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Abstract

This study investigated the effect of the addition of *Lepidium meyenii* (Maca) to the freezing extender on the post-thaw quality of dog semen. Ten canine ejaculates were frozen following a two-step protocol using a tris-glucose-citrate egg yolk extender with or without the addition of 10 µl/mL of aqueous extract of Maca (Maca and ctrl groups, respectively). Prior to (fresh semen) and after freezing (T0) sperm motility, kinetic parameters, viability and mitochondrial membrane potential (MMP), as well as the levels of malondialdehyde (MDA) were evaluated. In addition, sperm motility, kinetic parameters, viability and MMP were examined up to 2 h of incubation of 37°C after thawing (T1 and T2) to evaluate thermo-resistance.

The addition of Maca reduced MDA concentration at T0 ($p<0.05$) and increased total motility, the percentage of sperm with medium velocity and WOB at T1. Progressive motility decreased ($p< 0.05$) at T1 in the ctrl group, whereas it was

not affected in Maca group at any time point. In addition, the percentage of hyperactivated spermatozoa remained constant at T1 in the ctrl, while in the Maca group an increase ($p<0.05$) of this parameter was recorded. Although no differences were found for MMP between groups at any time points, a decrease of viable sperm with low MMP was observed in ctrl group between T0 and T1 and in Maca group between T1 and T2.

The addition of Maca prior freezing reduced the extent of lipid peroxidation and activated canine sperm motility and hyperactivation after thawing.

Keywords

antioxidant, cryopreservation, dog, sperm motility, lipid peroxidation

Introduction

Cryopreservation of canine semen has become an increasingly popular technology. The outcome of artificial insemination with frozen semen is dictated by the quality of semen after thawing. Cryopreservation determines extensive oxidative damage to all cellular components resulting in a decreased semen quality, indicated by reduced viability, motility, and DNA integrity, and increased acrosome damage, and apoptosis, interfering with the fertilization capability (Kim et al. 2010).

In order to reduce cryopreservation-induced oxidative stress, extender supplementation with antioxidants has been proposed (Mahiddine and Kim 2021). Lepidium meyenii (Maca) is an Andean plant of the Brassicaceae family native of Peru, known for its antioxidant properties (Fu et al. 2021). Oral administration of Maca improves semen quality and hence sperm cryotolerance in bulls and stallions (Clément et al. 2010; Del Prete et al. 2018). A beneficial effect of Maca extract on *in vitro* fertilization outcome, associated to increased acrosome reaction and sperm motility, was also observed in mice (Aoki et al. 2019). It was recently demonstrated that the addition of Maca extract to the extender improves the quality of canine semen stored at 4°C (Del Prete et al. 2022).

The aim of this study was to evaluate the effect of the addition of aqueous extract of Maca in the extender prior freezing on the post-thaw quality of dog spermatozoa.

Material and Methods

One ejaculate was collected by artificial vagina from each of ten dogs (n=10) of different breeds (Rottweiler, English Setter, Shih Tzu, English Pointer, Miniature poodle), ranging between 2 and 8 (median of 5.5) years of age, housed in the FOOF breeder center (Caserta, Italy), where semen is routinely collected twice a week for inseminations purpose. Sperm rich fractions were examined for volume (mL) by aspiration into a 5-mL pipette, concentration using a Bürker counting chamber and for motility by Sperm Class Analyzer (SCA) system (Microptic SL, Veterinary Edition, Barcelona, Spain). A single-layer centrifugation (600 x g for 10 minutes) of raw semen with a cushion (Glucose 59.95 g, Sodium citrate tribasic dihydrate 3.7 g, Disodium EDTA 3.7 g, Sodium bicarbonate 1.2 g, in 1 L of deionized water) was used to partial eliminate seminal plasma as good as possible. After removal of supernatant, a two-step freezing protocol was performed. Semen was first diluted at room temperature (RT: 20-25°C) with TCG (Tris 2.4 g, Citric Acid 1.4 g, Glucose 0.8 g, Penicillin G Sodium Salt 0.06 g, Streptomycin 0.1 g and distilled water to 100 ml) with 20% egg yolk and 3% glycerol to reach a concentration of 400 x 10^6 sperm/mL, and cooled over 30 minutes to 4°C. Samples were then diluted 1:1 (v/v)

to 200 x 10^6 sperm/mL with either TCG with 20% egg yolk, 7% glycerol and 1% Equex STM without (Ctrl) or with the addition of 10 µl/mL of aqueous extract of Maca (Maca) and equilibrated for additional 5 minutes at 4°C.

