



EFFECT OF SUPPLEMENTATION OF FREEZING MEDIA WITH ISOESPINTANOL ON FUNCTIONAL INTEGRITY OF EQUINE SEMEN

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Abstract

Adding antioxidants to semen extender has become one of the major strategies to reduce the damage caused by freezing to stallion sperm. Isoespintanol (Iso) is a natural antioxidant that is known as a good free radical scavenger. The aim of this research was to evaluate the effect of supplementation of freezing media with Iso on functional integrity of equine semen. Semen from five Colombian Creole horses was frozen with 0, 10, 20, 30, 40 and 50 μ M Iso. Post-thawing motility and kinetics, structural membrane integrity (SMI), normal morphology (NM) and functional membrane integrity (FMI) of sperm were evaluated. Seminal quality (SQi) and seminal velocity (SVi) indexes were calculated. Through a flow cytometric analysis, mitochondrial membrane potential ($\Delta\Psi$ M), viability and DNA integrity of frozen-thawed semen were assessed. General linear models were adjusted, and means comparisons were performed using Tukey's test. The significance level was $P < 0.05$. No differences for post-thaw sperm quality parameters were found, except for total motility, which showed a reduction by adding 40 μ M Iso ($P < 0.05$). However, the estimation of indexes showed that supplementation with 20 or 30 μ M Iso before semen freezing, can increase post-thaw SQi and SVi values of some horses. A higher proportion of high- $\Delta\Psi$ M sperm for 20 μ M Iso was found. It is concluded that Iso can improve the quality and mitochondrial activity of frozen-thawed equine semen, however its effect can vary according to the horse and in high concentrations it could have detrimental effects.

Key words: cryopreservation, antioxidants, semen analysis, mitochondria, stallion

Significant progress in the process of frozen-thawed stallion semen and, consequently, fertility has been achieved over the last decade (Alvarenga et al., 2016). However, compared to other mammalian species, stallion semen has a greater sensitivity in the freezing process, showing higher rates of oxidative stress and plasma membrane damage (Contreras et al., 2020). Spermatozoa are susceptible to attack by oxidative stress because of their deficiency in the levels of intracellular antioxidant enzymes that is a result of their low amount of cytoplasm (Ghallab et al., 2017). Furthermore, these freezing procedures are also known to reduce the enzymatic and nonenzymatic antioxidants of semen (Filho et al., 2009). Therefore, adding antioxidants to the semen extender has become a major strategy to reduce the damage caused by freezing (Shojaeian et al., 2018; de Albuquerque Lagares et al., 2020). In natural antioxidants and organic substances with antioxidant properties, some studies

have found an alternative to improve the post-thaw parameters of stallion semen (Nouri et al., 2018; Nasirabadi et al., 2019). Other research has focused on the effect of antioxidants in low-quality semen or poor freezer stallions (Nouri et al., 2018; Neuhauser et al., 2019), because some stallions appear to be more or less susceptible than others to cryoinjuries during standard semen cryopreservation, so there is a need to develop new strategies to improve sperm cryopreservation, mainly for stallions with poor sperm freezing ability (Carneiro et al., 2018).

The current research evaluates the use of isoespintanol (Iso; 2,5-dimethoxy-3-methyl-6-propan-2-ylphenol), a natural antioxidant, for freezing stallion semen. Iso is extracted from *Oxandra cf xylopioides* (Annonaceae) leaves, it is known as a good free radical scavenger and for its ability to inhibit lipid peroxidation and strengthen antioxidant enzymatic action (Rojano et al., 2008 b). In a previous study, Iso had positive results on motility, vi-

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tality and membrane integrity of frozen-thawed stallion sperm (Restrepo and Rojano 2018 a). Furthermore, Iso has the ability to reduce ROS production in stallion semen (Restrepo and Rojano, 2018 b). However, little is known about the effect of this molecule on other aspects, such as kinetics, mitochondrial membrane potential and DNA integrity of stallion semen. The aim of this research was to evaluate the effect of supplementation of freezing media with isoespintanol on functional integrity of equine semen.

Material and methods

Animals and semen collection

The present study included five Colombian Creole horses (*Equus caballus*) located in Antioquia (Colombia). Two ejaculates were collected from each horse a week apart, for a total of 10 weeks. The animals were active breeding stallions whose age ranged from 4 to 10 years, their fertility had been confirmed by live offspring and their body condition scores ranged from 7 to 8 (using a scale from 1 to 9). Semen samples were collected using a teaser mare and a Missouri model artificial vagina (Minitube, Tiefenbach, Germany). The gel fraction of the ejaculate was removed through filtration, and the sperm was diluted at a ratio of 1:2 using an EquiPlus extender (Minitube, Tiefenbach, Germany) at 32°C. Before processing, the sperm was transported for 2 h at 5°C in an Equitainer (Hamilton Biovet, Ipswich, MA, USA). This research was carried out in compliance with the regulations for animal experimentation, established by the ethics committee of the Politécnico Colombiano Jaime Isaza Cadavid. The investigation was in accordance with Colombian Law 84 of December 1989, Chapter VI (On the use of live animals in experiments and research), Article 26 and Law 1774 of 2016, Article 1.

