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Research Article

Cryoprotective and Antioxidant Effects of Maca *(Lepidium meyenii)* Supplementation on Post-Thaw Quality of Ram Sperm

Taygun GÖKDEMİR [1(*)], Nail Tekin ÖNDER [1], Muhammet Can KILIÇ [1], Oğuzhan ŞAHİN [1], Lale BAŞER [2], Yavuz ÖZTÜRKLER [1], Savaş YILDIZ [1,3]

[1] Kafkas University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, TR-36100 Kars - TÜRKİYE
[2] Kafkas University, Faculty of Veterinary Medicine, Department of Biochemistry, TR-36100 Kars - TÜRKİYE
[3] Nakhchivan State University, Faculty of Natural Sciences and Agriculture, Department of Veterinary Medicine, Nakhchivan, AZERBAIJAN

[(*)] **Corresponding author:**
Taygun Gökdemir
Phone: +90 474 242 6836
E-mail: tygokdemir@hotmail.com

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Abstract

The aim of this study was to evaluate the effects of Maca *(Lepidium meyenii)* supplementation on the freezing process of ram semen. Semen samples were collected from Tuj breed rams using an electroejaculator method and subsequently pooled and divided into four equal volumes. Samples were diluted with a Tris-based extender either without Maca (control) or supplemented with Maca at concentrations of 10 µL (M1), 20 µL (M2), and 30 µL (M3). After freezing, semen samples were thawed at 37°C for 30 sec for analysis. Post-thaw evaluations included sperm motility and kinematic parameters (Computer-Assisted Semen Analysis; CASA), hypoosmotic swelling test (HOST), acrosome integrity, mitochondrial membrane potential (MMP), lipid peroxidation (measured as malondialdehyde, MDA), and intracellular antioxidant status (glutathione, GSH). Data were analyzed using one-way ANOVA and Tukey's post hoc test. After thawing, supplementation with 30 µL of Maca significantly increased total semen motility from 36.14% to 46.21% and progressive motility from 33.56% to 43.91% ($P<0.05$). Maca, especially at this concentration, also significantly enhanced post-thaw motility, membrane integrity, and antioxidant status compared to the control group ($P<0.05$). In conclusion, it was determined that Maca added to the semen extender had a positive effect on ram semen.

Keywords: Antioxidant, Maca, Lepidium meyenii, Spermatozoa, Cryopreservation, Ram

Introduction

Sperm freezing and technological methods provide various benefits to animal husbandry, contributing to the preservation of livestock, the conservation of genetic material, and the continuation of its use. However, during cryopreservation, sperm is exposed to oxidative, osmotic, chemical, and physical stress, primarily in the form of ice crystal formation. Consequently, these changes negatively affect sperm quality and fertility [1]. The reason for the negative effect is the increased production of reactive oxygen species (ROS) [2].

High ROS levels cause DNA damage by leading to lipid peroxidation and negatively affect sperm motility and membrane integrity [3]. The role of cryoprotectants is crucial in reducing the stress and problems that occur during cryopreservation. Cryoprotectants aim to reduce increased ROS levels and increase sperm viability and motility [4,5]. Therefore, the selection and amount of cryoprotectant during cryopreservation is crucial for freezing success.

Plants produce various chemical compounds, including alkaloids, phenolic compounds, and terpenoids. Resveratrol, a good example of these compounds, is a polyphenolic compound used as an antioxidant in humans and domestic animals. Furthermore, these natural metabolites inhibit ROS and also have antimicrobial effects [6]. Therefore, plant extracts are a good alternative to traditional antioxidants in sperm freezing. Maca *(Lepidium meyenii)* is a plant from the Brassicaceae family that grows in the Andes. Its root contains high amounts of sucrose [7] and phytochemicals [8,9]. It also has benefits such as bioactive products, anti-stress antioxidant effects, cytoprotection against natural and oxidative stress, spermatogenesis [10,11], libido in animals and cytoprotection against [12,13] which are closely associated with the upregulation and activity of superoxide dismutase (SOD), a key enzyme in cellular antioxidant defense mechanisms.

These effects are attributed to superoxide dismutase enhancing enzymatic degradation and directly interacting with free radicals [14,15]. Furthermore, Ohta et al.[16] state that Maca may increase the bioavailability of testosterone or testosterone receptors and improve the response of Sertoli cells to follicle-stimulating hormone (FSH), which may have a positive effect on sperm capacity.

