using

Table 1. *Effect of sex on high CRE group*

Sex	Study Group (a)		High CRE Group (b)		(b/a)	P Value §
Male	1.133	33.8%	76	31.3%	6.9%	0.7877
Neutered male	550	16.5%	38	15.6%	6.8%	0.6098
Female	993	29.7%	68	28.0%	6.7%	0.3989
Spayed female	609	18.2%	60	24.7%	9.9%	< 0.01
No sex recorded	62	1.9%	1	0.4%	7.3%	0.1301
Total	3.347	100%	243	100%	7.3%	

§ Fisher's exact test
Male was compared with the entire analysis population excluding dogs of entire Male, and Neutered male with the entire analysis population excluding dogs of Neutered male. The comparative controls for entire Male and Neutered male were therefore different

Table 2. *Effect of age on high CRE group. Number of dogs for which CRE test results were available, and with CRE levels within the measurement range*

Age	Study Group (a)		High CRE group (b)		(b/a)	P Value §
Young	213	6.4%	3	1.2%	1.4%	0.0001
Adult	1.105	33.0%	42	17.3%	3.8%	< 0.0001
Senior	2.030	60.6%	198	81.5%	9.8%	< 0.0001
Total	3.347	100%	243	100%	7.3%	

§ Fisher's exact test

Table 3. *Proportions of dogs with high CRE group among breeds represented by at least 50 dogs. It shows the rates of high CRE group in breeds represented by at least 50 dogs within the study population (dogs for which CRE test results were available)*

Breed	Study Group (a)	High CRE Group (b)	(b/a)	P Value §
American Cocker Spaniel	72	3	4.2%	0.4846
Cavalier King Charles Spaniel	64	9	14.1%	< 0.05
Welsh Corgi *	137	16	11.7%	0.0612
Golden Retriever	86	7	8.1%	0.6743
Shih Tzu	190	11	5.8%	0.5625
Shetland Sheepdog	78	11	14.1%	< 0.05
Dachshund	471	16	3.4%	< -0.001
Chihuahua	217	19	8.8%	0.3460
Pug	59	2	3.4%	0.3184
Papillon	91	11	12.1%	0.0959
Beagle	62	5	8.1%	0.8046
French Bulldog	69	4	5.9%	0.8159
Poodle	257	10	3.9%	< -0.05
Pomeranian	104	3	2.9%	0.0844
Maltese	96	4	4.2%	0.3171
Miniature Schnauzer	96	10	10.4%	0.2246
Yorkshire Terrier	142	1	0.7%	< -0.001
Labrador Retriever	131	14	10.7%	0.1194
Mixed Breed	217	21	9.7%	0.1278
Shiba Dog	173	16	9.2%	0.2842
Other**	537	50	9.3%	0.0457
Total	3.347	243	7.3%	

§ Fisher's exact test
** Welsh Corgi: Pembroke Welsh Corgi, Cardigan Welsh Corgi*
*** Including dog breeds of less than 50 dogs*

the same health insurance enrollment data to investigate the prevalence of disease of 18 diagnostic categories [11]. Although such studies are valuable analyses of big data from veterinary medicine, unlike analyses of veterinary medical data such as test results, their results are not immediately applicable to the diagnosis and treatment of patients in clinical practice.

Previous studies have reported that some breeds of dog are genetically susceptible to glomerular disease, a known cause of renal dysfunction. However, because most of these studies have focused on large breeds [12], with almost no studies addressing the breeds and sex at higher risk in small dog breeds that account for the majority of those kept in cities, they have not been particularly useful for clinicians in inner-city area. In this study, we used the hospital electronic customer management system of a secondary veterinary medical institution in the metropolitan district of Tokyo that treats approximately 1.000 new patients per year as a database covering a period of 8 years and 6 months, enabling us to conduct an analysis covering mainly senior small dogs. Since various factors are involved in the rise and fall of CRE level, it is not appropriate to immediately suspect CKD from the elevation. In addition, in recent years, symmetric dimethylarginine (SDMA) has been found to be an index of decreased renal function that appears prior to the elevation of CRE [13]. However, SDMA is difficult to test in the hospital, so it is outsourced to an external laboratory and is not a routine test. It is necessary for clinical veterinarians to be informed of the high and low CRE levels that can be tested in the hospital during routine blood tests, and it is important that this trend is clarified.

