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Research Article

Re-freezing of Cryopreserved Equine Semen: Effect of Glycerol Concentration, Cholesterol, and α-Tocopherol on Sperm Quality

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Abstract

The present study evaluated the effect of glycerol concentration, treatment with cholesterol andα-tocopherol loaded into methyl-β-cyclodextrin, and sperm cell concentration on the quality of equine spermatozoa after two cycles of cryopreservation. Two experiments were conducted. The first trial utilized 18 ejaculates collected from six stallions. Each ejaculate was evaluated initially using computer assisted sperm analysis (CASA) and flow cytometry (FCM), and spermatozoa were subsequently frozen in a commercial diluent (containing 2.5% glycerol: control) or in one of four experimental diluents (each supplemented with 1%, 2%, 3% or 4% glycerol). Frozen samples were thawed and evaluated again prior to a second freeze-thaw cycle. The glycerol concentration that produced the best result was further investigated in experiment 2 where spermatozoa previously frozen in 2.5% glycerol were thawed and refrozen in one of three sperm cell concentrations (1, 5, 05 10 x106 spermatozoa/mL) treated or not treated with cholesterol and α-tocopherol incorporated into methyl-β-cyclodextrin. Semen analysis (CASA and FCM) was repeated after the second freezing and thawing. Results revealed that the 2.5% concentration of glycerol was optimal for refreezing equine spermatozoa. Centrifugation (900xg for 10 minutes) of the equine semen before refreezing resulted in a sperm loss rate of 12% but was associated with good viability and acrosomal integrity. Treatment of semen with cholesterol and α-tocopherol loaded into methyl-β-cyclodextrin before refreezing improved ($P<0.05$) the post-thaw quality of spermatozoa (motility, membrane and acrosome integrity, and oxidative status) refrozen at 1×10^6 spermatozoa/mL, thus allowing production of 100 to 300 ICSI straws from a standard 0.5 mL equine artificial insemination straw.

Keywords: Cryopreservation, Sperm, Glycerol, Methyl-B-cyclodextrin, Stallion, Vitamin E

Introduction

In equines, conventional *in vitro* fertilization (IVF) has been less successful than in many other species. This lower success rate constrains embryo production and has led researchers and practitioners to utilize an alternative fertilization technique called intra-cytoplasmic sperm injection (ICSI) [1]. To use ICSI, mares must undergo egg retrieval (also known as ovum pick-up [OPU]) to harvest immature oocytes that will be matured *in vitro* prior to fertilization via ICSI. This *in vitro* embryo production procedure has proven very effective and has become widely used in mares. However, with the increased use of ICSI, another issue has arisen - namely the cryopreservation of a small number of male gametes needed for ICSI [2].

Unlike artificial insemination (AI) where the inseminate dose is approximately 400 million spermatozoa/mL [3], ICSI requires only a single spermatozoon recruited from a small quantity of cells. Thawing an entire AI straw for the minimal needs of ICSI would be a waste of semen, particularly for the limited supplies of semen available globally from stallions of very high genetic and sporting value [4]. Thus, the ability to thaw stallion spermatozoa cryopreserved in straws at a concentration between 100-300 million spermatozoa/mL and subsequently refreeze them in so-called ICSI straws at a much lower concentration (1-10 million spermatozoa/mL) would facilitate greater gains in embryo production [5,6].

In horses, refreezing of stallion semen was first reported in 2006, although those procedures resulted in immobile sperm. After ICSI with immotile sperm, embryonic

development to the blastocyst stage was achieved, but with a low conception rate compared with that obtained using motile spermatozoa resulting from a single freeze-thaw cycle [7]. Several studies were performed after that initial report, but to date no one has succeeded in obtaining satisfactory quality (motility and integrity) of spermatozoa after refreezing. In fact, the re-freezing process exposes spermatozoa to damage - causing a deterioration of their motility, integrity and metabolism. This damage leads to a considerable reduction in fertilizing capacity, particularly for the equine spermatozoon which is sensitive to cold temperatures due to a low cholesterol:phospholipid membrane ratio compared with other species [5,8]. Lesions following freezing are due to osmotic stress acting in conjunction with oxidative stress through free radical elimination [9]. Therefore, treatment of the spermatozoa with cholesterol and α-tocopherol before re-freezing could increase the cryo-resistance of the equine sperm cell by stabilizing the plasma membrane and preventing the accumulation of free radicals (oxidative stress).

Cryoprotective agents (e.g., glycerol) protect spermatozoa during cryopreservation by minimizing their exposure to osmotic stress, stabilizing the plasma membrane, and reducing the deleterious effect of free radicals [10,11]. However, glycerol also has a dose-dependent toxic effect on spermatozoa, particularly during prolonged and/or repeated exposure during refreezing. For this reason, glycerol concentration in the freezing diluent should be optimized [12,13].

The present study evaluated the effect of glycerol concentration, final sperm cell concentration, and treatment with cholesterol and α-tocopherol loaded into methyl-β-cyclodextrin before re-freezing on the quality (motility, viability-integrity, oxidative stress) of equine sperm through two cycles of cryopreservation.

Material and Methods

Ethical Approval

All animal studies were conducted with the utmost regard for animal welfare, and all animal rights issues were appropriately observed. No animal suffered during the course of the work. All experiments were conducted according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

Study Area

The experiments were performed in the laboratories at BIOMERA Platform, Blida University 1 (Blida, Algeria).

Animals

Six clinically healthy purebred Arabian stallions of proven fertility were used for semen collection (fertility was assessed through results of AI with fresh semen). Stallions were chosen from one of two different farms (Haras Hocine El Mansour Mostaganem and the Sahel Hadjout Equestrian Farm), and they ranged from four to nine years of age. Although semen analysis is performed routinely on all stallions housed on those two farms, the six stallions were chosen at random from among those with satisfactory semen characteristics in order to avoid any potential bias/sampling effect.

Semen Collection

During the week preceding their use for experiments, stallions underwent semen collection three times to empty their extra-gonadal reserve of spermatozoa with the aim of ensuring a constant quality of semen during the experiments. During the experiments, semen was collected at least twice per week from each stallion. Semen collections from the same stallion were not carried out on two consecutive days to maintain an adequate sperm cell concentration. Stallions were, therefore, collected alternately in groups of two. Semen collection was done using a closed artificial vagina (Colorado type) at the beginning of the afternoon on a phantom in the presence of a mare in heat, or directly on the mare for stallions not trained to a phantom.

Once collected, semen was immediately filtered into a graduated container using filter paper or sterile double gauze to remove impurities and the "gel" which contains seminal fluid that is highly toxic for spermatozoa during cryopreservation. The artificial vagina and all materials that came in contact with semen were preheated at 37°C to avoid any possible thermal shock. The volume of the filtered ejaculate was measured, and the ejaculate was then aliquoted into four 50-mL tubes pre-warmed to 37°C.

Initial Assessment of Semen

Initial quality of the ejaculate was assessed using three criteria: 1) macroscopic evaluation of the physicochemical parameters of the ejaculate (volume**,** color, appearance, smell); 2) assessment of the sperm cell concentration using an "Improved Neubauer" chamber after a 1:100 dilution (10 µL of semen and 990 µL of 4% NaCl solution); 3) assessment of motility by placing a drop (10 µL) between a slide and a coverslip and visually examining it under a microscope by looking at several fields and making an average estimation (expressed as a percentage or using a corresponding grid from 0 to 5) of the spermatozoa with slow and fast rectilinear movement (Haras Nationaux, 2009).