Raw semen was centrifuged on a cushion at 600 x g for 10 minutes to partial eliminate the seminal plasma. After removal of supernatant, a two-step freezing protocol was performed. Semen was first diluted at room temperature (RT: 20-25°C) with TCG (Tris 2.4 g, Citric Acid 1.4 g, Glucose 0.8 g, Penicillin G Sodium Salt 0.06 g, Streptomycin 0.1 g and distilled water to 100 ml) with 20% egg yolk and 3% glycerol to reach a concentration of 400 x 10^6 sperm/mL, and cooled over 30 minutes to 4°C. Samples were then diluted 1:1 (v/v) to 200 x 10^6 sperm/mL with either TCG with 20% egg yolk, 7% glycerol and 1% Equex STM without (Ctrl) or with the addition of 10 µl/mL of aqueous extract of Maca (Maca) and equilibrated for additional 5 minutes at 4°C. The preparation and composition of the aqueous extract of Maca were previously described (Del Prete et al. 2022). Diluted semen was frozen on liquid nitrogen (LN_2) after 10 min suspension on vapor and stored for 4 weeks. Two straws per sample were thawed in a 37°C water bath for 30 sec.

Motility, kinetics, membrane integrity and mitochondrial membrane potential (MMP), as well as the levels of malondialdehyde (MDA), indicator of lipid peroxidation, were evaluated before freezing (fresh semen) and immediately post-thaw (T0). To evaluate post-thaw sperm longevity, thermal-resistance test was conducted, evaluating samples at 1 (T1) and 2 h (T2) of incubation at 37 °C for motility and kinetics, sperm membrane integrity and mitochondrial membrane potential.

Semen evaluation

Motility was assessed using Sperm Class Analyzer (SCA) system (Microptic SL, Veterinary Edition, Barcelona, Spain) installed on a camera-equipped light microscope system (Eclipse E200, Nikon, Japan). The parameters included were: total motility (%), progressive motility (%), the percentage of sperm subpopulations (rapid, medium and slow), average path velocity (VAP; µm/s), straight-line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), straightness (STR; %) and linearity (LIN; %), amplitude of lateral head (ALH; µm), wobble (WOB= VAP/VCL; %), beat cross frequency (BCF ; beats/s). All particles sized between 10 and 80 μm^2 were considered spermatozoa and classified as progressive motile in case of STR > 75 %. SCA system set-up was described in a previous study (Del Prete et al., 2018). For the evaluation, three µl of semen were placed in a 20 µm chamber (Leja, Nieuw-Vannep, Netherlands) pre-warmed at 37 °C. The evaluation was carried out at 100X magnification on a pre-warmed microscope stage and at least 500 spermatozoa in five randomly selected fields were assessed.

Mitochondrial transmembrane potential and membrane integrity were assessed by triple fluorescent labelling with propidium iodide (PI; Invitrogen™, Eugene, Oregon, USA), SYBR green-14, and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi- dazolylcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR, USA).

Thirty µL of each sample were incubated with 3 µl of SYBR green solution (5 µl SYBR-14 a10x + 15 µl PBS), 2 µl of PI solution (10 mg/ml) and 2 µl JC-1 solution (1 mg/ml) at 37 °C in the dark for 15 min. At least 200 cells per sample were evaluated using confocal microscopy (Leica DM6B; Leica Microsystems, Wetzlar, German). Three sperm populations were identified: dead sperm (PI+), viable sperm with high MMP (HMMP) and viable sperm with low MMP (LMMP).