CRE levels have been reported to increase with age [8], and our results in this study were consistent with this finding. CKD is a kidney disease that is characterized by structural renal damage with symptoms persisting for at least 3 months [8], and in a study of related factors, O'Neill et al.[6] found no significant difference between males and females. In the present study, sex was classified into entire male, neutered male, entire female, and spayed female, and the rate was significantly higher CRE levels in spayed females than in the other sexes. It is difficult to imagine that spayed females would show high CRE levels for other reasons such as muscle mass, which tends to increase CRE. We were unable to derive a clear answer to this question from the results in the present study, and further investigation is required.

With respect to the significant differences between different breeds of dog, Shelties have been reported to be at increased genetic risk of glomerular disease [12], and this may be related to the high proportion of Shelties with high CRE group seen in this study. The high rate of high CRE seen in Cavalier King Charles Spaniels has also been reported in a British study [6]. This may be connected to the fact that this breed is at high risk of mitral insufficiency [7]. Poodles have also been reported to be susceptible to glomerular disease [12], although the relevance of this to the significantly lower CRE levels in our study is unclear. A diet with restricted protein and phosphate content in stage 1 is also reportedly effective in slowing IRIS stage progression [14]. Incorporating these preventive measures into the health management of spayed female dogs and those breeds with significantly high CRE levels in this study while they are still healthy may lead to the early detection of CKD and to slowing of its progress.

In the present study, the dogs were grouped according to their serum CRE levels, and the related factors were analyzed using electronic medical records as a database. The results suggested that there were differences in the proportion of dogs with high and low CRE levels depending on gender and breed. Although CRE levels are considered to be one of the useful indicators of renal function, comprehensive data such as urinalysis, imaging tests, and blood pressure measurements are necessary for the diagnosis of CKD. A limitation of the database used in our animal hospital is that it is incapable of reflecting data that are managed in analog form, such as in paper medical records, or that are not linked to the customer management system. As a system, it is expected that all medical records will be comprehensively digitized so that the results of further data analysis can be utilized in clinical practice.

It is now common for medical data to be put to clinical use in human medicine, and in companion animal medicine this process is also underway in the UK and Australia. Efforts by individual veterinarians are insufficient to make valuable data widely available for clinical use. Backup from the hardware perspective will also be essential, for example, by enabling the generation of electronic medical records from voice data.

Because the institution where this study was performed is a secondary veterinary medical institution, the study subjects had been referred by their local veterinarians. Not only were all the study subjects were proved difficult to diagnose or treat by the local veterinarians, but the institution was located in the metropolitan district of Tokyo. It is thus unlikely that the study population of this study perfectly reflected the status of pet ownership in Japan as a whole. Nevertheless, the data from secondary veterinary care facilities in the Tokyo metropolitan area, where many small dogs are located, were analyzed over a period of 8 years and 6 months. As a result, it is significant that some parameters related to high and low CRE values, one of the important indexes for health management, were clarified.

In the present study, the factors associated with high CRE levels were advanced age, spayed female, and a number of different breeds. Although the development

of frameworks of cooperation between universities and animal hospitals is a major issue in terms of both hardware and organizational issues, the present study is highly significant, we successfully identified findings from electronic medical records data that will be useful in clinical practice.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author (R Tanaka) on reasonable request.

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Competing Interests

The authors declared that there is no conflict of interest.

Authors' Contributions

AU reviewed all the data and wrote a full chapter of the paper. LH is a native speaker of British English and edited the full text. RT participated as a representative of the Animal Medical Center of TUAT, assessing validity from overall clinical laboratory values and overseeing from the clinician's perspective. NT was responsible for planning, determining work algorithms, selecting categories, deciding on data handling, and overseeing data handling and analysis from a data analysis perspective. TT assessed the feasibility of handling test results and was responsible for analysis and statistical method selection decisions and statistics. TI was responsible for the Animal Medical Center of TUAT at the start of the study.