Preparation of Solutions Loaded into Methyl-β-Cyclodextrin

Cholesterol and α-tocopherolwere incorporated into methyl-β-cyclodextrin (CLC: cholesterol loaded methyl-

β-cyclodextrin; TLC:α-tocopherol loaded methyl-β-cyclodextrin, respectively) according to previously reported methods [15,16]. Two solutions were prepared for treatments to be applied to spermatozoa prior to re-freezing at the following concentrations:10.86 mg/mL (CLC) and 0.92 mg/mL (TLC). A third solution (CLC and TLC combined) was prepared by mixing the CLC and TLC solutions in equal proportion (v/v). All treatment solutions were filtered using a syringe filter and subsequently stored at +4°C until use. The CLC treatment dose was 5 mg/100x10^6 spermatozoa, and the TLC treatment dose was 0.1 mM/100x10^6 spermatozoa. The dose of the combination treatment (CLC+TLC) consisted of a half dose of each of the two treatments.

Semen Dilution and Temperature Management

The diluents used for these experiments was INRA96® (IMV, L'Aigle, France). To avoid any potential unintentional atmospheric alteration of the diluent after opening the 200 mL vial, the INRA96® was aliquoted into 5, 10, 20, and 50 mL quantities and frozen at -18°C (for ≥24 h). On each day of semen collection, the needed volume of INRA96® was thawed at room temperature and then warmed to 37°C in a water bath. The warmed INRA96® was slowly added to the semen for a 1:3 dilution (i.e., 3 volumes of INRA96® per 1 volume of semen).

Tubes containing the diluted sperm were placed in a water bath at 22°C for 10 min to initiate the cooling process. For further slow cooling, tubes with diluted semen were placed in plastic containers filled with 22°C water for subsequent placement into a 4°C refrigerator for 30 min. This procedure enabled cooling of the semen to occur slowly, avoiding temperature shock to the spermatozoa. After 30 min, the semen doses were packaged in insulated Equitainers and transported at 4°C to the BIOMERA platform in Blida. Average transport time was 3 h.

Upon arrival at BIOMERA, semen was layered on top of 2 mL of centrifugation cushion designed for equine spermatozoa (MaxiFreeze, IMV, L'Aigle, France) in a 10mL tube. Sperm cells were concentrated by centrifugation (SIGMA 3-30KS centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 900 x g for 10 min. This centrifugation protocol was expected to result in loss of approximately 15% of spermatozoa [17].

After removing the supernatant (first diluents D1 + seminal plasma), the sperm pellet was reconstituted using the freezing diluent (2nd diluent D2). We calculate the theoretical volume of D2 based on the sperm concentration measured before centrifugation (C1) to obtain a final concentration of 100x10^6/mL (C2) according to the relation (C1*V1=C2*V2). Half of the theoretical volume of D2 (½ Vol of D2) was added per tube, and the spermatozoa were resuspended via gentle pipetting by hand, then the final sperm concentration of the reconstituted sample was determined as described previously. The volume of D2 remaining to be added will be calculated as described previously based on the concentration measured after centrifugation in order to correct the volume for losses due to centrifugation.

Straw Filling, Freezing, and Thawing

After 120 min of equilibration time at 4°C, straws individually labeled using a printer (Domino A420i, Domino Printing, Cambridge, UK) were filled and sealed with a filling machine (MRS I DualV2, IMV, L'Aigle, France). The straws were spread on a freezing rack, and the rack was placed in an automatic freezer (MicroDigitcool, IMV, L'Aigle, France; Curve: Start at 4°C; - 60°C/min up to -100°C; -30°C/min to -140°C). At the end of the freezing cycle, straws were immersed directly in liquid nitrogen (LN2). Any straw that floated in LN2 due to insufficient filling was eliminated.

After 72 h of storage, straws were placed into a water bath at 37°C for 30 seconds for thawing.

Semen Evaluation

Motility Analysis by Computer-Assisted Sperm Analysis (CASA): Pre- and post-thaw sperm motility was determined via computer-assisted sperm analysis (CASA). To prepare spermatozoa for analysis, semen was diluted to a concentration of 25 million spermatozoa/mL using a commercial buffer solution (EasyBuffer B, IMV, L'Aigle, France) and subsequently incubated in water bath (MEMMERT incubator, Memmert, Büchenbach, Germany) at 37°C for 10 min. Spermatozoa were loaded into a 20 µm deep counting chamber (Leja®, LEJA Product BV, Netherlands) and analyzed with a Hamilton Thorne motility analyzer system (IVOS® II, Beverly, Massachusetts, USA) in accordance with a previously described technique [18]. The IVOS®II technical configurations shown in *Table 1*. The two parameters evaluated were percentages of motility (MOT) and progressive motility (PROG).

Viability, Acrosomal Integrity, and Sperm Oxidative Status Assessment by Flow Cytometry (FCM): Sperm viability, acrosomal integrity, and oxidative status were assessed via flow cytometry (Millipore GuavaEasyCyte HT Plus, Merck KgaA, Darmstadt, Germany) using ready-to-use rapid kits in the form of microplates containing fluorochromes in their wells. After several steps of sperm dilution and preparation, sperm were added to the fluorochrome-containing wells, incubated, and loaded directly into the flow cytometer for analysis. A sample of fluorescently labeled cells is aspirated into a uniquely proportioned microcapillary flow cell. A diode laser excites the cells and each cell emits signals

Table 1. *Parameters for computer-assisted sperm analysis of equine spermatozoa using a Hamilton Thorne IVOS II CASA analyzer*

Parameter	Value
Frame acquired	30
Frame rate (Hz)	60
Minimum contrast	60
Minimum cell size (pixels)	6
Minimum static contrast	25
Straightness cut-off (STR, %)	75
Average path velocity cut-off PM (VAP, µm/s)	50
VAP cut-off static cells (VAP, µm/s)	20
Cell intensity	100
Static head size	0.55-2.04
Static head intensity	0.45-1.70
Static elongation	11-99

that are individually detected by photomultipliers and a photodiode. The excitation laser provides up to five simultaneous detection parameters, including three fluorescent colors plus forward and side scatter for size and complexity determination. Software modules show all relevant data and results immediately.

The percentage of living spermatozoa with an intact acrosome was determined with the kit "Fluorochromes: FITC/PNA/PI" (VIAB-ACRO). A new combination of three stains was developed by IMV. Two of the stains monitor the integrity of the acrosome and of the membrane, simultaneously. The third fluorochrome is sperm specific and thus allows to remove debris from the analysis.

The oxidation status was determined by measuring the level of three intracellular Reactive Oxygen Species (hydrogen peroxide "H_2O_2", hypochlorous acid "HOCl", and peroxynitrite "$ONOO^-$") of the sperm through the use of flow cytometry, by combining cell and viability markers, as described previously in the principle of this technique. Physical levels of ROS are required for normal sperm functions. However, when in excess, ROS can be harmful. Oxidative stress occurs in spermatozoa when intra and extra cellular levels of ROS exceed the available antioxidant capacity.