Considering the reported bioactive properties of Maca *(Lepidium meyenii)* and its potential influence on oxidative balance and cellular integrity, this study was designed to evaluate the dose-dependent effects of Maca supplementation on ram sperm cryopreservation. The objective is to assess its impact on post-thaw sperm quality parameters using established analytical approaches and to contribute comparable data to the existing body of research.

Material and Methods

Ethical Approval

This study was conducted following the approval of the Animal Research Ethics Committee of Kafkas University (KAÜ-HADYEK/2024-196), Kars, Türkiye.

Animals

Five fertil rams aged between 2 and 4 years old, were used at the Prof. Dr. Ali Rıza AKSOY Education, Research and Application Farm of the Veterinary Faculty of Kafkas University, kept under the same housing, care and feeding conditions.

Preparation of Semen Extender and Aqueous Extract of Maca

Tris-citric acid-fructose-egg yolk-glycerol basic diluent was used in the study [17]. Composition of main diluent are detailed in *Table 1* [17]. To obtain a maca solution, 5 g of maca powder (commercially available, business registration number:TR-34-K-000495) was mixed with 100 mL of distilled water and stirred automatically in water bath at 70°C for 3 h. The mixture was centrifuged at 4000 rpm for 10 min and repeated into a water bath at 70°C for 2 h again. The solution was filtrated and stored in a refrigerator at 5°C to use in semen extenders. The preparation and characterization of the maca powder (including macamides and glucosinolates) and its aqueous extract were based on the methodology described by Del Prete et al. [18], *(Table 2)* where the extract composition and active compounds were previously defined. Accordingly, the extract used in this study can be considered standardized in terms of its bioactive content. The selected doses were determined based on this reference and were chosen considering their previously reported biological relevance.

Table 1. *Composition of the main semen diluent*

Component	Concentration	Function
THAM (Tris; trishydroxymethyl-aminomethane)	27.1 g/L	Buffer; pH stabilization
Citric acid	14.0 g/L	Buffer; ionic balance
D-fructose	10.0 g/L	Energy source
Penicillin G	0.3 g/L	Antibiotic (Gram-positive)
Dihydrostreptomycin	0.4 g/L	Antibiotic (Gram-negative)
Egg yolk	20 % (v/v)	Membrane stabilization; cryoprotection
Glycerol	6 % (v/v)	Cryoprotectant; prevents ice crystal formation

Semen Collection

Semen was collected using the electro-ejaculator method twice a week respectively and 10 times in total. The collected sperm was placed in a 32°C water bath and brought to the laboratory, where mass activity (≥3 out of 0-5, 5X magnification), motility (70%) (using a phasecontrast (brightfield (A), Ph 2, 40X magnification) microscope (Nikon Eclipse-E400, Tokyo, Japan equipped with a heated slide set at a temperature of 37°C) and sperm concentration $1.5x10^9$ (counting was performed under a microscope at 40X magnification using a Thoma slide) assessments were selected for cryopreservation.

Sperm Dilution and Freezing

The pooled semen samples were divided into four equal groups: a control group (C) and three treatment groups supplemented with maca at concentrations of 10 µL (M1), 20 µL (M2), and 30 µL (M3), and subsequently diluted. The diluted semen samples were drawn into 0.25 mL straws and equilibrated (horizontally) at 4°C for 120 min. Following equilibration, the straws were frozen in liquid

Table 2. *Analysis of the powder and maca aqueous extract*

Items	Analysis of maca types	
	Maca Powder (µg/L)	Aqueous Extract of Maca (µg/L)
5-oxo-6E,8E octadecadienoic acid (Macaen)	69.53	17.89
N-(3-hydroxy-benzyl)-2Zfivecarbon acrylamide	614.29	157.99
N-benzyl-5-oxo-6E,8Eoctadecadienamide (MI 7)	46.08	61.81
N-benzyloctadecanamide (MI 16)	53.96	28.89
Macalines or Lepilidines	59.03	13.31
Methyltetrahydro hydridecarboline carboxylic acid	47.17	3.63
1-dibenzyl-2-propane-4,5-dimethylimidazilium		1.25

nitrogen vapour (The temperature range of −100°C to −140°C corresponds to an average value of approximately −120°C) and then transferred to liquid nitrogen for storage until evaluation.

Semen Thawing and Evaluation

The frozen semen samples were thawed at 37°C for 30 s, after which sperm motility and kinematic parameters, the hypo-osmotic swelling test (HOST), acrosome integrity (A), mitochondrial membrane potential (MMP), malondialdehyde (MDA) levels, and glutathione (GSH) activities were analyzed. Sperm motility and kinematic motion parameters were assessed using portable CASA (Androscope, Minitube, D). For the HOST was determined under a phase-contrast microscope (Nicon Eclipse E400, JP). HOS test was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails, and was performed by incubating 40 µL of semen with 960 µL of a 100 mOsm/L hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37°C for 60 min [19].