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CASE REPORT

Biceps Tendon Rupture in Two Beetal Goats

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Abstract

Biceps tendon rupture is a rare condition that has been previously reported in athletic animals. In the present case study, biceps tendon rupture was diagnosed in two Beetal goats. Tendon rupture was indicated by performing a biceps test while diagnostic confirmation was achieved by arthrography and ultrasonography. Bicep tendon appeared hyperechoic but it was noted with disruption and swollen (slightly hypoechoic). Moreover, it was moderately in-homogenous fibrillar structure. While some parts of the tendon has normal echo structure. Tenotomy was performed to mitigate the aforementioned rupture. The follow-up of cases were done for six weeks after the surgery, whereby both goats demonstrated complete recovery. Despite the infrequency of biceps tendon rupture in pasture animals, analysis of diagnostic procedures and surgical stabilization was undertaken for its mitigation is imperative for the clinical understanding of the affection.

Keywords: *Arthrography, Biceps tendon rupture, Tendon fixation, Caprine, Ultrasonography*

İki Beetal Keçisinde Biseps Tendon Rupturu

Öz

Biseps tendon rupturu, daha önce atletik hayvanlarda bildirilen nadir bir durumdur. Bu vaka çalışmasında iki Beetal keçisinde biseps tendon rupturu teşhis edildi. Tendon rupturu biseps testi ile belirlendi ve artrografi ve ultrasonografi ile tanı doğrulandı. Biseps tendonu hiperekoik görünümdeydi ve kopma ve şişkinlik (hafif hipoekoik) dikkat çekiyordu. Ayrıca orta derecede homojen olmayan fibriler yapıdaydı. Tendonun bazı kısımları normal eko yapısına sahipti. Tendon rupturunu redükte etmek için tenotomi yapıldı. Ameliyattan sonra altı hafta boyunca olguların takibi yapıldı, bu sayede her iki keçide de tam iyileşme görüldü. Mera hayvanlarında biseps tendon rupturu nadir olmasına rağmen, hastalığın hafifletilmesi için gerçekleştirilen tanı prosedürleri ve cerrahi stabilizasyonun analizi, hastalığın klinik olarak anlaşılması için zorunludur.

Anahtar sözcükler: *Artrografi, Biseps tendon rupturu, Tendon fiksasyonu, Keçi, Ultrasonografi*

INTRODUCTION

Biceps tendon originates from the supraglenoid tubercle and passes distally at the cranio-medial side of the humeral head between the inter-tubercular grooves. Humerus glides proximally and distally along the biceps tendon after flexion and extension of the shoulder joint. Shoulder joint instability as a consequence of biceps tendon rupture can cause grade 4 lameness. Moreover, any trauma to biceps tendon sheath rupture is frequently associated with

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rupture of biceps brachii tendon [1], bicipital tenosynovitis, avulsion fracture, partial or complete tendon tear, trans-humeral ligament rupture [2], and medial displacement of the tendon. Such cases have been well reported in dogs [1], cats [3], and humans [4]. However, literature is scarce about biceps tendon rupture (partial or complete) in goats. The aim of this report is to describe the diagnostic and surgical procedure for biceps tendon rupture encountered in two Beetal goats.

Case History

Case 1

A 3-year-old, female Beetal goat weighing 29 kg was presented to the Veterinary Teaching Hospital, Cholistan University of Veterinary and Animal Sciences, Bahawalpur with grade 4/5 right leg posture shown in *(Fig. 1)*. Anamnesis revealed that the animal had been lame for the last three months following an unknown trauma. The goat was treated with non-steroidal anti-inflammatory drugs to relieve symptoms. Yet, severe lameness persisted. A general clinical examination was conducted. The patient was observed to be active and attentive. The capillary refill time was 1 second. Chest auscultation was normal (no crackles sound) and breathing rate was 25 breaths/min. The heart rate was 80 beats/min and no heart murmurs were noted. Rectal temperature was 38.4°C. Blood samples were collected from jugular vein in vacuum blood collection tubes. Blood was analyzed for hemato-biochemical parameters. Values for these parameters have been given in *Table 1*. Orthopedic examination revealed severe pain in the shoulder joint upon hyperextension, hyper-flexion, and palpation of shoulder joint on the medial side. Hyperflexion test for the biceps tendon was positive whereby the limb was observably straight instead of exhibiting a natural retro-curvatum *(Fig. 2-A,B)*. Neurological examination showed no abnormalities. Radial and ulnar nerves were found with normal reflexes. Before contrast radiography, shoulder joint ultrasonography was performed to view different shoulder joint structures. Ultrasound examination showed partial rupture of the biceps tendon *(Fig. 3-A)*. Medio-lateral radiographs of right and left shoulder joints were taken. Firstly, performed plain radiographic examination but we could not rule out without contrast radiograph. For that iohexol contrast radiographic media was used with dose rate 4 mL per animal. Positive contrast radiography of the right shoulder was done as well. Partial rupture of the biceps tendon was noted due to unequal percolation of contrast medium.