Experimental Design

Experiment 1- Evaluation of Glycerol Concentration and Effect of Centrifugation: Ejaculates from stallions were collected and processed as described earlier. After initial assessments (T0), ejaculates were split in half and allocated to the control (INRAFreeze®, IMV, L'Aigle, France; contains 2.5% glycerol [G]) or to one of four experimental diluents (INRA96®+1%G, INRA96®+2%G, INRA96®+3%G, INRA96®+4%G) prepared by supplementing INRA96® with 1%, 2%, 3%,or 4% G, respectively. Spermatozoa were evaluated (CASA & FCM) after centrifugation (T1), equilibrated at 4°C for 120 min, and packaged in 0.5 mL straws before being frozen with an automatic freezer (Curve: Start at 4°C; - 60°C/min up to -100°C; -30°C/min to -140°C) and subsequently stored in LN_2 for three days. Three straws from each group were thawed (+37°C for 30 sec) in five different tubes, evaluated (CASA& FCM; T2), and then centrifuged (900xg for10 min on cushion) and resuspended in ½ volume of the corresponding diluent. Sperm concentration was assessed after centrifugation and a volume of diluent calculated was added to have a final concentration of $10x10^6$ spz/mL in the five tubes. A new evaluation (CASA & FCM) was performed (T3) before spermatozoa were refrozen exactly as described for the first freezing cycle. Three straws/treatment group were thawed and evaluated after the second freezing and thawing event (CASA & FCM; T4).

A priori, it was decided that the G concentration giving the best result would be retained and used as the basis for experiment 2 *(Fig. 1)*.

Experiment 2- Effect of Cholesterol andα-Tocopherol, and Sperm Cell Concentration: Based on results from experiment 1, semen frozen in INRA-Freeze® was used for experiment 2. Six straws (3 mL total volume of semen) were thawed as described in experiment 1 and then analyzed (CASA and FCM) at T0. The thawed ejaculates were centrifuged (900xg for 10 minutes) and resuspended before being aliquoted into one of three tubes at different dilutions (1:10; 1:20, 1:100) corresponding to three final sperm concentrations ($10x10^6$ spermatozoa/mL; $5x10^6$spermatozoa/mL; $1x10^6$ spermatozoa/mL, respectively). Each of the 3 tubes subsequently was aliquoted into two tubes - one containing the combined CLC+TLC treatment and the other serving as an untreated control. The six tubes were equilibrated, and semen was packaged in straws, frozen, stored, and then thawed and analyzed (CASA and FCM) exactly as described in experiment 1 *(Fig. 2)*.

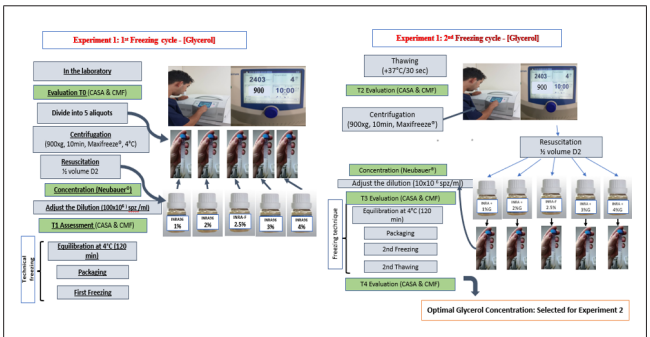

Fig 1. Schematic chart illustrating the experiment 1

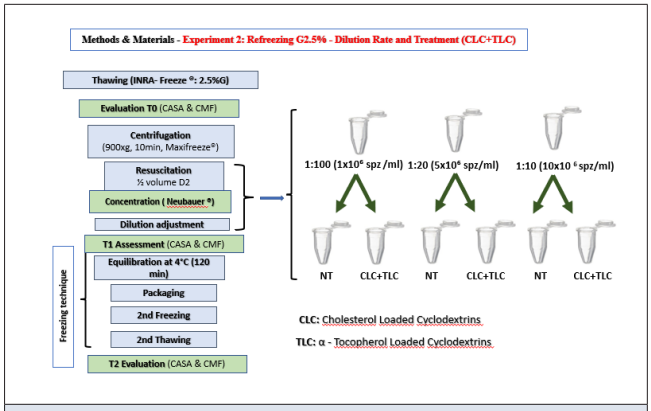

Fig 2. Schematic chart illustrating the experiment 2

Statistical Analysis

The SPSS statistical software was used for analysis of data, including the generation of descriptive statistics (mean, standard deviation, standard error of the mean); testing if response variables were normally distributed; performance of a logarithmic transformation if data were not normally distributed; conduct of an analysis of variance (ANOVA) and mean separation tests (Tukey and Duncan tests; Post hoc). The significance level was set at 5% ($P<0.05$), and results are presented as Mean±SEM (standard error of the mean).

Results

Initial evaluation of the semen immediately after collection showed a mean concentration of 155.5±4.21 x10^{6}spermatozoa/mL and an average mass motility score of 3.5 corresponding to around 70% of spermatozoa with slow and fast rectilinear movement.

Experiment 1- Evaluation of Glycerol Concentration and the Effect of Centrifugation

Sperm parameters were evaluated at five different time points during experiment 1. The initial analysis after 3 h of transport (T0) *(Table 2)*, after the first centrifugation (T1) *(Table 3)*, after the first thawing (T2) *(Table 4)*, before the second freezing (T3) *(Table 5)*, and after the second thawing (T4) *(Table 6)*.

Table 2. *Initial sperm parameters (n=18 ejaculates) after three h of transport (T0- Exp1)*

Parameter‡	Value*
Concentration (x10^{6}spermatozoa /mL)	155.5±4.21
VIAB/ACRO (%)	54.0±2.30
ROS (%)	23.8±0.73
MOT(%)	78.6±1.55
PROG (%)	54.6±2.33

‡ VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility
** Expressed as mean ± standard error of the mean (SEM)*

The comparison of sperm parameters between the first and the second thawing in different cryopreservation solutions showed a decrease ($P<0.05$) in motility parameters as well as in the percentage of spermatozoa that were alive with intact plasma and acrosomal membranes *(Fig. 3)*. Comparing glycerol concentrations, data revealed that the only difference observed was in MOT; the 2.5% glycerol concentration was better than the 1% and 4% but not different than the 2% and 3%.

For the diluent INRA-Freeze®, the percentage of motile (MOT), progressively motile (PROG), and viable spermatozoa with intact acrosomes dropped considerably after refreezing with values of 51.40 vs. 9.17%; 42.74 vs. 7.63%; 52.84 vs. 21.39%, respectively. Oxidative damage to spermatozoa increased ($P<0.05$) from 26.39 to 39.83%.

The effect of centrifugation on spermatozoal parameters during the refreezing cycles was also examined. Centrifugation decreased ($P<0.05$) sperm cell concentration with a loss rate of 12.81% in the second centrifugation (refreezing). For motility (MOT and PROG) and acrosome viability, no differences ($P>0.05$) were observed due to centrifugation *(Fig. 4; Table 5)*.

Experiment 2- Effect of Cholesterol and α-Tocopherol, and Sperm Cell Concentration

A comparison of sperm parameters between the first and second thawing in each of the three sperm cell concentrations (1x10^6; 5x10^6; 10x10^6) is presented in *Table 7* and *Table 8*. At the two highest sperm cell concentrations, there was no difference ($P>0.05$) between concentrations in sperm motility, sperm integrity, or oxidative stress level; however, those sperm parameters were lower ($P<0.05$) at a concentration of 1x10^6 spermatozoa/mL than at the other two concentrations.