Sperm concentration of malondialdehyde (MDA) was measured using the thiobarbituric acid (TBA) procedure as previously described (Esterbauer and Cheeseman 1990). Concentrations of MDA were calculated using a calibration curve ranged between 0.5–2 pmoles/mL. The results were expressed as nmol/L of proteins.

Statistical analysis

Data were analyzed with Statistical Package for Social Sciences (SPSS IBM® Statistics version 27.0, IBM Corporation, Armonk, NY, USA). Because of non-normal distribution (Shapiro-Wilk test), results were expressed as median and interquartile range (IQR) and Wilcoxon non-parametric test was used to compare groups at each time point (Fresh semen, Post-thaw, T1, T2). The effect of storage time on semen parameters in each group (Ctrl or Maca) was evaluated Friedman test, post hoc analysis with Wilcoxon's signed-rank test was used to compare individual storage times.

Results

Fresh semen volume ranged between 0.4 and 12 mL (median of 2.4 mL), with a median concentration of 312.5 x 10^6 sperm/ml (IQR: 269,5- 462 sperm/mL). Fresh semen presented a viability of 84 (79.3-85) % and HMMP of 38.5 (36-44.8)%.

Sperm motility and kinetic parameters of fresh semen and ctrl and Maca-treated semen post-thaw (T0, T1 and T2) are shown in Table 1. As expected, a reduction ($p \leq 0.05$) in total motility in both groups was observed post-thaw at T0 and T1 compared to fresh semen. Furthermore, total motility decreased ($p \leq 0.05$) at T1 and T2 compared to T0 (Table 1). Progressive motility decreased ($p \leq 0.05$) at increasing post-thawing times in the ctrl group, whereas it was not affected in the Maca-treated group. The percentage of hyperactivated spermatozoa remained constant during the four time points in

the ctrl, while increased ($p \leq 0.05$) in the Maca-treated semen at T1 and T2. The pattern of the motile-sperm subpopulations (rapid, medium, slow) during thermal stress was similar in the two groups.

Most of the sperm kinetic parameter followed the same pattern in the two groups over time (Table 1). However, in the ctrl group VSL decreased ($p \leq 0.05$) at T2, whereas in the maca-treated group remained at values similar to fresh semen at T0, T1 and T2. The LIN decreased ($p \leq 0.05$) in the ctrl group at T0, with a further reduction ($p \leq 0.05$) detected at T1 and T2; in contrast, in maca-treated group no decrease was observed after freezing and post-thaw incubation times.

Total motility and the percentage of sperm with medium velocity were higher ($p \leq 0.05$) in Maca-treated semen than in the ctrl after 1 hour of incubation at 37°C (T1; Table 1). At the same time point, WOB was remarkably higher ($p \leq 0.05$) in Maca-treated group than in the ctrl (Table 1). There were no other differences in semen kinetic parameters between the two groups, as shown in Table 1.

Table 1. *Motility and kinetic parameters assessed using Sperm Class Analyzer (SCA) in ctrl and Maca-treated (10 µL/mL of aqueous extract of Maca) dog semen (n = 10) before freezing (fresh semen), post-thaw (T0) and after 1 (T1) and 2 h (T2) of incubation at 37°C.*