Semen freezing and thawing

Collected ejaculates were centrifuged at 800 X g for 10 minutes (Mikro 220R; Hettich, Vlotho, Germany). Then, the pellet was resuspended using an EquiPlus extender (Minitube, Tiefenbach, Germany) supplemented with 5% centrifuged egg yolk (CEY) and 5% N,N-dimethylformamide (Sigma-Aldrich, Eugene, OR, USA) until reaching a concentration of 100×10^6 spermatozoa/ml (Usuga et al., 2018). The CEY was obtained according to the methods given by Nouri et al. (2013). Iso was extracted from an ether extract of *Oxandra cf xylopioides* as a crystalline solid, then it was treated with 2.0 mL of acetic anhydride-pyridine (1: 1) for 24 hours. The mixture was diluted with distilled water and extracted three times with CH_2Cl_2 . It was concentrated under reduced pressure and the acetylated compound purified on silica gel eluting with petroleum ether- CH_2Cl_2 (8: 2) (Rojano et al., 2008 a). Using gas chromatography – mass spectrometry, an Iso purity > 99% has been determined (González-Arbeláez et al., 2020). The same isoespintanol batch was

used in the study. The sperm suspension was split into six aliquots, which were randomly assigned to one of six treatments: 0 (control), 10, 20, 30, 40 and 50 μM Iso; these were then packaged in 0.5 mL straws (MRS1 Dual V2; IMV Technologies, L'Aigle, France). The straws were cooled at 5°C for 30 minutes in a conventional refrigerator and then exposed to liquid nitrogen vapours for 15 min, using a straw rack located 4 cm above the level of liquid nitrogen added to a styrofoam box. Finally, the straws were stored in a liquid nitrogen tank (IMV Technologies, L'Aigle, France). After a week of storage, the cryopreserved straws were thawed by plunging them into a water bath at 37°C for 1 min.

Semen quality assessment

Sperm motility and kinetics were assessed by the Sperm Class Analyzer computer system (Microptic S.L, Barcelona, Spain). An Eclipse E200 phase-contrast microscope (Nikon Inc., Tokyo, Japan) with a digital camera (Scout SCA780; Basler) was used. A specific configuration was established for the software: 20 x 20 mm glass slide, optics in ph (–), drop of 7 mL, equine species, thermal plate at 37°C and a particle size of 20 to 72 mm, capture velocity 25 fps. A minimum of 500 spermatozooids were evaluated in five observation fields. The structural membrane integrity (SMI) was evaluated using a Live/Dead Sperm Viability kit (Invitrogen, Eugene, OR, USA) (Gamboa et al., 2010). A drop of 50 μL of semen was mixed with 0.3 μL of SYBR-14 at a final concentration of 6 μM and incubated for 8 min at 35°C. Following this, propidium iodide (PI) (0.48 mM) was added to the mixture and incubated for 8 min at 35°C. Samples of 7 μL of semen were placed on a glass slide and covered with a cover slip. Then, 200 spermatozoa were counted using an Eclipse E200 microscope with HBO fluorescence (Nikon Inc., Tokyo, Japan). The normal morphology (NM) was assessed using the modified eosin–nigrosin test (Brito et al., 2011). A droplet of semen and a droplet of eosin–nigrosin (Sigma-Aldrich, Eugene, OR, USA) were placed on a microscope slide, mixed, smeared and placed on a warming plate at 37°C. Subsequently, 200 spermatozoa were assessed individually using an Eclipse E200 phase-contrast microscope (Nikon Inc., Tokyo, Japan). The functional membrane integrity (FMI) was assessed using the hypo-osmotic swelling test (HOST) (Neild et al., 1999). To provide a concentration of 100 mOsmol/L, 20 μL of semen were added to 200 μL of a hypo-osmotic 5.4% sucrose solution. This mixture was incubated at 38.5°C for 30 min. Then, 200 spermatozoa were evaluated using an Eclipse E200 phase-contrast microscope (Nikon Inc., Tokyo, Japan).

Flow cytometric assessment of frozen-thawed semen

Mitochondrial membrane potential ($\Delta\psi\text{M}$) was assessed with an adapted protocol (Zamzami et al., 1996). A tube was used to deposit 10 μL of frozen-thawed semen, 250 nM of 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3), Invitrogen D273, Eugene, OR, USA) and

1 mg/mL of propidium iodide (PI, Invitrogen, Eugene, OR, USA) was added. Sperm viability was evaluated with a combination of SYBR-14 (0.2 µM) and PI (1 mg/mL) (Live/Dead Sperm Viability Kit; Invitrogen L7011, Eugene, OR, USA), that were added to 10 µL of frozen-thawed semen in 500 µL of phosphate buffered saline (PBS) (Vasconcelos-Franco et al., 2014). The DNA integrity was evaluated with the combination of the fluorochromes acridine orange (AO, Invitrogen, Eugene, OR, USA) and PI (Invitrogen, Eugene, OR, USA) using an adapted protocol (Lazaros et al., 2011). A stock solution of AO was prepared at 16.6 µg/mL in PBS. Then, 0.5 µL of AO was added to 10 µL of frozen-thawed semen in 500 µL PBS for a final AO concentration of 0.016 µg/mL. Additionally, 10 µL of PI were added for a final concentration of 1 mg/mL. Sperm DNA fragmentation was determined using a solid phase laser at 488 nm. AO fluorescence was detected at 530/15 nm for nonfragmented DNA and 585/42 nm for fragmented DNA. For the three tests, an LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA, USA) was used, samples were incubated in the dark at 37°C for 20 min, 10,000 events were evaluated, and flow cytometry data were analyzed using FlowJo version 10.5 (FlowJo, LLC, USA) software.

Data analysis

General linear models were fitted for each dependent variable and fixed effects of the ejaculate nested within the equine (Ej(Eq)) and treatment were included. Calculation of seminal indexes was performed to comprehensively express the sperm quality obtained from the individual parameters. A seminal quality index (SQi) was calculated and expressed as mean and per horse, according to the following formula: $SQ_i = \sum Sp / 100n$, where Sp (sperm parameter) is each of the parameters evaluated in semen and n is the number of parameters (Ortiz et al., 2015). For kinetic parameters, a seminal velocity index

(SVi) was calculated. For this, the method reported by Núñez-Martínez et al. (2006) was used. A principal component analysis (PCA) was performed, from which the eigenvectors to weigh each variable were found to establish the SVi equation. The normal distribution of the variables was validated with the Kolmogorov–Smirnov test. A total of 120 straws of frozen-thawed semen were evaluated. Means were compared by Tukey's test. Results were expressed as mean ± standard error of the mean (SEM). The significance level used for all assessments was $P < 0.05$. All analyses were conducted using SAS Version 9.2. Software (SAS Inst. Inc., Cary, NC, USA).