Treatment of spermatozoa with a combination of cholesterol and α-tocopherol loaded into cyclodextrin (CLC+TLC) improved ($P<0.05$) motility, integrity and oxidative status *(Table 9)*, with the exception of motility (MOT and PROG) in the highest sperm cell concentration (10x10^6 spermatozoa/mL) which was unaffected.

Discussion

Conventional sperm selection techniques used in reproductive biotechnologies rely on centrifugation. Investigations that evaluated the influence of centrifugation on stallion sperm quality revealed conflicting results [19,20]. In the present study, stallion spermatozoa underwent two centrifugations with a standard protocol (900xg for 10 min), as it was deemed essential when preparing

Table 3. *Initial sperm parameters (n=18 ejaculates) after the first centrifugation (T1- Exp1)*

Parameter‡	Diluent*				
	INRA-Freeze	**INRA96+1% G**	**INRA96+2% G**	**INRA96+3% G**	**INRA96+4% G**
Concentration (x10^6 spermatozoa/mL)	132.63±3.49^a	130.43±3.45^a	127.95±3.44^a	132.63±3.49^a	132.34±3.38^a
VIAB/ACRO (%)	53.40±2.28^a	51.87±2.22^a	52.84±2.25^a	51.23±2.19^a	50.29±2.16^a
ROS (%)	23.54±0.72^a	23.35±0.71^a	23.79±0.73^a	23.06±0.71^a	22.64±0.69^a
MOT (%)	78.40±1.49^a	75.79±1.45^{bc}	76.56±1.44^a	73.04±1.38^{ab}	68.29±1.33^c
PROG (%)	53.64±2.2^a	52.61±2.24^a	53.13±2.24^a	50.69±2.15^a	49.21±2.08^a

** G denotes glycerol; values are expressed as mean ± standard error of the mean (SEM); means with unlike superscripts within a row are different (P<0.05)*
‡ VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

Table 4. *Sperm parameters (n=18 ejaculates) after the first thawing with different levels of glycerol added to diluents (T2-Exp 1)*

Parameter ‡	Diluent *				
	INRA Freeze	**INRA96+1%G**	**INRA96+2%G**	**INRA96+3%G**	**INRA96+4%G**
VIAB/ACRO (%)	52.84±2.25^a	45.02±1.43^b	51.32±2.23^{ab}	50.13±1.43^a	44.01±1.43^c
ROS (%)	26.90±0.67^a	33.78±0.51^b	30.88±0.84^c	29.10±0.51^{ac}	33.21±0.64^{cb}
MOT(%)	51.40±1.14^a	35.55±0.68^b	46.06±0.81^a	46.65±0.92^a	31.93±0.77^c
PROG(%)	42.74±1.68^a	30.79±0.73^b	41.97±1.10^a	40.68±1.34^a	29.25±0.90^c

** G denotes glycerol; INRAFreeze contains 2.5% G; values are expressed as mean ± standard error of the mean (SEM); meanswith unlike superscripts within a row are different (P<0.05)*
‡ VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

Table 5. *Sperm parameters after the second centrifugation with the different levels of glycerol added to the diluents before the second freezing (T3-Exp 1)*

Parameter‡	Diluent *				
	INRA-Freeze	**INRA96+1%**	**INRA96+2%**	**INRA96+3%**	**INRA96+4%**
Concentration (x10^6 spermatozoa/mL)	113.13±3.17	111.43±3.12	109.76±3.08	108.25±3.08	106.51±3.05
VIAB/ACR (%)	45.75±2.02	44.44±1.97	45.26±2.00	43.89±1.95	43.08±1.92
ROS (%)	35.85±1.24	36.90±1.28	36.23±1.25	37.36±1.29	38.04±1.32
MOT (%)	50.46±1.11	48.62±1.07	49.53±1.09	47.73±1.05	46.86±1.03
PROG (%)	41.96±1.65	40.44±1.60	41.19±1.63	39.70±1.58	38.98±1.56

** G denotes glycerol; INRAFreeze contains 2.5% G; values are expressed as mean ± standard error of the mean (SEM); means with unlike superscripts within a row are different (P<0.05)*
‡ VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

equine semen for freezing and refreezing [21]. The first centrifugation enabled elimination of the seminal plasma (containing a lot of frost) with known deleterious effects on sperm quality as well as on the centrifugation diluent. The second centrifugation allowed removal of the original freezing medium whose composition had presumably been altered and potentially loaded with toxins; it was replaced with fresh freezing medium for the second cycle of cryopreservation [22]. However, centrifugal force exposed spermatozoa to the risk of mechanical shock, likely altering their quality and increasing risk of cell loss in the supernatant [23]. Thus, we evaluated the motility and integrity parameters, as well as sperm cell concentration, before and after each centrifugation to assess any effect of centrifugation on semen quality. As the main aim of this study is to split a frozen 0.5 mL straw into as many ICSI subunits of good quality as possible after thawing and refreezing, the benefit of repeated centrifugation before the second freezing seems questionable.

Results of the first experiment showed a decrease in sperm cell concentration in the second centrifugation (refreezing) with a loss rate of 12.81%. This loss rate is lower than the 22% loss rate reported previously using the same centrifugation protocol [23]. With respect to motility (MOT and PROG) and acrosomal integrity, centrifugation had no effect. Although the centrifugation protocol caused a loss of spermatozoa, it preserved motility and sperm integrity satisfactorily. This result is contrary to a previous study

Table 6. Sperm parameters (n=18 ejaculates) after the second thawing with different levels of glycerol added to diluents (T4-Exp 1)					
Parameter‡	**Diluent ***				
	INRA-freeze	**INRA96 + 1%G**	**INRA96 +2%G**	**INRA96 + 3%G**	**INRA96 +4%G**
VIAB/ACRO (%)	21.39±1.02[a]	18.90±0.77[b]	21.17±1.01[a]	20.52±0.99[a]	17.68±0.63[b]
ROS (%)	39.83±1.22[a]	47.57±1.63[be]	44.50±1.65[cd]	45.88±1.70[d]	50.44±1.59[e]
MOT (%)	9.17±0.22[a]	7.79±0.25[b]	9.00±0.22[a]	8.67±0.21[ab]	7.38±0.28[cb]
PROG(%)	7.63±0.32[a]	6.35±1.18[b]	7.49±0.31[a]	7.22±0.31[ab]	6.17±0.17[cb]

** G denotes glycerol; INRAFreeze contains 2.5%G; values are expressed as mean ± standard error of the mean (SEM); means with unlike superscripts within a row are different (P<0.05)*
‡ VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

[24] where various centrifugation protocols (300× g for 5 min, 300× g for 10 min, 1500× g for 5 min, 1500× g for 10 min) impaired motility and increased oxidative damage to cryopreserved stallion spermatozoa, even at a weak force for a short time. More recently, colloidal centrifugation and filtration techniques were compared with conventional simple centrifugation following cryopreservation, and no discernible improvement in semen quality was observed when using these alternate techniques [25]. On the contrary, Gutiérrez-Cepeda et al.[26] found that colloidal centrifugation optimized the efficiency of cryopreservation, as it allowed to increase the number of ejaculates suitable to be frozen, especially when dealing with individuals or breeds for which initially low sperm quality prevents or limits their inclusion in sperm cryopreservation programs.