	Total motility (%)		**Progressive motility (%)**		**Hyperactive (%)**	
Fresh semen	86.4 (79.8-93)[a]		25.8 (20-39.7)[a]		2.8 (0.5-4.1)[a]	
Post-thaw	*Ctrl*	*Maca*	*Ctrl*	*Maca*	*Ctrl*	*Maca*
T0	31.2 (25.6-60.1) [b]	40.4 (28.5-58) [b]	7.4 (1.5-14.9) [a]	9.3 (0.6-23) [a]	0.5 (0-2.9) [a]	1.8 (0-6.7) [a]
T1	23.8 (15.9-36.3) [c]	33.1 (18-40.1) [*, c]	4.7 (0-7.9) [b]	5.5 (0.4-12.1) [a]	1.9 (0-11.1) [a]	6.8 (1.7-16.9) [b]
T2	25.6 (12.7-32.8) [c]	26.8 (10.5-42) [c]	0.5 (0-7.1) [b]	3.6 (0.4-9.6) [a]	4.3 (0-12.3) [a]	10 (0.7-17.9) [b]
	Rapid (%)		**Medium (%)**		**Slow (%)**	
Fresh semen	16.4 (4.6-32) [a]		26.2 (23.2-29.8) [a]		35.8 (32.1-45.4)[a]	
Post-thaw	*Ctrl*	*Maca*	*Ctrl*	*Maca*	*Ctrl*	*Maca*
T0	5.7 (0-16.1) [a]	8.5 (1.7-20.7) [a]	2.1 (1.6-6.5) [b]	4.1 (2.6-6.9) [b]	25.5(19.4-32.2) [a]	26.9 (21.3-29.1) [a]
T1	4.7 (0-10.6) [a]	4.5 (1.4-16.1) [a]	1.4 (0.4- 2.8) [b]	2.3 (0.7-4) [*, b]	17.7 (12.2-22.1) [b]	19.2 (13-25.5) [b]
T2	1.1(0-10) [a]	4.6 (0.4-15.8) [a]	0.9 (0-2.1) [b]	2.1 (0.2-3.7) [b]	19.5 (11.9-22.3) [b]	21 (9.8-23.8) [b]
	VCL (µm/s)		**VSL(µm/s)**		**VAP(µm/s)**	
Fresh semen	70.8 (57.5-82.9) [a]		40.1 (33.4-46.6)[a]		50.5 (45.8-60.9)[a]	
Post-thaw	*Ctrl*	*Maca*	*Ctrl*	*Maca*	*Ctrl*	*Maca*
T0	51.4 (28.7-71.8) [a]	26.9 (15.8-33.7)[a]	34.2(20.1-40.9)[b]	40.1 (24-52.7) [b]	25.5(19.4-32.2) [a]	26.9 (21.3-29.1) [a]
T1	49.9 (25.2-77.6) [a]	21.1 (5.9-31)[ab]	28 (10.5-42.1)[b]	31.7 (15.7-50.3) [b]	17.7 (12.2-22.1) [b]	19.2 (13-25.5) [b]
T2	33.5 (24-79.7) [a]	9.3 (4.3-34.1) [b]	15.3 (8.9-46.1)[b]	29.5 (11.7-48.6) [b]	19.5 (11.9-22.3) [b]	21 (9.8-23.8) [b]
	LIN (%)		**STR (%)**		**WOB(%)**	
Fresh semen	51.5 (49.9-53.6) [a]		70.1 (66.9-73.7) [a]		70.7 (66.5-72.4)[a]	
Post-thaw	*Ctrl*	*Maca*	*Ctrl*	*Maca*	*Ctrl*	*Maca*
T0	37.2 (33.1-46.2) [b]	46 (23.1-51.7) [a]	64.1 (57-67) [a]	70.2 (52.5-71.9) [a]	55.2 (50.3-63.2) [b]	62.9 (41.8-66.9) [b]
T1	27.2 (17.7-32.4) [c]	30.8 (16.8-34.4) [a]	57.1 (48.1-59.8)[a]	59 (38.5-62.9) [a]	43.4 (36.9-51.4) [c]	48.4 (44.6 -53.1)[*b]
T2	24.8 (9.9-33.3) [c]	23.1 (15.1-34.4) [a]	55 (35.8-61.6) [a]	53.9 (47.8-64.3) [a]	46 (31.4-51.1) [c]	43.5 (31.3-51.5) [b]
	ALH (µm)		**BCF (beat/s)**			
Fresh semen	1.7 (1.3-1.9) [a]		11.4 (9.1-12.7) [a]			
Post-thaw	*Ctrl*	*Maca*	*Ctrl*	*Maca*		
Post	1.3 (0.9-1.7) [a]	1.5 (1.1-1.8) [a]	6.1 (4.6-8.9) [a]	7.9 (4.6-11.5) [a]		
T1	1.4 (0.9-1.9) [a]	1.4 (1.2-2) [a]	5 (2-7.6) [a]	5.8 (3-8.7) [a]		
T2	1.1 (0.9-2) [a]	1.4 (0.9-2.3) [a]	3.4 (0.9-7.6) [a]	5.2 (2-7.7) [a]		

All values are expressed as median and interquartile range (IQR); asterisk indicates statistical difference between groups at p≤ 0.05; the letters indicate statistically differences at p≤ 0.05 between time points within each group.