Results

Isoespintanol effect on post-thaw semen quality

The results for the mean quality of fresh equine semen from the ten collected ejaculates were: total motility (TM) of $91.7 \pm 1.7\%$, progressive motility (PM) of $57.6 \pm 4.4\%$, SMI of $80.4 \pm 4.5\%$, NM of $75.4 \pm 7.9\%$ and FMI of $57.2 \pm 2.1\%$. The coefficients of variation for all fresh semen quality parameters were below 25%. The stallion influenced the variability of the sperm parameters because the Ej(Eq) effect was significant in all statistical models ($P < 0.05$). For the seminal quality assessment, no differences for the mean of post-thaw individual parameters were found, except for TM, which showed a reduction by adding 40 µM Iso ($P < 0.05$) (Table 1).

The eigenvectors were derived from the component of the first principal component, which explained 74% of the variance. The resulting equation based on the PCA was as follows:

$$\text{Seminal velocity index (SVi)} = (VCL \times 0.503) + (VAP \times 0.495) + (VSL \times 0.505) + (ALH \times 0.132) + (BCF \times 0.477)/100$$

Table 1. Post-thaw quality of frozen-thawed semen supplemented with isoespintanol

	0 µM	10 µM	20 µM	30 µM	40 µM	50 µM
TM	61.58 ± 4.32 ab	54.95 ± 4.47 abc	57.84 ± 4.69 abc	63.65 ± 4.03 a	48.45 ± 4.35 c	51.38 ± 4.12 bc
PM	20.38 ± 3.86 a	19.20 ± 2.97 a	22.29 ± 3.22 a	23.12 ± 3.10 a	20.11 ± 3.55 a	21.02 ± 3.71 a
VCL	48.98 ± 3.43 a	47.48 ± 2.78 a	51.21 ± 3.51 a	50.72 ± 3.29 a	49.23 ± 3.26 a	51.84 ± 3.82 a
VAP	28.38 ± 2.87 a	25.69 ± 2.26 a	28.62 ± 2.78 a	28.70 ± 2.61 a	27.75 ± 2.69 a	29.24 ± 3.09 a
VSL	20.48 ± 2.21 a	20.08 ± 1.85 a	21.90 ± 2.34 a	22.06 ± 2.10 a	21.39 ± 2.17 a	22.70 ± 2.65 a
ALH	3.12 ± 0.83 a	2.17 ± 0.07 a	2.28 ± 0.10 a	2.46 ± 0.21 a	2.19 ± 0.08 a	2.28 ± 0.09 a
BCF	6.35 ± 0.56a	6.50 ± 0.50 a	6.88 ± 0.50 a	6.80 ± 0.51 a	6.67 ± 0.49 a	7.12 ± 0.56 a
SMI	46.59 ± 3.10 ab	45.59 ± 3.71 ab	49.95 ± 3.32 a	50.86 ± 2.71 a	43.50 ± 2.46 ab	42.41 ± 3.30 b
NM	75.36 ± 2.03 a	77.55 ± 1.40 a	75.14 ± 2.05 a	74.36 ± 1.84 a	75.36 ± 1.43 a	75.59 ± 2.04 a
FMI	31.45 ± 3.26 a	32.50 ± 2.90 a	33.73 ± 2.72 a	34.23 ± 2.53 a	32.05 ± 2.16 a	31.68 ± 3.19 a

TM: total motility. PM: progressive motility. VCL: curvilinear velocity. VAP: average path velocity. VSL: straight line velocity. ALH: lateral head displacement. BCF: beat cross frequency. SMI: structural membrane integrity. NM: normal morphology. FMI: functional membrane integrity. Results are shown as mean ± standard error of the mean (SEM). Different letters between treatments (rows) indicate statistical difference ($P < 0.05$).

Seminal indexes showed that supplementation with 20 or 30 μM Iso before semen freezing, increased the post-thaw SQi and SVi values in two horses, while in another horse the higher concentrations of Iso produced a reduction of SQi. Likewise, a decrease of the mean SQi values was observed for the highest concentrations of Iso, although it was only statistically lower for 40 μM Iso ($P < 0.05$) (Table 2).

Flow cytometric assessment of frozen-thawed semen

Frozen-thawed semen evaluation showed that Iso influences the mitochondrial activity of sperm; thus, a higher proportion of cells with high- $\Delta\Psi\text{M}$ for 20 μM and with low- $\Delta\Psi\text{M}$ for 10 μM compared with the control were found ($P < 0.05$) (Table 3). Figure 1 presents examples of the flow cytometric analysis of $\Delta\Psi\text{M}$ of frozen-thawed semen.

Table 2. Indexes for quality evaluation of frozen-thawed semen supplemented with isoespintanol