Flow Cytometric Analysis

The analysis was performed using Attune NxT Acoustic Focusing Cytometer (Invitrogen, USA). The fluorescein isothiocyanate-conjugated peanutagglutinin (PNA)/ propidium iodide (PI) dual-staining technique was used to evaluate A. The fluorescence was quantified using a 480 nm excitation wavelength with a 10 nm excitation bandwidth. The emitted light was filtered using a 530/30 nm filter (BL-1) and a 695/40 nm filter (BL-3). The measurements were recorded using Attune NxTsoftware v2.7 (Thermo Fisher, USA). Following the utilization of forward and side scatter light signals to isolate the cell population, the mean fluorescence intensity of the analyzed spermcells was quantified. The experiment contained a total of 10.000 sperm cells, with a flow rate of 12.5 µL/min. Rhodamine123/PI was used to evaluate MMP. All flow cytometric analys is performed as previously described by Önder et al. [20].

Biochemical Analysis

Thawed semen samples were subjected to spermatological analyses after centrifugation them at 800 g for 10 min, resulting in the separation of the supernatant. Semen samples were used to measure reduced GSH [21] and MDA [22] levels in the biological samples obtained by using spectrophotometer (Epoch, Biotek, USA). As an MDA standard, 1, 1, 3, 3- Tetramethoxypropane was used, and the results were reported as nmol/mL protein. GSH analysis, the samples underwent precipitation using a 10% solution of trichloracetic acid, followed by centrifugation at a speed of 1000 g for 5 min. The reaction mixture consisted of 0.5 mL of semen supernatant, 2 mL of tris hydroxymethyl aminomethane buffer (0.4 M; pH 8.9), and 0.1 mL of l 5,5'-dithio-bis-2-nitrobenzoic acid. The solution was maintained at room temperature for a duration of 5 minutes, and subsequently measured at a wavelength of 412 nm using the spectrophotometer.

Statistical Analysis

The statistical analysis was conducted using IBM SPSS version 20 (SPSS 20.0 Windows - SPSS, Chicago, IL, USA). The Shapiro Wilk test was used to assess for normality. The data were shown as mean ± standard error. The statistical significance of the differences between subdivided groups was determined using one-way ANOVA followed by Tukey. Statistical significance was determined for P values below 0.05.

Results

Total and progressive motility [%] are detailed in *Table 3*, kinematic parameters [%] are detailed in *Table 4*, A, MMP, and HOST [%] results are detailed in *Fig. 1*, and MDA [mM/L] and GSH [mg/dL] results are detailed in *Fig. 2*.

The final total motility in the control, M1, M2, and M3 groups was 36.14, 36.26, 41.17, and 46.21, respectively, while progressive motility was found to be 33.56, 35.86, 38.95, and 43.91 in the control, M1, M2, and M3 groups, respectively. Groups containing Maca, particularly the M3 group, were significantly different from the control group ($P<0.05$). The M3 group was also significantly different from the LIN and STR parameters in terms of kinematic movements ($P<0.05$). The M3 group also achieved the best results in terms of A (74.24%) and MMP (72.06%) ($P<0.05$). Overall, the Maca groups yielded better results compared to the control group, with the M3 group showing significant differences from all other groups in all parameters ($P<0.05$). Total and progreessive motility (%) are detail in *Table 3*, kinematic parameters (%) are presented in detail in *Table 4*, whereas the results for A, MMP, and HOST (%) are shown in *Fig. 1*. The MDA levels and GSH activities (%) are summarized in *Fig. 2*. The highest MDA level was detected in the control group at 10.76±1.46, while the lowest level was detected in the M3 group at 2.66±0.33 ($P<0.05$). GSH levels were highest in the M3 group at 2.60±0.05, while the lowest level was

Table 3. *Total and Progressive Motility different thawing groups*

Groups	Total Motility (%)	Progressive Motility (%)
C (control)	36.14±0.03[a]	33.56±0.34[a]
M1 (10 µL maca)	39.26±0.02[b]	35.86±0.35[b]
M2 (20 µL maca)	41.17±0.003[c]	38.95±0.10[c]
M3 (30 µL maca)	46.21±0.008[d]	43.91±0.36[d]

a, b, c, d: Groups with different letters in the sperm trait are statistically different (P<0.05)