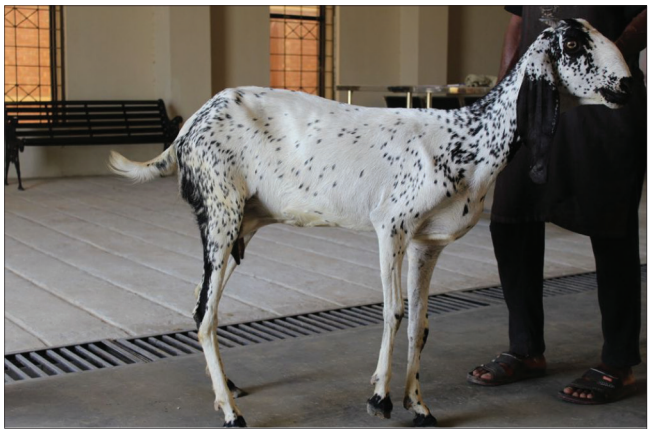


Fig 1. Beetal goat with non-weight bearing grade 4 lameness on the right forelegæ

Case 2

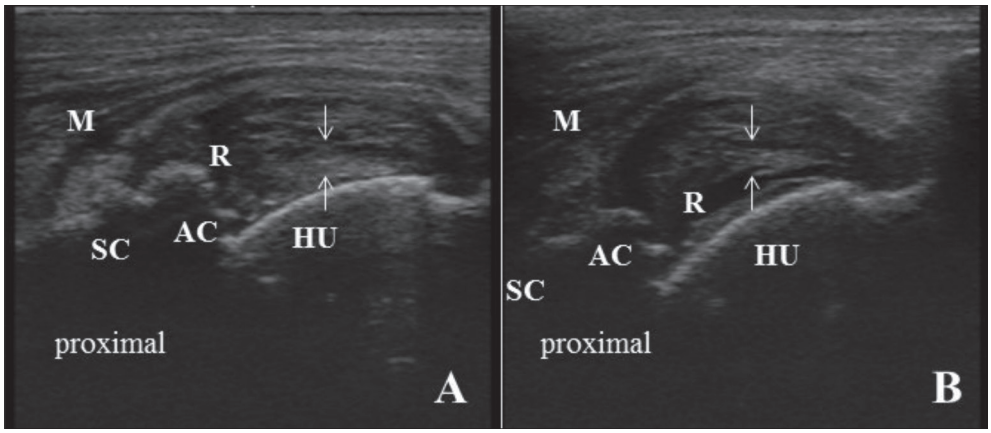
A 34 kg, 4-year-old female Beetal goat with a good body condition was presented to the Veterinary Teaching Hospital, Cholistan University of Veterinary and Animal Sciences,

Table 1. *Values of Hematology and Blood Chemistry analysis of goat cases*

Parameters		Case 1	Case 2	Reference Values
Hematology	Hemoglobin (Hb) (g/dL)	7.91	8.16	8.0-12.0
	Erythrocytes (RBC) (x10^6/µL)	13.2	14.6	8.0-18.0
	Total leucocytic count (TLC) (× 10^3/µL)	11	12.5	4.0-13.0
	Packed cell volume (PCV) (%)	33.71	37.04	22.0-38.0
	Mean corpuscular Hb concentration (MCHC) (pg)	30.01	35.61	30.0-36.0
	Platelets (/µL)	271.5	275.09	300-600
	Mean corpuscular volume (MCV) (fL)	12.9	14.7	16.0-25.0
Blood Chemistry	Aspartate transaminase (AST) (IU/L)	92.6	97.33	66-230
	Alkaline phosphatase (ALP) (IU/L)	173.19	179.3	61-283
	Alanine transaminase (ALT) (IU/L)	40.16	45.21	15-52
	Gamma-glutamyl transferase (GGT) (IU/L)	42.94	46.12	20-50
	Albumin (ALB) (IU/L)	3.06	3.92	2.7-3.9
	Total bilirubin (mg/dL)	0.1	0.1	0.1-0.2
	Cholesterol (mg/dL)	77.86	111.46	65-136
	Urea Nitrogen (mg/dL)	17.83	18.7	13-28
	Creatinine (mg/dL)	0.562	0.163	0.7-1.5