As shown in Fig. 1a, the percentage of viable sperm with HMMP decreased at post-thaw (T0) and at T1 compared to fresh semen, with a further decrease recorded at T2 in both groups (P<0.05). Viable sperm with LMMP did not differ between fresh and post-thaw (T0) semen in both groups. However, a decrease of viable sperm with LMMP was observed in ctrl group between T0 and T1 and in Maca group between T1 and T2 (Fig. 1b). No differences were found between Maca and ctrl groups at any time points.

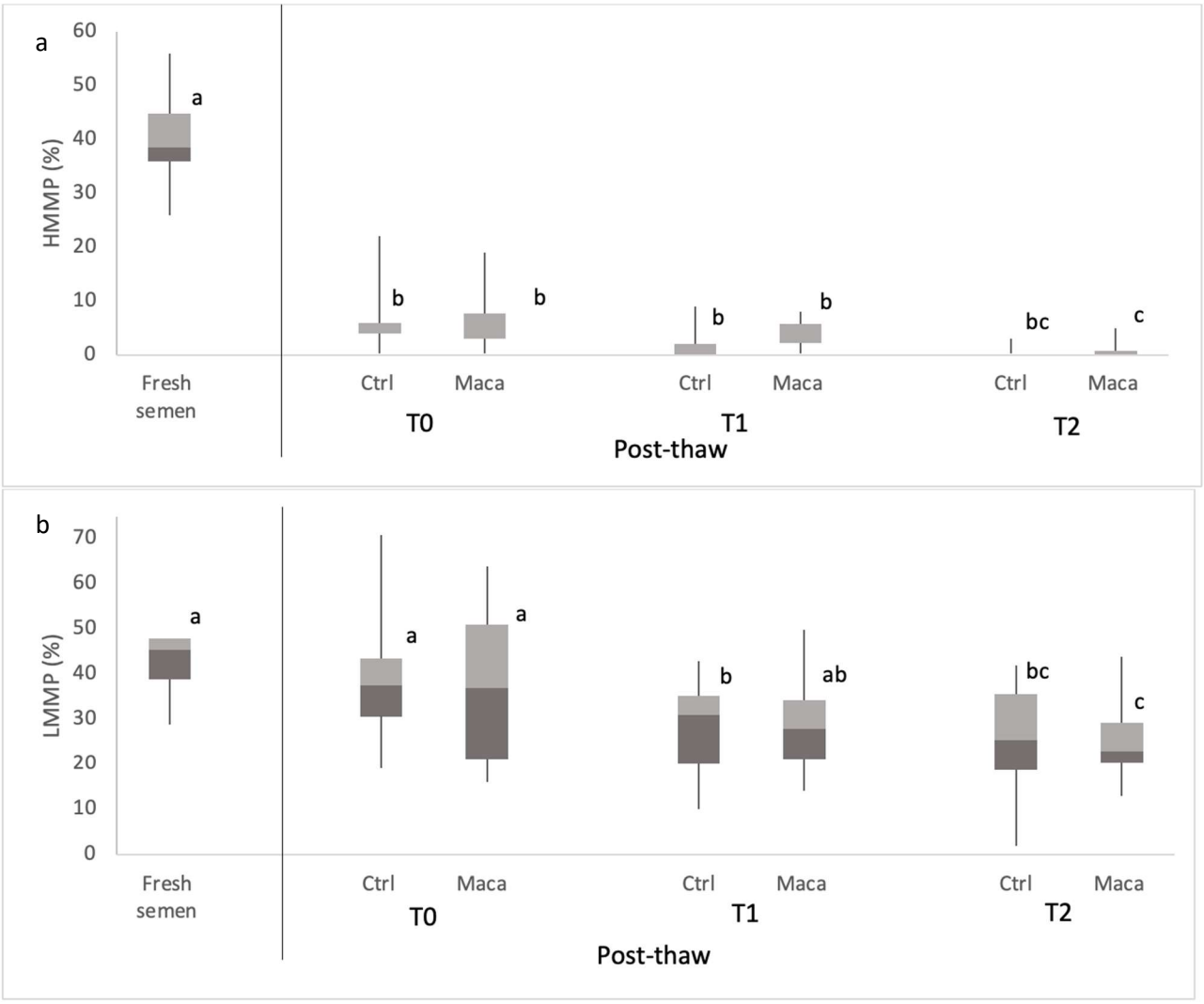

Fig. 1 The percentage of viable sperm with high mitochondrial potential (HMMP: SYBR-14+/PI-/JC-1+) (a) and with low mitochondrial membrane potential (LMMP; SYBR-14+/PI-/JC-1-) (b) in ctrl and Maca-treated dog semen (n = 10) at different time points, before freezing (fresh semen), immediately after thawing (T0) and after 1 (T1) and 2 h (T2) of incubation at 37°C. For each

box, the central line represents the median, the edges represent the IQR (25th and 75th percentiles), the whiskers represent the extreme points; the letters (a-c) indicate significant differences at p≤ 0.05 between time points and groups.