	Index	0 μM	10 μM	20 μM	30 μM	40 μM	50 μM
Equine 1	SQi	0.53 $\pm$ 0.05 a	0.52 $\pm$ 0.05 a	0.52 $\pm$ 0.04 a	0.53 $\pm$ 0.04 a	0.53 $\pm$ 0.03 a	0.53 $\pm$ 0.04 a
	SVi	0.58 $\pm$ 0.09 a	0.56 $\pm$ 0.11 a	0.59 $\pm$ 0.10 a	0.61 $\pm$ 0.08 a	0.56 $\pm$ 0.09 a	0.59 $\pm$ 0.09 a
Equine 2	SQi	0.59 $\pm$ 0.01 a	0.53 $\pm$ 0.02 ab	0.55 $\pm$ 0.03 ab	0.58 $\pm$ 0.03 ab	0.48 $\pm$ 0.03 b	0.50 $\pm$ 0.01 ab
	SVi	0.86 $\pm$ 0.02 ab	0.69 $\pm$ 0.02 b	0.83 $\pm$ 0.06 ab	0.83 $\pm$ 0.05 ab	0.82 $\pm$ 0.03 ab	0.92 $\pm$ 0.05 a
Equine 3	SQi	0.40 $\pm$ 0.04 b	0.41 $\pm$ 0.06 ab	0.42 $\pm$ 0.06 ab	0.49 $\pm$ 0.03 a	0.37 $\pm$ 0.03 b	0.37 $\pm$ 0.03 b
	SVi	0.46 $\pm$ 0.04 a	0.41 $\pm$ 0.03 a	0.42 $\pm$ 0.01 a	0.43 $\pm$ 0.01 a	0.44 $\pm$ 0.04 a	0.44 $\pm$ 0.04 a
Equine 4	SQi	0.41 $\pm$ 0.04 b	0.42 $\pm$ 0.02 b	0.49 $\pm$ 0.05 a	0.43 $\pm$ 0.02 b	0.44 $\pm$ 0.02 ab	0.47 $\pm$ 0.02 ab
	SVi	0.43 $\pm$ 0.03 b	0.57 $\pm$ 0.05 ab	0.64 $\pm$ 0.05 a	0.60 $\pm$ 0.06 a	0.54 $\pm$ 0.07 ab	0.59 $\pm$ 0.05 ab
Equine 5	SQi	0.44 $\pm$ 0.03 a	0.44 $\pm$ 0.04 a	0.42 $\pm$ 0.04 a	0.44 $\pm$ 0.02 a	0.44 $\pm$ 0.02 a	0.39 $\pm$ 0.05 a
	SVi	0.32 $\pm$ 0.02 a	0.33 $\pm$ 0.03 a	0.31 $\pm$ 0.03 a	0.32 $\pm$ 0.02 a	0.32 $\pm$ 0.01 a	0.30 $\pm$ 0.02 a
Mean	SQi	0.47 $\pm$ 0.02 abc	0.46 $\pm$ 0.02 abc	0.48 $\pm$ 0.02 ab	0.49 $\pm$ 0.01 a	0.43 $\pm$ 0.01 c	0.44 $\pm$ 0.02 b
	SVi	0.52 $\pm$ 0.04 ab	0.50 $\pm$ 0.03 b	0.55 $\pm$ 0.04 ab	0.54 $\pm$ 0.04 ab	0.53 $\pm$ 0.05 ab	0.56 $\pm$ 0.05 a

SQi: seminal quality index. SVi: seminal velocity index. Results are shown as mean $\pm$ standard error of the mean (SEM). Different letters between treatments (rows) indicate statistical difference ($P < 0.05$).

Table 3. Mitochondrial membrane potential ($\Delta\Psi\text{M}$) of frozen-thawed semen supplemented with isoespintanol

	0 μM	10 μM	20 μM	30 μM	40 μM	50 μM
High- $\Delta\Psi\text{M}$ (%)	19.34 $\pm$ 1.30 b	20.27 $\pm$ 1.53 ab	22.14 $\pm$ 1.48 a	19.85 $\pm$ 1.03 ab	19.95 $\pm$ 1.26 ab	20.41 $\pm$ 1.75 ab
Medium- $\Delta\Psi\text{M}$ (%)	3.82 $\pm$ 1.27 a	3.70 $\pm$ 0.62 a	2.78 $\pm$ 0.51 a	3.53 $\pm$ 0.69 a	2.83 $\pm$ 0.52 a	4.20 $\pm$ 1.72 a
Low- $\Delta\Psi\text{M}$ (%)	0.11 $\pm$ 0.03 b	0.26 $\pm$ 0.07 a	0.11 $\pm$ 0.02 b	0.11 $\pm$ 0.03 b	0.10 $\pm$ 0.02 b	0.11 $\pm$ 0.02 b
PI+ (%)	64.08 $\pm$ 2.09 a	64.08 $\pm$ 2.01 a	64.31 $\pm$ 1.87 a	65.14 $\pm$ 2.00 a	66.36 $\pm$ 2.06 a	63.79 $\pm$ 2.30 a

High- $\Delta\Psi\text{M}$: spermatozoa with high mitochondrial membrane potential. Medium- $\Delta\Psi\text{M}$: spermatozoa with medium mitochondrial membrane potential. Low- $\Delta\Psi\text{M}$: spermatozoa with low mitochondrial membrane potential. PI+: non-viable spermatozoa. Results are shown as mean $\pm$ standard error of the mean (SEM). Different letters between groups (rows) indicate statistical difference ($P < 0.05$).