Fig 2. Biceps test was performed on right (A) and left forelegs (B). An imaginary line was drawn from the head of the humerus to radius ulna and from radius ulna to carpal joint. The biceps tendon ruptured foreleg did not show any curve formation at the elbow joint (A) but there was clear curved formation at the elbow joint in healthy biceps tendon foreleg (B)

Fig 3. Longitudinal ultrasound scan of the medial aspect of the shoulder joint (A and B) demonstrating partial rupture of the biceps tendon. Disruption of normal fibrillar pattern is indicated by non-homogenous margins of tendon stump (arrows). The following structures were identified including hyperechoic line of scapula (SC), anechoic articular cartilage (AC), joint recess (R), hyperechoic humerus bone (HU) and supraspinatus muscle (M)



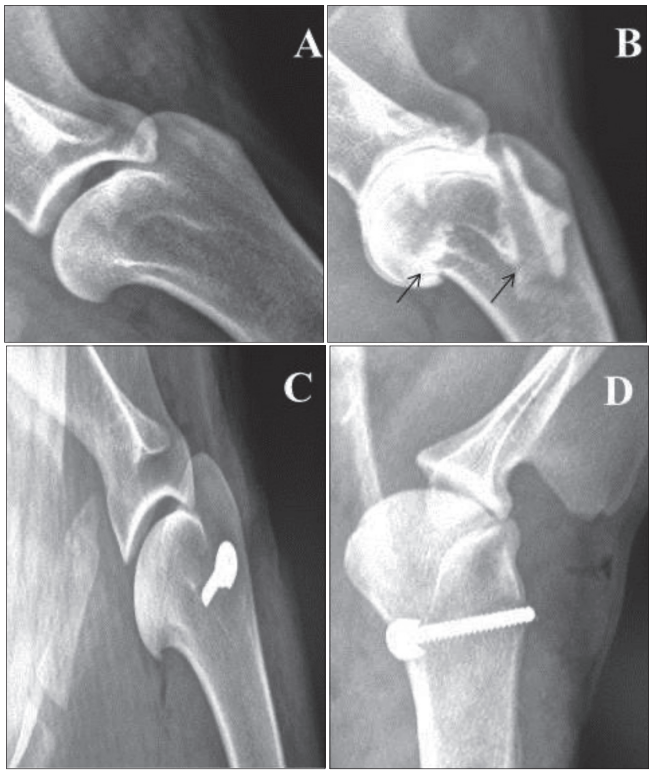


Fig 4. Medio-lateral positive contrast arthrography of the left shoulder joint (A) demonstrating moderate irregularity and insufficient filling of the biceps tendon sheath *(arrows),* Medio-lateral radiograph of the contra-lateral right shoulder joint demonstrating normal joint (B), Post-operative medio-lateral radiograph of the left foreleg showing nail position (C), Cranio-caudal radiographic view demonstrating nail length and position at humeral head after biceps tendon tenotomy (D)

Bahawalpur with severe left foreleg lameness (Grade 4/5). The goat had suffered severe trauma after a collision with another animal. Lameness could not subside, despite one week of analgesic therapy. Clinical exam was conducted whereby the patient was found to be active and attentive. Capillary refill time was less than 1 second. Chest auscultation was normal (no crackles sound) and breathing rate was 27 breaths/min. Heart rate was 76 beats/min and no heart murmurs were noted. Rectal temperature was 38.6°C. Venous blood sample analysis showed no abnormalities in complete blood cell count and blood chemistry *(Table 1)*. Orthopedic examination demonstrated severe pain in the shoulder region when the limb was hyperextended or hyper-flexed. The hyper-flexion test was positive and straight leg was noticed instead of natural curvature in the limb. Neurological examination showed no abnormalities. Radial and ulnar nerves reflexes were normal. Before radiography, ultrasonography of the biceps tendon was performed and showed distorted structure of the tendon *(Fig. 3-B)*. Mediolateral radiographs of the right and left shoulder joints were done. The left shoulder positive contrast radiograph was also done *(Fig. 4-A)*. Rupture of the biceps tendon was noted due to unequal distribution of contrast medium.