The loss of spermatozoa in the present investigation, although lower than other studies, may have resulted from the low speed/force used (900xg for 10 min) which in turn did not allow all of the sperm to enter the sperm pellet prior to reconstitution. High speeds/forces of centrifugation (1200, 1800, 2400xg) were associated with lower sperm losses (23.0, 7.4, and 2.1%, respectively) but also with reduced sperm quality following mechanical shock [23].

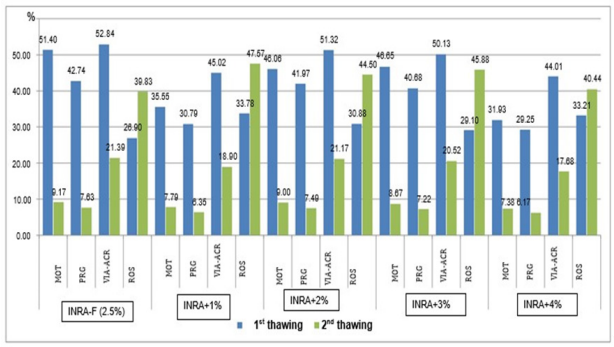

Fig 3. Motility (MOT), progressive motility (PRG), viability with an intact acrosome (VIA-ACRO), and oxidative damage due to reactive oxygen species (ROS) in frozen-thawed equine spermatozoa after the first and the second thawing. Spermatozoa were cryopreserved in diluents containing one of five different levels of glycerol: 2.5% for INRA-Freeze (INRA-F); 1%, 2%, 3%, and 4% for INRA+1, INRA+2, INRA+3, and INRA+4, respectively. All values within a diluent and sperm parameter, all values are different (P<0.05)

The preservation of sperm motility and integrity after centrifugation in our study could be explained by the use of a centrifugation cushion which prevented the percussion shock of the spermatozoa against the wall of the centrifuge tube. This protective effect has been widely reported in the literature [21,22,27]. An additional measure to combat mechanical shock to the sperm was applied in our study; the rotor brake of the centrifuge was deactivated, thus allowing the centrifuge to stop gradually (sudden stoppage increases the mechanical shock inflicted on spermatozoa).

Sperm motility and integrity were compared between the first and the second freezing cycle in the present study, and results showed that equine sperm suffered significantly through this double freezing procedure. Excessive degradation of sperm quality after re-freezing has been reported in the literature [7,28]. Degradation after the second freeze/thaw cycle could be explained by the action of cold temperature and glycerol on cells already weakened by the first cycle of cryopreservation. In a different experiment using INRA82, Sielhorst et al.[29] reported the first study evaluating sperm fertility after three freezing cycles of stallion spermatozoa. These authors found a pregnancy

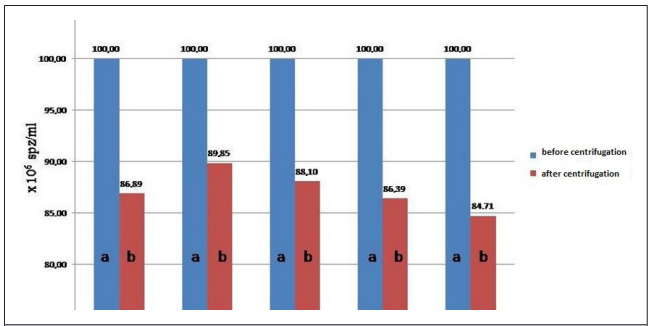

Fig 4. Effect of centrifugation on sperm cell concentration during the second freezing of equine spermatozoa (spz) in different cryopreservation diluents (Exp1). (INRA-F = INRA-Freeze containing 2.5% glycerol, INRA+2 = INRA96® + 2% glycerol, INRA+3 = INRA96® +3% glycerol, INRA+4 = INRA96® + 4% glycerol. Sperm cell concentrations in each diluent were set equivalent to 100% prior to centrifugation. Means within a diluent with unlike superscripts are different (P<0.05; n=18 ejaculates)

Table 7. *Spermatic parameters (n=18 ejaculates) after the first thawing (T0-Exp 2)*

Parameter‡	1:10 Ejaculate Dilution
VIAB/ACRO (%)	52.39±2.14
ROS (%)	28.62±0.92
MOT (%)	50.39±1.11
PROG (%)	41.91±1.66

‡VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

Table 8. *Spermatic parameters after the second thawing (T1-Exp 2) in the three dilutions studied (P<0.05; n=18)*

Parameter‡	Dilution		
	1:100	1:20	1:10
VIAB/ACRO (+) (%)	14.05±1.07[a]	20.56±0.98[b]	20.97±1.00[b]
ROS (%)	43.19±1.62[a]	28.92±1.83[b]	27.35±1.61[b]
MOT(%)	6.07±0.37[a]	8.99±0.21[b]	8.81±0.21[b]
PROG(%)	5.03±0.36[a]	7.48±0.31[b]	7.34±0.31[b]

‡VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

rate of 40% with semen frozen once and 10% with double-frozen semen. They revealed after the third freeze/thaw cycle, that the sperm quality decreased to less than 10% motile and membrane-intact cells, which resulted in no pregnancy after conventional AI. A reduction in sperm quality after refrigeration (4°C) is considered normal, as the decreased temperature can induce changes that may have a detrimental impact on semen quality and fertility [25]. In another study, Leisinger et al.[28] confirmed that DNA denaturability was not affected by semen packaging method and was only affected by thaw number, increasing at post-thaws 5 and 6.

Fertilization, the most fundamental success criterion to assess after sperm cryopreservation, requires the functional integrity of the sperm plasma membrane to be maintained throughout the freezing and thawing processes. This functional integrity is correlated with motility [30]. Cold shock, osmotic pressure change, ice crystal formation, and generation of reactive oxygen species (ROS) can all cause changes in the lipid composition of the cell membrane [31]. These changes negatively affect sperm motility, viability, mitochondrial membrane function, DNA integrity, and fertilizing ability of spermatozoa [32].

The appropriate thawing rate can be influenced by many aspects of the cryopreservation procedures, including diluent type, glycerol concentration, and freezing curve [33]. Glycerol (G) is the most commonly utilized membrane-permeable cryoprotectant which restricts intracellular ice crystal formation [34]. However, it also exerts a dose-dependent toxic effect on spermatozoa-especially with prolonged and/or repeated exposure to refreezing [12]. The optimal concentration of G to use in diluents is not standardized yet, and published works revealed contradictory data [35]. For this reason, we evaluated the effect of five different concentrations of G (1%, 2%, 2.5%, 3% and 4%) in the freezing medium for optimization purposes. Results from experiment 1 revealed that the control medium containing 2.5% G offered better preservation of motility (MOT and PROG) and membrane and acrosomal integrity (VIAB-ACRO) as well as better protection against oxidative stress (ROS) compared with other G concentrations during two freezing cycles. Concentrations of 1% and 4% G gave poorer results than those obtained with 2.5% G for all parameters studied compared. It seems that 1% G is insufficient to effectively protect the spermatozoa from intracellular ice crystal formation and poorly preserves the motility, integrity, and oxidative status of the sperm. On the other hand, a 4% concentration of G would be at the lower limit of toxicity. Interestingly, the 2% and 3% concentrations of G gave numerically poorer results compared to the control diluent for all the parameters studied, but these differences were not significant.