As shown in Fig. 2, lipid peroxidation did not differ between fresh and post-thaw (T0) semen in both groups; however, the MDA concentration at T0 was lower in Maca-treated semen than in the ctrl ($p<0.05$).

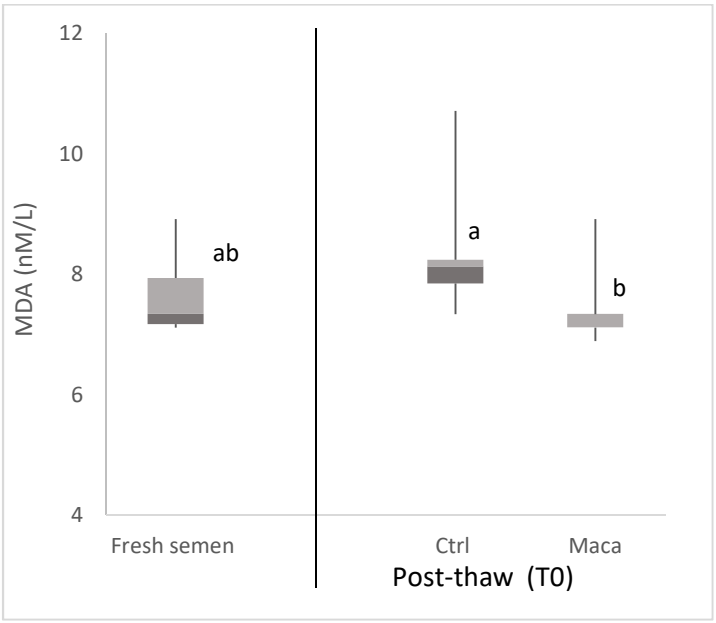

Fig. 2 Lipid peroxidation before freezing (Fresh semen) and immediately post-thaw (T0) in Control (Ctrl) and Maca-treated (Maca) dog semen (n = 10). For each box, the central line represents the median, the edges represent the IQR (25th and 75th percentiles), the whiskers represent the extreme points; the letters (a,b) indicate significant differences at p≤ 0.05 between time points and groups.

Discussion

This study showed a potential beneficial effect of Maca on sperm cryotolerance, indicated by reduced post-thaw lipid peroxidation, increased total motility, medium velocity and WOB after 1 h post-thawing incubation, as well as by the pattern of temporal decrease of other quality-related parameters.