Surgical Procedure

For both goats the day after admission, the patients were scheduled for surgical stabilization of the biceps tendon. Food was withdrawn for 24 h but water was allowed until 30 min before anaesthesia. Before anesthetic induction,

amoxycillin 15 mg/kg body weight (Injection Amoxycillin 500 mg by Yanzhou Xier Kangtai Pharma. Co., Ltd. China) was administered intravenously and ketoprofen 1.1 mg/kg body weight (Injection KetoJect 100 mg/mL by Selmore Pharma Pvt. Ltd. Pakistan) was injected intramuscularly. The goat was administered midazolam 0.2 mg/kg IV (Injection Midazom 1 mg/mL by Akhai Pharma. Pakistan) as pre-anesthesia. After ten minutes, anesthesia was induced with ketamine hydrochloride B.P. 1 mg/kg IV (Injection Ketasol 100 mg/2 mL by Indus Pharma. Pakistan) and Propofol 1 mg/kg IV (Injection Propofol 10 mg/mL by Abbott Laboratories Pakistan) [5]. Once sedated, goat was positioned in sternal recumbency and intubated with an 8 mm internal diameter, cuffed endotracheal tube. After that, isoflurane 1-2 L/min (Isoflurane liquid 2% W/V by Akhai Pharma Pakistan) oxygen inhalation anesthesia was maintained. The concentration of isoflurane was maintained to achieve sufficient aesthetic depth after assessing ventro-medial positioning of eyeball and absence of blinking reflexes. Cardio-pulmonary system was regularly monitored. Throughout the surgical procedure, patient received an intravenous solution of 0.9% NaCl at 10-mL/kg/h (Unisol-NS^{R} UNISA Pharmaceutical Industry Limited Pakistan). Electrocardiogram lead II, respiration rate, pulse rate, oxygen concentration, and end-tidal partial pressure of carbon dioxide was monitored throughout the surgical procedure [6]. Normothermia was maintained by placing a heating pad underneath the patient.

After preparation, the patient underwent surgical stabilization of the biceps tendon. A tenotomy was performed to relieve strain on the shoulder joint after fixing the biceps tendon with the humeral head. Incision was made along the cranio-medial aspect of the shoulder joint over the greater tubercle. The incision was extended medially until it reached mid-humeral diaphysis. The fascia was incised at the lateral border of the brachiocephalic muscle. Superficial and deep pectoral muscles from the humerus were incised and retracted medially. While the supraspinatus muscle retraction was done laterally. Coracobrachialis muscle was resected to expose the subscapularis muscle tendon. After that, supra-scapularis muscle was incised as well. Furthermore, the transverse humeral ligament was incised to expose the biceps tendon. The joint capsule was nicked while the tendon was freed and moved medially. A bone screw and spiked washer were used with a boring machine to secure the biceps tendon with the humerus bone. The joint capsule and muscles were sutured with absorbable Poliglecaprone 25 monofilament (Monocryl Suture 2/0 Allumer Medical Pvt. Ltd. Pakistan) suture material. Skin was sutured with nNylon (Ethilon 2/0 Allumer Medical Pvt. Ltd. Pakistan) suture material. The suturing was performed in simple interrupted pattern.

After general anesthesia recovery, postoperative pain was evaluated by the gentle palpation of the surgical site. Though the goat did not show any sign of pain yet, the leg was flexed to assess normal shoulder joint curve formation. There was a normal joint curve formation after surgery. The postoperative radiographs were captured to check nail position at the humeral head *(Fig. 4-C,D)* Anti-inflammatory treatment was administered with ketoprofen 1.1 mg/kg body weight (Injection KetoJect 100 mg/mL by Selmore Pharma Pvt. Ltd. Pakistan) intramuscularly in 24 h for 5 days. Amoxycillin 15 mg/kg body weight (Injection Amoxycillin 500 mg by Yanzhou Xier Kangtai Pharma. Co., Ltd. China) was injected IM 2 times a day for 7 days.