The 2.5% concentration of G seems to be the optimal concentration of G to preserve the functionality, integrity

Table 9. *Spermatozoal parameters after the second thawing in the three sperm cell concentrations with (CLC+TLC) or without (NT) treatment (T1-Exp 2) (P<0.05; n=18 ejaculates)*

Parameter‡	$1x10^6$ Spermatozoa/mL		$5x10^6$ Spermatozoa/mL		$10x10^6$ Spermatozoa/mL	
	NT	CLC+TLC	NT	CLC+TLC	NT	CLC+TLC
VIAB/ACRO (%)	14.05±1.07[a]	29.08±1.09[b]	20.56±0.98[a]	32.26±1.74[b]	20.97±1.00[a]	27.37±1.35[b]
ROS (%)	43.19±1.62[a]	32.93±1.04[b]	28.92±1.83[a]	21.48±1.59[b]	27.35±1.61[a]	22.85±1.59[b]
MOT (%)	6.07±0.37[a]	13.00±0.88[b]	8.99±0.21[a]	18.50± 0.41[b]	8.81±0.21[a]	11.45±0.26[a]
PROG (%)	5.03±0.36[a]	11.79±1.07[b]	7.48±0.31[a]	11.94±0.59[b]	7.34±0.31[a]	8.83±0.39[a]

‡VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

and oxidative balance of equine spermatozoa through two cycles of cryopreservation. Although the results of the present study are similar to those of another study [36] where the best concentration of G ranged from 2.4 to 2.8%, other researchers [17,37] found the most suitable concentration of G was 4.0%. In the same context, Hoffmann et al.[38] reported that concentrations of 2-3% of the cryoprotectants with freezing extender showed the highest motility rates after freeze-thaw, both for 'good' and 'poor' freezers. On the other hand, Gonzalez-Castro et al.[39] found that G concentration (2, 4, 6 or 8%) did not affect post-thaw motility. The discrepancies in results of various studies make it difficult to establish an optimal concentration of G. Different results across studies may be due to the variation in diluent composition, quality control in the laboratory, individual stallion effects, or other factors.

The effect of final sperm cell concentration on motility and integrity of twice-frozen equine spermatozoa was investigated in the second study. Three final concentrations of spermatozoa ($10x10^6$, $5x10^6$, $1x10^6$ spermatozoa/mL) were compared, and no differences in sperm motility, integrity, or oxidative damage were observed between sperm concentrations of $10x10^6$or $5x10^6$ spermatozoa/ml. It is common for most straws of stallion semen to possess between 100 and $300x10^6$ spermatozoa/mL, so the dilution rates used in the present study allowed the production of 10-20, 20-40, or 30-60 ICSI-dosed straws from a single conventional AI straw having $100x10^6$, $200x10^6$, or $300x10^6$ spermatozoa/mL, respectively.

The lowest sperm concentration evaluated ($1x10^6$ spermatozoa/mL) would enable production of between 100 and 300 ICSI straws from a single conventional AI straw. However, the control diluent without the added CLC+TLC treatment yielded poorer sperm motility, integrity and oxidative status in comparison with the other two concentrations. No definitive explanation for this result is known. Regardless of the reasons that may explain our data, it would be interesting to develop a semen treatment capable of improving the results by increasing the resistance of spermatozoa to refreezing.

Given the paucity of cholesterol in the plasma membrane of the equine spermatozoon and the increase of oxidative stress caused by accumulated ROS, we hypothesized that a pre-refreezing treatment of semen based on cholesterol and an antioxidant such as α-tocopherol may be beneficial in the prevention of cryoinjury generated by reactive oxygen species (ROS). Treatment of sperm with a combination of cholesterol and α-tocopherol loaded into cyclodextrin (CLC+TLC) at the half dose of 2.5 mg CLC per $100x10^6$ spermatozoa and 0.05 mM TLC per $100x10^6$ spermatozoa before refreezing allowed, under the conditions of the current experiment, a considerable improvement in sperm motility, integrity and oxidative damage in the three dilutions studied (with the exception of the motility in the 1:100 dilution [$1x10^6$ spermatozoa/mL]).

The lowest sperm cell concentration studied, which is of the greatest interest in terms of production of ICSI straws, resulted in a marked improvement in the percentages of motile (MOT), progressive (PROG) and viable spermatozoa with intact acrosomes. These results could be explained by the protective and the stabilizing action of cholesterol on the equine sperm plasma membrane that is poor in cholesterol [8], as well as by the antioxidant action of α-tocopherol - particularly for cells suffering with disruption of their metabolism and free radical elimination mechanism. Our result agrees with that of other studies [40,41]. Specifically, α-tocopherol can stop the lipid peroxidation chain reaction in biomembranes, protecting the cell from damage to the plasma and acrosomal membranes [42]. In addition, CLCs are believed to prevent the rearrangement of phospholipids by cholesterol addition and to increase membrane fluidity at low temperatures [43].

In addition to the protective action of cholesterol and α-tocopherol, the effectiveness of this treatment can also be explained by the ability of methyl-β-cyclodextrin to increase the solubility (in an aqueous medium) of cholesterol and α-tocopherol (lipid molecules) thereby improving their penetration into the sperm plasma membrane. The beneficial effects of CLC and TLC observed in the current study agree with other previous investigations [42-44]. Some reports revealed that CLC, either loaded with cholesterol or unloaded but in the presence of a high cholesterol content diluent, improved post-thaw motility, viability, and fertility but inhibited post-thaw acrosome reaction of equine and bovine semen [45-47].

To further improve results, it would be interesting to enhance this treatment in the future by optimizing the dose and duration of treatment immediately before both the first and second freezing. It would also be interesting to evaluate the benefit of this treatment without re-centrifugation before refreezing. More sperm parameters to include in the future, DNA integrity, is very crucial in term of ICSI and in IVF procedures.

Refreezing of equine spermatozoa in the control diluent is possible, but with a considerable reduction in sperm quality parameters (motility, integrity and oxidative status). Treatment of spermatozoa before refreezing with cholesterol and α-tocopherol loaded cyclodextrins seems to improve all quality parameters of refrozen sperm cells. Further investigations into the exact biological mechanism and the separate effect of each substance (cholesterol and α-tocopherol) on equine sperm cryopreservation is warranted.

Declarations

Data Availability Statement: The data that support the findings of this study are available from the corresponding author (NM) upon reasonable request.

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Ethics Approval: All experiments were conducted according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA. 14).

Conflict of Interest: The authors declare no competing interests. None of us has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Declaration of Generative Artificial Intelligence (AI): The article and tables and figures were not written/ created by AI and AI assisted technologies.

Author Contribution: RB, OM: Writing– original draft, Methodology, Formal analysis, Conceptualization, Supervision. CRY: Writing–Review& editing. NM: Writing-review & editing, Investigation, Supervision.