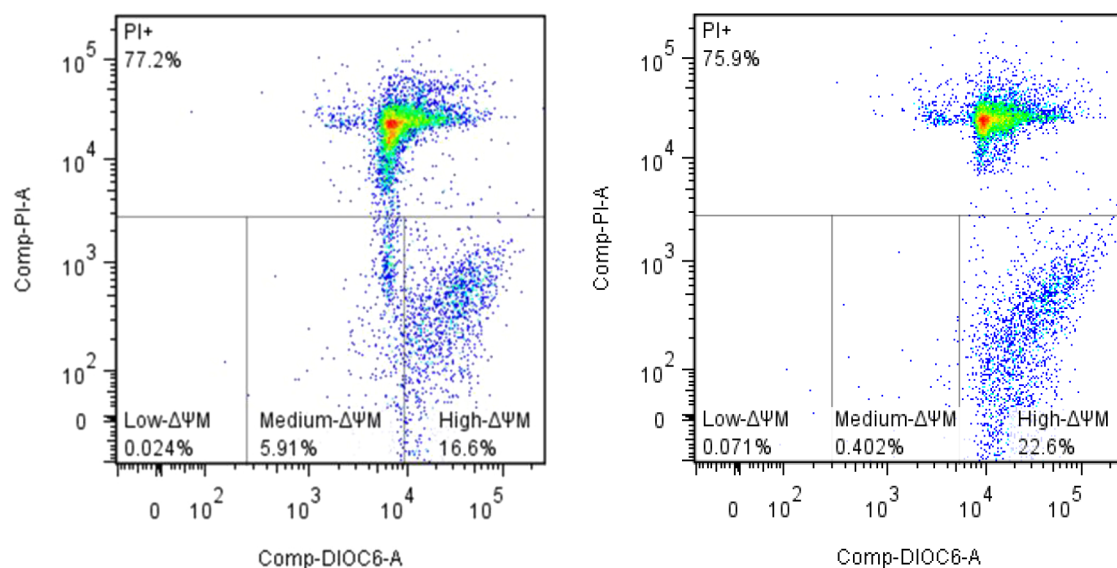
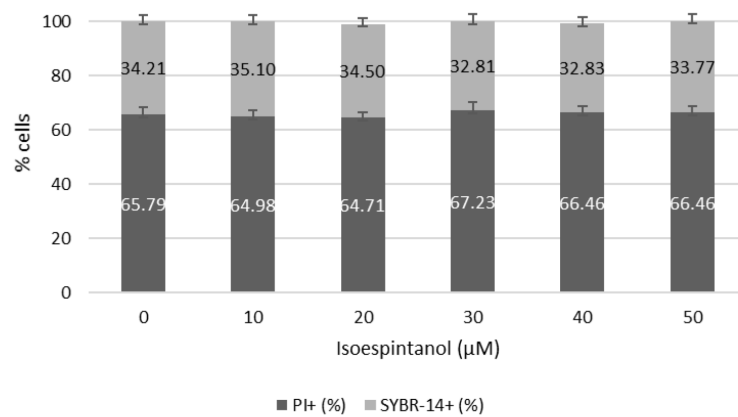
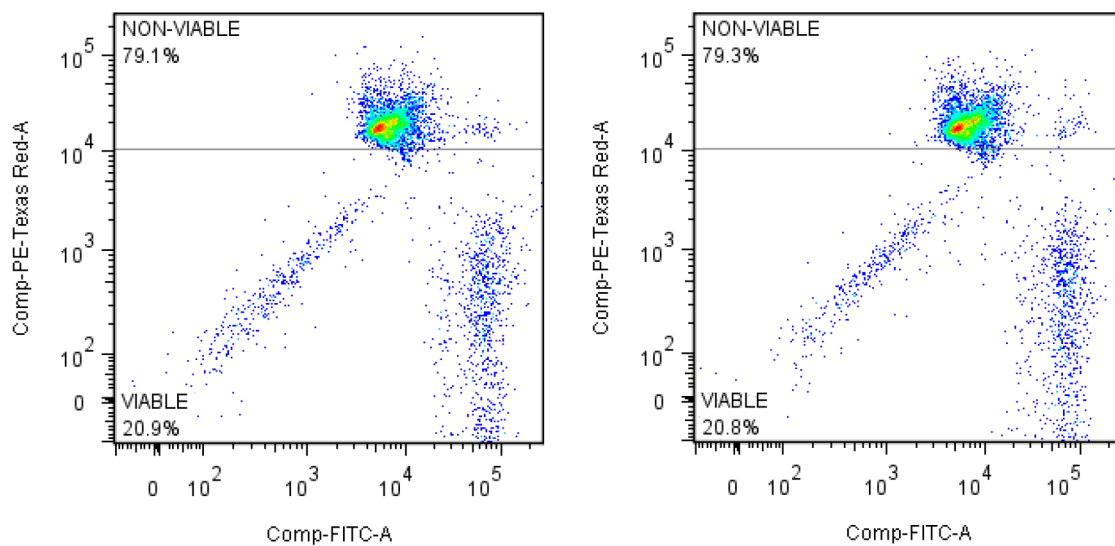


Figure 1. Flow cytometric analysis of mitochondrial membrane potential (DiOC6/PI) of frozen-thawed semen. Left: example of analysis of semen frozen without isoespintanol (control). Right: example of analysis of semen frozen with 20 μM of isoespintanol. Low- $\Delta\Psi\text{M}$: spermatozoa with low mitochondrial membrane potential. Medium- $\Delta\Psi\text{M}$: spermatozoa with medium mitochondrial membrane potential. High- $\Delta\Psi\text{M}$: spermatozoa with high mitochondrial membrane potential. PI+: non-viable spermatozoa



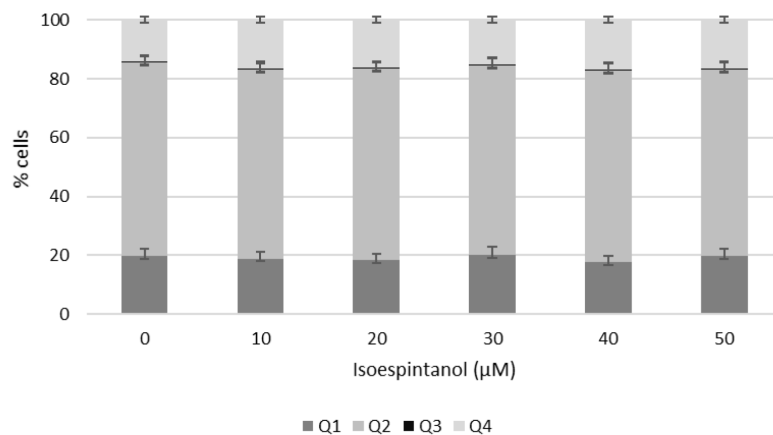
PI+: non-viable spermatozoa. SYBR-14+: viable spermatozoa. No statistical differences were found ($P > 0.05$).