Table 4. *Kinematic parameters in different thawing groups*

Groups	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	DCL (µm)	DSL (µm)	DAP (µm)	ALH (µm)	BCF (Hz)	LIN (VSL/VCL)	STR (VSL/VAP)
C (control)	70.78±0.19^{a}	52.60±0.30^{a}	54.19±0.43^{a}	26.22±0.005^{a}	20.77±0.04^{a}	21.37±0.008^{a}	1.08±0.01^{a}	7.10±0.04^{a}	0.78±0.005^{ab}	0.92±0.02^{a}
M1 (10 µL maca)	72.56±0.56^{ab}	53.69±0.23^{ab}	55,65±0.23^{b}	26,63±0.24^{a}	21.38±0.31^{ab}	21,53±0.04^{a}	1.12±0.03^{ab}	7.72±0.24^{a}	0.77±0.03^{a}	0.95±0.005^{a}
M2 (20 µL maca)	74.29±0.35^{b}	55.17±0.08^{b}	57.01±0.40^{bc}	27,75±0.21^{b}	22.04±0.20^{b}	22,02±0.03^{b}	1.18±0.01^{b}	8.60±0.17^{b}	0.86±0.01^{ab}	0.95±0.02^{a}
M3 (30 µL maca)	77.28±0.64^{c}	57.11±0.55^{c}	58.11±0.06^{c}	28,96±0.33^{c}	23.02±0.03^{c}	22,99±0.06^{c}	1.17±0.01^{ab}	8.95±0.09^{b}	0.87±0.01^{b}	0.96±0.003^{a}

a, b, c, d: Groups with different letters in the sperm trait are statistically different (P<0.05). VCL: Velocity of Curvilinear, VSL: Velocity of Straight Line, VAP: Velocity of Avarage Path, DCL: Distance Curvilinear Line, DSL: Distance Straight Line, DAP: Distance Average Path, ALH: Amplitude of Lateral Head Displacement, BCF: Beat Cross Frequency, LIN: Linearity, STR: Straightnes

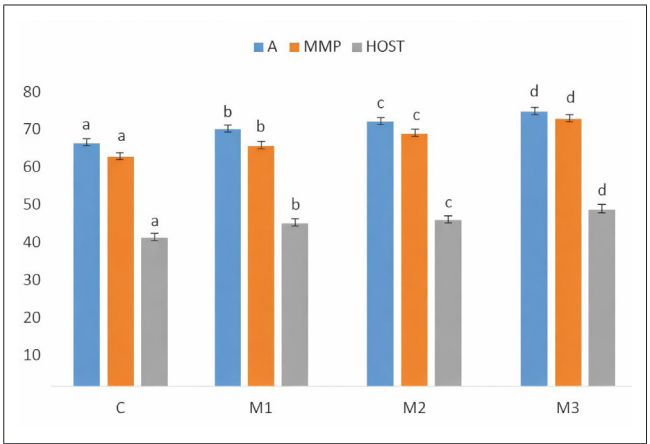

Fig 1. Acrosome Integrity (A) (%), Mitochondrial Membrane Potential (MMP) (%) and HOS test (%) in different thawing groups. a, b, c, d: Groups with different letters in the sperm trait are statistically different (P<0.05)

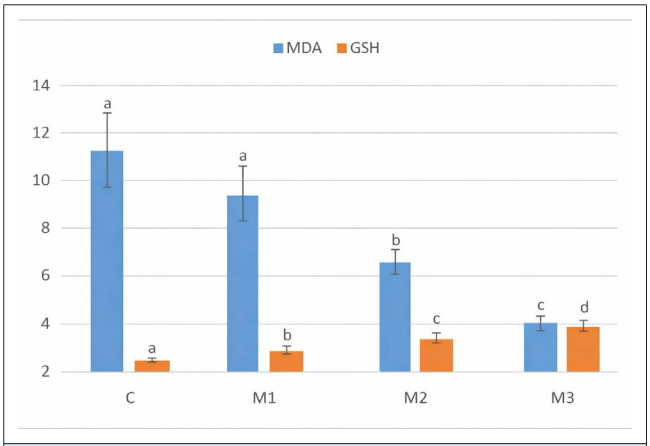

Fig 2. MDA [mM/L] and GSH [mg/dL] parameters in different thawing groups. a, b, c, d: Groups with different letters in the sperm trait are statistically different (P<0.05)

found in the control group at 0.36±0.05 (P<0.05). The highest values of A, MMP and HOST were determined in the M3 group, with values of 74.24±0.38, 72.06±0.52 and 47±0.42, respectively (P<0.05).