REFERENCES

1. **Felix MR, Turner RM, Dobbie T, Hinrichs K:** Successful *in vitro* fertilization in the horse: Production of blastocysts and birth of foals after prolonged sperm incubation for capacitation. *Biol Reprod,* 107 (6): 1551-1564, 2022. DOI: 10.1093/biolre/ioad028
2. **Lazzari G, Colleoni S, Crotti G, Turini P, Fiorini G, Barandalla M, Landriscina L, Dolci G, Benedetti M, Duchi R, Galli C:** Laboratory production of equine embryos. *J Equine Vet Sci,* 89:103097, 2020. DOI: 10.1016/j.jevs.2020.103097
3. **McCue PM, Moore AI, Bruemmer JE:** Refreezing stallion spermatozoa for assisted reproduction. *Reprod Fertil Dev,* 16 (2): 176-177, 2004. DOI: 10.1071/RDv16n1Ab109
4. **Morse-Wolfe B, Bleach E, Kershaw C:** An investigation of equine sperm quality following cryopreservation at low sperm concentration and repeated freeze-thawing. *J Equine Vet Sci,* 120:104167, 2023. DOI: 10.1016/j.jevs.2022.104167
5. **Galli C, Duchi R, Colleoni S, Lagutina I, Lazzari G:** Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. *Theriogenology,* 81 (1): 138-151, 2014. DOI: 10.1016/j.theriogenology.2013.09.008
6. **Gonzalez-Castro RA, Carnevale EM:** Association of equine sperm population parameters with outcome of intracytoplasmic sperm injections. *Theriogenology,* 573, 114-120, 2018. DOI: 10.1016/j.theriogenology.2018.06.027
7. **Choi YH, Love CC, Varner DD, Hinrichs K:** Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. *Theriogenology,* 65 (4): 808-819, 2006. DOI: 10.1016/j.theriogenology.2005.04.035
8. **Ricker JV, Linfor JJ, Delfino WJ:** Equine sperm membrane phase behavior: The effects of lipid-based cryoprotectants. *Biol Reprod,* 74 (2): 359-365, 2006. DOI: 10.1095/biolreprod.105.046185
9. **Ball BA:** Oxidative stress, osmotic stress and apoptosis: Impacts on sperm function and preservation in the horse. *Anim Reprod Sci,* 107 (3/4): 257-267, 2008. DOI: 10.1016/j.anireprosci.2008.04.014
10. **Alharbi YM, Ali M, Alharbi MS:** Impact of the antioxidant hydroxytyrosol on the quality of post-thawed stallion semen. *Vet Med Int,* 2024:6558480, 2024. DOI: 10.1155/2024/6558480
11. **Sharafi M, Borghei-Rad SM, Hezavehei M, Shahverdi A, Benson JD:** Cryopreservation of semen in domestic animals: A review of current challenges, applications, and prospective strategies. *Animals (Basel),* 12 (23):3271, 2022. DOI: 10.3390/ani12233271
12. **Álvarez C, Gil L, González N, Olaciregui M, Luño V:** Equine sperm post-thaw evaluation after the addition of different cryoprotectants added to INRA 96® extender. *Cryobiology,* 69 (1): 144-148, 2014. DOI: 10.1016/j.cryobiol.2014.06.008
13. **Bucak MN, Keskin N, Ili P, Bodu M, Akalin PP, Öztürk AE, Özkan H, Topraggaleh TR, Sari F, Başpınar N, Dursun S:** Decreasing glycerol content by co-supplementation of trehalose and taxifolin hydrate in ram semen extender: Microscopic, oxidative stress, and geneexpressionanalyses. *Cryobiology,* 96, 19-29, 2020. DOI: 10.1016/j.cryobiol.2020.09.001
14. **Milovanov VK:** The Biology of Reproduction and the Artificial Insemination of Animals. Moscow: Seljhozizdat, 1962.
15. **Purdy PH, Graham JK:** Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. *Cryobiology,* 48 (1): 36-45, 2004. DOI: 10.1016/j.cryobiol.2003.12.001
16. **Koontz JL, Marcy JE, O'Keefe SF, Duncan SE:** Cyclodextrin inclusion complex formation and solid-state characterization of the natural antioxidants alpha-tocopherol and quercetin. *J Agric Food Chem,* 57 (4):1162-1171, 2009. DOI: 10.1021/jf802823q
17. **Cochran JD, Amann RP, Froman DP, Pickett BW:** Effects of centrifugation, glycerol level, cooling to 5°C, freezing rate and thawing rate on the post-thaw motility of equine sperm. *Theriogenology,* 22 (1): 25-38, 1984. DOI: 10.1016/0093-691X(84)90470-9
18. **Belala R, Kebbal S, Bouguetof C, Medjkoune M, Mecherouk C, Mimoune N:** Morphometric parameters of canine spermatozoa: Comparison between conventional microscopy and CASA system. *Med Weter*, 81 (1): 23-31, 2025. DOI: 10.21521/mw.6969
19. **Len JA, Jenkins JA, Eilts BE, Paccamonti DL, Lyle SK, Hosgood G:** Immediate and delayed (after cooling) effects of centrifugation on equine sperm. *Theriogenology,* 73 (2): 225-231, 2010. DOI: 10.1016/j.theriogenology.2009.09.003
20. **Len JA, Beehan DP, Lyle SK, Eilts BE:** Cushioned versus noncushionedcentrifugation: Sperm recovery rate and integrity. *Theriogenology,* 80 (6): 648-653, 2013. DOI: 10.1016/j.theriogenology.2013.06.009
21. **Bliss SB, Voge JL, Hayden SS, Teague SR, Brinsko SP, Love CC, Blanchard TL, Varner DD:** The impact of cushioned centrifugation protocols on semen quality of stallions. *Theriogenology,* 77 (6): 1232-1239, 2012. DOI: 10.1016/j.theriogenology.2011.10.031
22. **Waite JA, Love CC, Brinsko SP, Teague SR, Salazar Jr JL, Mancill SS, Varner DD:** Factors impacting equine sperm recovery rate and quality following cushioned centrifugation. *Theriogenology,* 70 (4): 704-714, 2008. DOI: 10.1016/j.theriogenology.2008.04.047
23. **Hoogewijs M, Rijsselaere T, De Vliegher S, Vanhaesebrouck E, De Schauwer C, Govaere J, Thys M, Hoflack G, Van Soom A, de Kruif A:** Influence of different centrifugation protocols on equine semen preservation. *Theriogenology,* 74 (1): 118-126, 2010. DOI: 10.1016/j.theriogenology.2010.01.022
24. **Marzano G, Moscatelli N, Di Giacomo M, Martino NA, Lacalandra GM, Dell'Aquila ME, Maruccio G, Primiceri E, Chiriacò MS, Zara V, Ferramosca A:** Centrifugation force and time alter CASA parameters and oxidative status of cryopreserved stallion sperm. *Biology (Basel),* 9 (2):22, 2020. DOI: 10.3390/biology9020022
25. **Zabala SM, Serres C, Montero N, Montero N, Crespo F, Lorenzo PL, Pérez-Aguilera V, Galán C, Domínguez-Gimbernat M, Oliet A, Moreno S:** Strategies to reduce the use of antibiotics in fresh and chilled equine semen. *Animals (Basel),* 14 (2):179, 2024. DOI: 10.3390/ani14020179
26. **Gutiérrez-Cepeda L, Crespo F, Blazquez JC, Serres C:** Optimization of the equine-sperm freeze test in purebred Spanish horses by incorporating colloidal centrifugation. *Animals (Basel),* 13 (3):382, 2023. DOI: 10.3390/ani13030382

27. **Knop K, Hoffmann N, Rath D, Sieme H:** Effects of cushioned centrifugation technique on sperm recovery and sperm quality in stallions with good and poor semen freezability. *Anim Reprod Sci,* 89:294, 2005.