Figure 2. Post-thaw viability of semen frozen with isoespintanol



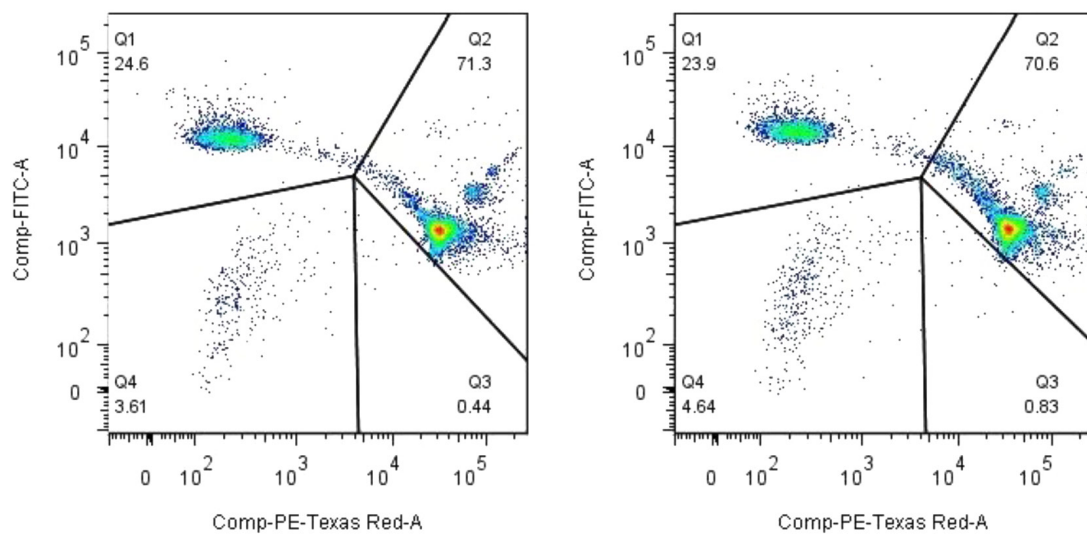
Left: example of viability analysis of semen frozen without isoespintanol (control). Right: example of viability analysis of semen frozen with 50 μM of isoespintanol. Non-viable: propidium iodide positive spermatozoa (PI+). Viable: SYBR-14 positive spermatozoa (SYBR-14+).

Figure 3. Flow cytometric analysis of viability (SYBR-14/PI) of frozen-thawed semen



DNA integrity was evaluated with acridine orange/propidium iodide dyes. Q1: viable spermatozoa with non-fragmented DNA. Q2: spermatozoa with fragmented DNA. Q3: abnormal spermatozoa. Q4: debris. No statistical differences were found ($P > 0.05$).

Figure 4. DNA integrity of semen frozen with isoespintanol



Left: example of DNA integrity analysis of semen frozen without isoespintanol (control). Right: example of DNA integrity analysis of semen frozen with 40 µM of isoespintanol. Q1: viable spermatozoa with non-fragmented DNA. Q2: spermatozoa with fragmented DNA. Q3: abnormal spermatozoa. Q4: debris.

Figure 5. Flow cytometric analysis of DNA integrity (AO/PI) of frozen-thawed semen

Iso had no effect on frozen-thawed sperm viability ($P > 0.05$) (Figure 2). Figure 3 shows examples of the flow cytometric analysis of the viability of frozen-thawed semen. Likewise, DNA integrity was not affected by the addition of Iso during sperm freezing ($P > 0.05$) (Figure 4). Similar levels of DNA fragmentation of frozen semen are shown in Figure 5.

Discussion

Numerous studies have suggested that sperm freeze-thaw cycles are associated with an increase in reactive oxygen species (ROS) production (Seifi-Jamadi et al., 2016). Supplementation with some antioxidants has been carried out to reduce the concentration of reactive species such as nitrite (NO_2^-), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radicals ($\cdot\text{OH}$) (Michael et al., 2007; de Albuquerque Lagares et al., 2020). The current study evaluated the use of Iso, a natural antioxidant to decrease oxidative stress caused by freezing, hence improving the post-thaw seminal quality. Iso has shown good antioxidant activity in assays such as ABTS⁺, DPPH, TBARS (lipid peroxidation), and a good antioxidant power expressed by its reducing ability $\text{Fe}^{3+}/\text{TPTZ}$ (Rojano et al., 2008 b).

The thawed semen quality results did not show a significant effect of Iso during the freezing of semen stallion, except for a reduction in TM by adding 40 µM Iso (Table 1). In this research, the calculation of seminal indexes allowed integrating the results of quality and kinetics of the spermatozoa. Previously, the importance of using indexes to express seminal quality and even freezability has been highlighted. The simultaneous use

of multiple parameters, including other aspects of sperm integrity and functionality, could be considered as a more objective method (Yeste et al., 2014). According to Ortiz et al. (2015), sperm-quality parameters can be improved and successfully integrated in the SQi. As has also been stated, the multivariate approach based on the use of computer-assisted sperm analysis (CASA)-derived data gives more information than the classical semen analysis based on medium values (Núñez-Martínez et al., 2006).

Seminal indexes showed that semen supplementation with 20 or 30 µM Iso, can increase the post-thaw SQi and SVi values in some horses, while higher concentrations of the antioxidant produced the reduction in the SQi of a stallion (Table 2). This result could be related to the highly intra-stallion variability in pre- and post-freezing parameters in horses (Ruiz et al., 2011). An investigation with canine semen also showed that there are significant differences between individuals for CASA-derived indexes used for the evaluation of sperm velocity and movement (Núñez-Martínez et al., 2006). Likewise, great variability is reported in the components and the antioxidant capacity of equine semen, both between horses and between ejaculates from the same animal, even this has been related to differences in the cryotolerance of sperm (Kareskoski and Katila, 2008; Gamal et al., 2016). Also, it has been reported that ejaculates from good and poor freezability stallion differ in their reactive oxygen species and superoxide dismutase (SOD) levels after cryopreservation (Yeste et al., 2015; Papas et al., 2019). Thus, according to the results obtained, it is possible that Iso only exerts a positive effect on freezing the semen of those horses or ejaculates with limited antioxidant activity.

The positive effect of Iso could be explained by its strong antioxidant activity, which has been attributed to its substituent effects and to the formation of intra- and intermolecular hydrogen bonds; furthermore, its antioxidant role could lie in the interruption of the second stage of the propagation chain of lipid oxidation by neutralising lipid radicals (ROO[•]) (Rojano et al., 2008 b). However, it was evident that higher levels of Iso supplementation can also affect the quality of thawed semen. Previously, it has been reported that molecules with antioxidant properties can have a pro-oxidant effect depending on its quantity and its neighbouring molecules (Carocho and Ferreira, 2013). Despite this, it has been reported that concentrations up to 80 µM Iso do not have genotoxic or cytotoxic effects (Marquez-Fernandez et al., 2018).