Sperm motility is the most commonly used indicator of semen quality before and after freezing-thawing process (Martìnez 2004). Motility is expression of structural and functional competence of spermatozoa, and it is necessary to reach and colonize the oviduct (Scott 2000). A beneficial effect of Maca on sperm motility of refrigerated canine semen was previously recorded (Del Prete et al. 2022). Likewise, it was reported that Maca improves motility of human sperm (Leiva-Revilla et al. 2022) and frozen-thawed bovine sperm (Aoki et al. 2019).

An interesting finding was the increase in hyperactivation and WOB of canine frozen-thawed sperm after 1 hour incubation at 37°C when Maca was used. It has been suggested that hyperactivated bovine sperm with high WOB are able to efficiently progress in a fluid similar to cervical and oviductal mucus, reaching the oocyte more quickly (Hyakutake et al. 2018). Therefore, we speculate that the use of a Maca-supplemented extender for canine semen cryopreservation, due to the increased hyperactivated spermatozoa, may improve fertilization rates. Meanwhile, if the hyperactivation is acquired too early, the sperm cells drain their energy and risk not reaching the oocyte, reducing the efficiency of *in vivo* fertilization. Improved sperm hyperactivation was already reported 3 hours after the addition of the same aqueous extract of Maca in cooled semen (Del Prete et al. 2022). This effect may be due to the presence of alkaloids in Maca known to increase cyclic adenosine monophosphate and intracellular calcium, important for the regulation of sperm movement (Wang et al. 2009).

Sperm motility depends on the energy supply provided by either oxidative phosphorylation or glycolysis and is correlated to inner mitochondrial potential in stallions and humans (Love et al. 2003). A decrease of mitochondrial membrane potential has been considered an early sign of apoptosis (Martin et al. 2004). In the present study freezing decreased sperm motility, that was associated with a decreased proportion of viable sperm with high mitochondrial membrane potential (HMM). This is in contrast to an earlier study, in which low sperm motility was found to be unexpectedly associated with a high proportion of sperm with HMM (Volpe et al. 2009).

Another interesting result of this study is the protective role of Maca against lipid membrane peroxidation of canine spermatozoa. Lipid peroxidation is caused by an increased production of ROS during the cryopreservation procedure (Lucio et al. 2016) and is a known marker of oxidative stress. Due to the high content of polyunsaturated fatty acids in

the membrane, dog spermatozoa are highly sensitive to lipid peroxidation and less resistant to cooling (Bencharif et al. 2008). Our results agree with those of a previous study reporting the beneficial effects of Maca on the plasma membrane of canine cooled sperm (Del Prete et al. 2022). This effect may be due to the antioxidant activity of phenolic compounds and specific alkamides contained in Maca (Tafuri et al. 2019). Phenols act as effective inhibitors of peroxidation, by chelating redox-active metal ions and inhibiting free-radical mediated events (Rice-Evans 2001). Maca-specific alkamides called 'Macamides' scavenge free radicals and thus protect sperm cells from oxidative damage (Tafuri et al. 2019).

In conclusion, the supplementation of the extender with 10 µl/mL of aqueous extract of Maca prior freezing could ameliorate the cold shock resistance of spermatozoa, improving the fertility of frozen-thawed canine sperm, but further studies are indeed required to investigate the effects on post-thaw quality and fertilizing ability.

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Authors' contributions

NC, BM, CDP: conceptualization and methodology; CDP, AC, SS, EI, RC, SD, EG and FL: investigation and data curation. CDP: statistical analysis and writing; NC, BM and BG: review, supervision and visualization; AC: editing of the manuscript. All authors read and approved the final manuscript.

Statements and Declarations

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Competing Interests

Ethics approval

All procedures were carried out in compliance with the code of ethics (D.lgs. 26 - 04/03/2014) and were approved by the Ethics Committee of the Department of Veterinary Medicine and Animal Productions at the University of Naples Federico II, Italy (prot. no. PG/2021/0057934 of 07/06/2021).

Consent to participate

A written inform consent was signed by the legal representative of the breeding center.

Consent to publish

Not applicable.

Conflict of interest

The authors have declared that there is no conflict of interest concerning this study.

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