28. **Leisinger CA, Pinto CRF, Cramer E, Love CC, Paccamonti DL:** Effects of repeated partial thaw and refreeze on post-thaw parameters of stallion semen cryopreserved in cryovials. *J Equine Vet Sci,* 49, 19-24, 2017. DOI: 10.1016/j.jevs.2016.10.006

29. **Sielhorst J, Hagen C, Behrendt D, Schuette B, Burger D, Martinsson G, Sieme H:** Effect of multiple freezing of stallion semen on sperm quality and fertility. *J Equine Vet Sci,* 40, 56-61, 2016. DOI: 10.1016/j.jevs.2016.01.014

30. **Önder NT, Gökdemir T, Kılıç MC, Şahin O, Yıldız S, Kaçar C, Demir MC, Öztürkler Y:** Insulin and bull sperm interactions during cryopreservation. *Kafkas Univ Vet Fak Derg*, 29 (4): 401-405, 2023. DOI: 10.9775/kvfd.2023.29623

31. **Ustuner B, Alcay S, Gokce E, Yilmaz MM, Aktar A, Huraydin O, Duman M, Onder NT, Akal E, Nur Z:** Lyophilized extender supplemented with rainbow trout *(Oncorhynchus mykiss)* seminal plasma improves cryopreservation of ram sperm. *Kafkas Univ Vet Fak Derg,* 28 (2): 255-260, 2022. DOI: 10.9775/kvfd.2021.26855

32. **Alçay S, Toker MB, Gökçe E, Önder NT, Üstüner B, Nur Z:** Long term incubation resilience of post-thaw ram semen diluted with lecithin-based extender supplemented with bovine serum albumin. *Kafkas Univ Vet Fak Derg,* 25 (3): 291-297, 2019. DOI: 10.9775/kvfd.2018.20843

33. **Esin B, Akar M, Tağrıkulu MD, Kaya C, Çevik M:** The effects of fast and slow thawing on spermatological parameters and detect [sic] of chromatin condensation by toluidine blue staining in frozen-thawed bull sperm. *Kafkas Univ Vet Fak Derg*, 28 (3): 307-313, 2022. DOI: 10.9775/kvfd.2021.26950

34. **İnanç ME, Olgac KT, Tekin K, Çil B, Alemdar H, Özen D, Uysal O:** Effect of cholesterol and 7-dehydrocholesterol on bull semen freezing with different rates of glycerol. *Kafkas Univ Vet Fak Derg,* 24 (6): 815-820, 2018. DOI: 10.9775/kvfd.2018.19958

35. **Wu Z, Zheng X, Luo Y, Huo F, Dong H, Zhang G, Yu W, Tian F, He L, Chen J:** Cryopreservation of stallion spermatozoa using different cryoprotectants and combinations of cryoprotectants. *Anim Reprod Sci,* 163, 75-81, 2015. DOI: 10.1016/j.anireprosci.2015.09.020

36. **Vidament M, Yvon JM, Couty I, Arnaud G, Nguekam-Feugang J, Noue P, Cottron S. Le Tellier S, Noel F,Palmer E, Magistrini M:** Advances in cryopreservation of stallion semen in modified INRA82. *Anim Reprod Sci*, 68(3/4):201-218, 2001. DOI: 10.1016/S0378-4320(01)00157-9

37. **Miroslava M, Horackova E, Vyvial M, Vinkler A, Krisova S, Bodeček Š, Sedlinská M:** Effects of glycerol concentration on the motility of equine spermatozoa after thawing. *Acta Vet Brno,* 86 (3): 263-268, 2017. DOI: 10.2754/avb201786030263

38. **Hoffmann N, Oldenhof H, Morandini C, Rohn K, Sieme H:** Optimal concentrations of cryoprotective agents for semen from stallions that are classified 'good' or 'poor' for freezing. *Anim Reprod Sci,* 125, 112-118, 2011. DOI: 10.1016/j.anireprosci.2011.03.001

39. **Gonzalez-Castro RA, Trentin JM, Carnevale EM, Graham JK:** Effects of extender, cryoprotectants and thawing protocol on motility of frozen-thawed stallion sperm that were refrozen for intracytoplasmic sperm injection doses. *Theriogenology,* 136, 36-42, 2019. DOI: 10.1016/j.theriogenology.2019.06.030

40. **Qamar AY, Naveed MI, Raza S, Fang X, Roy PK, Bang S, Tanga BM, Saadeldin IM, Lee S, Cho J:** Role of antioxidants in fertility preservation of sperm - A narrative review. *Anim Biosci,* 36 (3): 385-403, 2023. DOI: 10.5713/ab.22.0325

41. **Silvestre MA, Yániz JL, Peña FJ, Santolaria P, Castelló-Ruiz M:** Role of antioxidants in cooled liquid storage of mammal spermatozoa. *Antioxidants (Basel),* 10 (7):1096, 2021. DOI: 10.3390/antiox10071096

42. **Aguiar CS, Barros CHSC, Machado WM, Allaman IB, Leite Filho AO, Barbosa LP, Snoeck PPN:** Effect of different concentrations of Trolox® in association with docosahexaenoic acid on equine semen freezing. *Anim Reprod,* 19 (4):e20220010, 2022. DOI: 10.1590/1984-3143-AR2022-0010

43. **Ligocka Z, Partyka A, Bonarska-Kujawa D, Mucha A, Niżański W:** Addition of low concentration of cholesterol-loaded cyclodextrin (CLC) has a positive effect on cryopreserved canine spermatozoa evaluated by andrological and biophysical methods. *BMC Vet Res*, 20 (1):7, 2024. DOI: 10.1186/s12917-023-03851-6

44. **Kumar P, Mehta JS, Ravi SK, Dedar RK, Purohit GN, Legha RA, Tripathi BN, Talluri TR:** Cholesterol loaded cyclodextrin supplementation enhances the cholesterol-to-phospholipid ratio and diminishes oxidative stress in jack spermatozoa during cryopreservation. *J Equine Vet Sci,* 94:103237, 2020. DOI: 10.1016/j.jevs.2020.103237

45. **Kumar P, Mehta JS, Ravi SK, Talluri TR, Kumawat BL, Kumar T, Kumar S, Jhamb D, Dedar RK:** Protective efficacy of cholesterol-loaded cyclodextrin (CLC) against sperm morphological abnormalities in Marwari stallions. *Anim Reprod,* 4 (1): 3-10, 2023. DOI: 10.48165/aru.2023.4.1.2

46. **Jepsen RJ, Evans LE, Youngs CR:** Use of direct thaw insemination to establish pregnancies with frozen-thawed semen from a standard jack. *J Equine Vet Sci,* 30 (11): 651-656, 2010. DOI: 10.1016/j.jevs.2010.10.001

47. **Madison RJ, Evans LE, Youngs CR:** The effect of 2-hydroxypropyl-β-cyclodextrin on post-thaw parameters of cryopreserved jack and stallion semen. *J Equine Vet Sci,* 33 (4): 272-277, 2013. DOI: 10.1016/j.jevs.2012.07.021