Stallion spermatozoa rely primarily on oxidative phosphorylation to generate the energy required for motility and the maintenance of membrane integrity (Papas et al., 2019). A recent study on isolated cardiac mitochondria found that Iso decreases mitochondrial calcium (Ca²⁺) uptake and attenuates mitochondrial permeability transition pore openings in the inner mitochondrial membrane, which in turn is related to mitochondrial swelling and dysfunction (González-Arbeláez et al., 2020). The increase of mitochondrial Ca²⁺ induces mitochondrial permeability transition (MPT), in which a decrease in $\Delta\Psi$ and cessation of $\Delta\Psi$ -dependent functions occurs (Treulen et al., 2018). In accordance with the above and with the findings of this investigation, where Iso produced an increase in $\Delta\Psi$ (Table 3), it is possible to infer that Iso might regulate Ca²⁺ uptake and ROS production in mitochondria, which could consequently modify ATP synthesis and sperm motility depending on the concentration used. Likewise, other processes mediated for changes in intracellular calcium levels such as capacitation (Graham et al., 2004), could explain the effects of Iso in sperm kinetics and SVi.

It is widely known that the cryopreservation of sperm cells has deleterious effects in the plasma and acrosome membrane integrity (Treulen et al., 2019). It is also known that oxidative stress in stallion semen triggers the activation of several cell damage pathways that ultimately culminate in DNA fragmentation and cell death (Contreras et al., 2020). A flow cytometric analysis showed that Iso had no effect on the viability (Figure 2) and DNA integrity (Figure 4) of sperm. In a previous study, Iso has shown a protective effect of viability as evaluated by eosin-nigrosine staining of stallion semen (Restrepo and Rojano, 2018 a). An investigation with human polymorphonuclear cells showed that Iso in concentrations between 1 and 100 µM has no negative effects on viability (Dade et al., 2016). Moreover, Iso can be considered a good alternative antioxidant because it has been reported that at concentrations between 3 and 1620 µM, it does not have clastogenic effects on DNA (Marquez-Fernandez et al., 2018).

Conclusions

Isoespintanol can improve the quality and mitochondrial activity of frozen-thawed equine semen, however its effect can vary according to the horse and in high concentrations it could have detrimental effects.

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References

- Albuquerque Lagares M. de, Silva G.C. da., Cortes S.F., Luz S.B., de Resende A.C., Alves N. de C., Wenceslau R.R., Stahlberg R. (2020). Does coenzyme Q10 exert antioxidant effect on frozen equine sperm? *J. Equine Vet. Sci.*, 88: 102964.
- Alvarenga M.A., Papa F.O., Ramires Neto C. (2016). Advances in stallion semen cryopreservation. *Vet. Clin. North. Am. - Equine Pract.*, 32: 521–530.
- Brito L.F.C., Greene L.M., Kelleman A., Knobbe M., Turner R. (2011). Effect of method and clinician on stallion sperm morphology evaluation. *Theriogenology*, 76: 745–750.
- Carneiro J.A.M., Canisso I.F., Bandeira R.S., Scheeren V.F.C., Freitas-Dell'Aqua C.P., Alvarenga M.A., Papa F.O., Dell'Aqua J.A. (2018). Effects of coenzyme Q10 on semen cryopreservation of stallions classified as having good or bad semen freezing ability. *Anim. Reprod. Sci.*, 192: 107–118.
- Carocho M., Ferreira I.C.F.R. (2013). A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food. Chem. Toxicol.*, 51: 15–25.
- Contreras M.J., Treulen F., Arias M.E., Silva M., Fuentes F., Cabrera P., Felmer R. (2020). Cryopreservation of stallion semen: Effect of adding antioxidants to the freezing medium on sperm physiology. *Reprod. Domest. Anim.*, 55: 229–239.
- Dade M., Galeano P., Ríos J.L., Rojano B., Schinella G. (2016). Actividad apoptótica de derivados semisintéticos del isopentanol en células polimorfonucleares humanas. *Vitae*, 23: 11–17.
- Filho I.C.B., Pederzoli C.D., Sgaravatti A.M., Gregory R.M., Filho C.S.D. (2009). Skim milk-egg yolk based semen extender compensates for non-enzymatic antioxidant activity loss during equine semen cryopreservation. *Anim. Reprod.*, 6: 392–399.
- Gamal A., El S., Amal M., Abo E.M. (2016). Comparative blood and seminal plasma oxidant/antioxidant status of Arab stallions with different ages and their relation to semen quality. *Asian Pacific J. Reprod.*, 5: 428–433.
- Gamboa S., Rodrigues A.S., Henriques L., Batista C., Ramalho-Santos J. (2010). Seasonal functional relevance of sperm characteristics in equine spermatozoa. *Theriogenology*, 73: 950–958.
- Ghallab A.R.M., Shahat A.M., Fadl A.M., Ayoub M.M., Moawad A.R. (2017). Impact of supplementation of semen extender with antioxidants on the quality of chilled or cryopreserved Arabian stallion spermatozoa. *Cryobiology*, 79: 14–20.
- González Arbeláez L.F., Ciocci Pardo A., Fantinelli J.C., Rojano B., Schinella G.R., Mosca S.M. (2020). Isoespintanol, a monoterpene isolated from *Oxandra cf xylopioides*, ameliorates the myocardial ischemia-reperfusion injury by AKT/PKC ϵ /eNOS-dependent pathways. *Naunyn Schmiedeberg Arch. Pharmacol.*, 393: 629–638.
- Graham J.K., Alvarenga M.A., Squires E.L. (2004). Calcium influx into equine and bovine spermatozoa during. *Animal*, 1: 96–105.
- Kareskoski M., Katila T. (2008). Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity. *Anim. Reprod. Sci.*, 107: 249–256.
- Lazaros L.A., Vartholomatos G.A., Hatzis E.G., Kaponis A.I., Makrydimas G.V., Kalantaridou S.N., Sofikitis N.V., Stefos T.I.,