Discussion

Effective freezing of semen requires understanding the changes that occur during the process, and various studies have been conducted on this subject [23,24]. The primary objective in this process is to preserve and enhance spermatological characteristics during freezing and thawing [25]. In the present study, the aim of this study was to demonstrate whether the addition of Maca to the diluent increases the resistance of semen cells to cryodamage.

Semen motility is the most widely used indicator of semen quality both before and after cryopreservation [26]. It reflects the functional and structural competence of sperm cells [27]. Maca has been reported to improve sperm motility in human semen [28], bovine semen [29], chilled canine semen [18], as well as in experimental studies conducted in mice [30] and stallions [31]. In the present study, Maca supplementation improved sperm motility and kinematic parameters in a dose-dependent manner. Furthermore, one study demonstrated that secondary metabolites of maca, including macamides, macaenes, maca alkaloids, and glucosinolates, enhance superoxide dismutase and GSH levels and exhibit iron reducting antioxidant power, hydroxyl radical scavenging activity, and 2,2-diphenyl-1picrylhydrazyl (DPPH) free radical scavenging capacity [32]. These antioxidant properties may explain the beneficial effects of maca on male reproductive function. Denk et al. [33] examined the effects of quinic acid, a phenolic compound, on ram semen and particularly at 100 and 200 µg, significantly enhanced sperm motility and kinematic parameters, including Prog M, VCL, VAP, and VSL compared to the control. This study consistent with our study, highlighted the compound's positive effects on spermatological parameters. Despite the promising findings of the present study, several methodological limitations should be considered when interpreting the results. Firstly, sperm motility and kinematic parameters evaluated by CASA systems, although widely accepted, may be influenced by system settings, operator variability, and sample handling procedures.

One of the tests performed in our study was the HOS test, which evaluates the integrity of the sperm plasma membrane. This test is considered an important indicator of sperm fertilization capacity [34]. In the current study, HOST rates increased in parallel with the rise in maca dosage. Leiva-Revilla et al. [28] also reported an improvement in HOST in their study on the effect of Maca on frozen-thawed bovine sperm.

Polyunsaturated fatty acids are found in ram semen including acrosome membranes, mitochondrial membranes, and plasma membranes, all of which are extremely vulnerable to oxidative stress [34]. Oxidative stress increases ROS, leading to increased cell damage and functional losses in sperm [36]. Acrosome integrity is one of the determining factors for fertilization success. Spermatozoa with an intact acrosome can pass through the zona pellucida and fuse with the oocyte [37]. In this study, the addition of Maca to the diluent did not cause damage to the acrosome and mitochondrial membrane and showed therapeutic effects. This suggests that Maca may act as a protective effect on the membrane. Avdatek et al. [38] also reported that 1mM baicalein increases plasma membrane and acrosome integrity in ram sperm.

Malondialdehyde (MDA) is used to assess the intensity of oxidative stress due to lipid peroxidation [39], and GSH is used to determine the effectiveness of the antioxidant defense system [40]. The beneficial effects of maca on sperm are attributed to the presence of various secondary metabolites such as macaridin, macamicides, maca alkaloids, and glucosinolates [32]. In our study, the addition of maca to the semen diluent resulted in a significant difference in MDA and GSH levels, especially in 30 µL. In addition, oxidative stress markers such as MDA and GSH reflect lipid peroxidation and antioxidant capacity; however, they represent only a part of the complex redox balance within sperm cells.

Another limitation is that the study was conducted under in vitro conditions, and fertility trials were not performed. Therefore, the positive effects of maca supplementation on spermatological parameters should be interpreted cautiously, as improvements in laboratory parameters do not always translate into increased fertility outcomes.

In conclusion, Maca supplementation during ram semen cryopreservation showed measurable effects on post-thaw sperm quality, indicating that this plant-derived additive can influence the preservation performance of sperm cells under freezing–thawing conditions. The findings obtained in the present study demonstrate that the response was dose-dependent and that Maca contributed to the maintenance of key spermatological parameters associated with cryopreservation success. Accordingly, the study provides experimentally grounded evidence that Maca may serve as a biologically relevant supplement in ram semen extenders and offers data that can support the evaluation of antioxidant-based strategies in reproductive biotechnology.

Declarations

Availability of Data and Materials: Data and Materials are available from the corresponding author (T.G.).

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Author Contributions: TG planned the study. TG, MCK and OS performed the semen collection and freezing procedures. NTO performed the semen examination and flow cytometric analyses. LB performed the biochemical analyses. SY and YO ensured the study design, writing and overall control. All authors approved the final version of the manuscript and accept responsibility for the scientific integrity of the work in accordance with the journal's Ethical Principles and Authorship Policy.

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