The next day normal diet plan was started; however, the movement of the patient was restricted for one week. The follow-up was carried out in both cases for 6 weeks. After 6 weeks, the goats showed normal walking and running patterns. No postoperative complications were recorded in both cases.

Discussion

Biceps tendon rupture happens less frequently both in humans [4] and dogs [7]. The biceps tendon rupture either partial or complete was not reported before in the goat. Although, biceps brachii tendon and sheath rupture were reported in dogs [1,8]. Both of the cases here were reported in Beetal goats. Previously, prepubic tendon rupture was reported in Beetal goats [9]. Now, bicep tendon rupture is diagnosed in Beetal goats. In fact, Beetal goat is reared for meat purposes. It may be the reason of tendon rupture due to over body weight. Secondly, population of Beetal goats in Punjab is more as compared to other goat breeds.

The biceps test that is explained in German literature [10] was positive in both cases. Biceps test is very helpful to diagnose biceps tendon rupture. When the foreleg is flexed caudally by holding the carpal joint, there is a curve formation in normal joint. While in the ruptured case, there is no curve formation at the level of humerus bone, but it showed straight and upward leg movement. An imaginary line was sketched from shoulder joint to elbow joint and from elbow joint to carpal joint. A curved imaginary line formed in healthy joint while it was straight-line formation in biceps tendon ruptured case.

Previously, canine shoulder joint ultrasonography was considered less sensitive than arthroscopy. Research showed that ultrasonography with a 7.5 MHz linear transducer depicts a very good visualization of the biceps tendon and its related structures [11,12]. Here, ultrasonography of the biceps tendon with a 7.5 MHz linear transducer was performed in both goats. Longitudinal ultrasound images showed disruption of the normal fibrillar pattern. Previously, it was explained that pathological changes either of tendon sheath or tendon could be examined through ultrasonography. Ultrasonography, a best tool and technique, can diagnose tendon diseases including biceps tendon rupture (partial or complete), tendon luxation, tendo-vaginitis, corpora libera in tendon sheath [11,13]. Partial rupture of the biceps tendon was diagnosed in

seven dogs [11]. In both goats, partial rupture of the biceps tendon was noticed at the region of the supraglenoid tubercle where the biceps tendon originates. Bicep tendon appeared hyperechoic but it was noted with disruption and swollen (slightly hypoechoic). Moreover, it was moderately in-homogenous fibrillar structure. While some parts of the tendon have normal echo structure. Those findings were concurrent with the previous study that was performed on dogs [11].

Before and after surgery, radiographic images were captured. The positive contrast arthrography revealed the irregular contrast material margin at the anatomical site of the biceps tendon. After surgery, the position of bone screw was checked through radiographic image for its position and length. Contrary to plain radiography, contrast radiography has been considered the best additional technique to diagnose biceps tendon diseases. The most common finding after contrast radiography is the irregular distribution of contrast radiograph agents [1,14]. Positive contrast agent showed irregular distribution at the side of the biceps tendon in goat's biceps tendon. But it should not be neglected that false-negative results are detected after arthrography [14]. So, it's better to perform firstly biceps tendon test to confirm the diagnosis. Arthrography is less sensitive than ultrasonography to show the extent of tendon pathology [15].

In conclusion, biceps tendon rupture is a rare condition that requires surgical intervention in goats. The best way to diagnose biceps tendon rupture is the biceps tendon test, ultrasonography and arthrography examination. Tenotomy of biceps tendon remains the best choice to address biceps tendon rupture also in goats.

Study Limitation

The absence of arthroscopy findings is a study limitation.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author.

Ethical Statement

This study was approved by the Ethical Review Committee Cholistan University of Veterinary and Animal Sciences, Bahawalpur.

Conflict of Interest

There was no conflict of interest in regard to authors reporting their findings.

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Author Contributions

MS planned, designed, and supervised all procedure. Ultrasonography was done by MS and AA. X-ray was performed by MS and AHR. Hematology and blood chemistry analysis was performed by YRK and ON. Surgery was performed by MS, AA and AHR. Data was arranged by KH and ASA. X-ray and ultrasonographic images were evaluated by MS. For draft, images and tables were prepared by MS, MLS and KP. Original draft was written by MS. All authors have contributed to the revision and final proof-reading of the manuscript.

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