- Zikopoulos K.A., Georgiou I.A. (2011). Assessment of sperm chromatin condensation and ploidy status using flow cytometry correlates to fertilization, embryo quality and pregnancy following *in vitro* fertilization. *J. Assist. Reprod. Genet.*, 28: 885–891.
- Marquez-Fernandez M.E., Munoz-Lasso D.C., Lopez J.B., Zapata K., Mejia M.A.P., Alarcon C.L., Rojano B.A. (2018). Effect of isoespintanol isolated from *Oxandra cf. xylopioides* against DNA damage of human lymphocytes. *Pak. J. Pharm. Sci.*, 31: 1777–1782.
- Michael A., Alexopoulos C., Pontiki E., Hadjipavlou-Litina D., Saratsis P., Boscos C. (2007). Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology*, 68: 204–212.
- Nasirabadi M.H., Shirazi A., Kadivar A., Shams-Esfandabadi N., Mohebbi A. (2019). Sericin ameliorates the capacitation state and chromatin integrity of frozen-thawed stallion spermatozoa by reducing oxidative stress. *Avicenna J. Med. Biotechnol.*, 11: 245–252.
- Neild D., Chaves G., Flores M., Mora N., Beconi M., Agüero A. (1999). Hypoosmotic test in equine spermatozoa. *Theriogenology*, 51: 721–727.
- Neuhauser S., Gösele P., Handler J. (2019). Postthaw addition of autologous seminal plasma improves sperm motion characteristics in fair and poor freezer stallions. *J. Equine Vet. Sci.*, 72: 117–123.
- Nouri H., Towhidi A., Zhandi M., Sadeghi R. (2013). The effects of centrifuged egg yolk used with INRA plus soybean lecithin extender on semen quality to freeze miniature Caspian horse semen. *J. Equine Vet. Sci.*, 33: 1050–1053.
- Nouri H., Shojaeian K., Samadian F., Lee S., Kohram H., Lee J.I. (2018). Using resveratrol and epigallocatechin-3-gallate to improve cryopreservation of stallion spermatozoa with low quality. *J. Equine Vet. Sci.*, 70: 18–25.
- Núñez-Martínez I., Moran J.M., Peña F.J. (2006). A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: Changes after cryopreservation. *Reprod. Domest. Anim.*, 41: 408–415.
- Ortiz I., Dorado J., Acha D., Gálvez M.J., Urbano M., Hidalgo M. (2015). Colloid single-layer centrifugation improves post-thaw donkey (*Equus asinus*) sperm quality and is related to ejaculate freezability. *Reprod. Fertil. Dev.*, 27: 332–340.
- Papas M., Catalán J., Fernandez-Fuertes B., Arroyo L., Bassols A., Miró J., Yeste M. (2019). Specific activity of superoxide dismutase in stallion seminal plasma is related to sperm cryotolerance. *Antioxidants*, 8: 539.
- Restrepo G., Rojano B. (2018 a). Isoespintanol improves the post-thaw quality of stallion semen. *Indian J. Anim. Sci.*, 88: 50–54.
- Restrepo G., Rojano B. (2018 b). Antioxidant activity of isoespintanol and thymol in cryopreserved stallion semen. *Rev. Investig. Vet. del Perú*, 29: 205–216.
- Rojano B., Gaviria C., Gil M., Saez J., Schinella G., Tournier H. (2008 a). Actividad antioxidante del isoespintanol en diferentes medios. *VITAE, Revista De La Facultad De Química Farmacéutica*, 15: 173–181.
- Rojano B., Saez J., Schinella G., Quijano J., Vélez E., Gil A., Notario R. (2008 b). Experimental and theoretical determination of the antioxidant properties of isoespintanol (2-isopropyl-3,6-dimethoxy-5-methylphenol). *J. Mol. Struct.*, 877: 1–6.
- Ruiz L., Echegaray A., Lafuente A. (2011). Seminal freezing in pure breed Andalusian horse: Difference in individual stallions and correlation between pre and post-freezing sperm parameters. *Cryo-Letters*, 32: 473–476.
- Seifi-Jamadi A., Kohram H., Zare Shahneh A., Ansari M., Macías-García B. (2016). Quercetin ameliorate motility in frozen-thawed Turkmen stallions sperm. *J. Equine Vet. Sci.*, 45: 73–77.
- Shojaeian K., Nouri H., Kohram H. (2018). Does MnTBAP ameliorate DNA fragmentation and *in vivo* fertility of frozen-thawed Arabian stallion sperm? *Theriogenology*, 108: 16–21.
- Treulen F., Arias M.E., Aguila L., Uribe P., Felmer R. (2018). Cryopreservation induces mitochondrial permeability transition in a bovine sperm model. *Cryobiology*, 83: 65–74.
- Treulen F., Aguila L., Arias M.E., Jofré I., Felmer R. (2019). Impact of post-thaw supplementation of semen extender with antioxidants on the quality and function variables of stallion spermatozoa. *Anim. Reprod. Sci.*, 201: 71–83.
- Usuga A., Rojano B., Restrepo G. (2018). Association of the cysteine-rich secretory protein-3 (CRISP-3) and some of its polymorphisms with the quality of cryopreserved stallion semen. *Reprod. Fertil. Dev.*, 30: 563–569.
- Vasconcelos-Franco J.S., Chaveiro A., da Silva F.M. (2014). Effect of freezing rates and supplementation of α -tocopherol in the freezing extender in equine sperm cryosurvival. *J. Equine Vet. Sci.*, 34: 992–997.
- Yeste M., Estrada E., Rivera Del Álamo M.M., Bonet S., Rigau T., Rodríguez-Gil J.E. (2014). The increase in phosphorylation levels of serine residues of protein HSP70 during holding time at 17°C is concomitant with a higher cryotolerance of boar spermatozoa. *PLoS One*, 9: e90887.
- Yeste M., Estrada E., Rocha L.G., Marín H., Rodríguez-Gil J.E., Miró J. (2015). Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. *Andrology*, 3: 395–407.
- Zamzami N., Susin S.A., Marchetti P., Hirsch T., Gómez-Monterrey I., Castedo M., Kroemer G. (1996). Mitochondrial control of nuclear apoptosis. *J. Exp. Med.*, 183: 1533–1544.

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