Ciência Rural, Santa Maria, v.53:11, e20220508, 2023 http://doi.org/10.1590/0103-8478cr20220508
ISSNe 1678-4596
ANIMAL REPRODUCTION

Cooling of porcine semen in an extender supplemented with isoespintanol

Giovanni Restrepo Betancur[1*] Kelly Vanessa Zapata[1] Paola Andrea Colorado Vidal[1] Yudith Sánchez[1] Benjamín Alberto Rojano[1]

[1]Universidad Nacional de Colombia (UNAL), Medellín, Colombia. E-mail: grestre0@unal.edu.com. *Corresponding author.

ABSTRACT: Spermatozoa experience oxidative, osmotic, chemical, and thermal stresses when cooled, which degrade the quality and fertilizing capacity of the cells. Adding antioxidants to the sperm extender mitigates these alterations. This study evaluated the effect of isoespintanol (ISO) on boar semen subjected to cooling. Fifteen ejaculates from five boars (*Sus scrofa domestica*) were extended in Beltsville thawing solution (BTS) supplemented with 0 µM (control), 5 µM (ISO5), 10 µM (ISO10), 15 µM (ISO15), 20 µM (ISO20), 25 µM (ISO25), and 30 µM (ISO30) of ISO, which were then cooled for five days at 16 °C. Sperm kinetics, total motility (TM), and progressive motility (PM) were evaluated every 24 h using an IVOS computer-assisted sperm analysis (CASA) system. On day 1 and day 5 of cooling, a hypoosmotic test, spectrofluorometry, and flow cytometry were performed to evaluate the following: membrane functionality, measured as a function of hypoosmotic swelling (HOS); total antioxidant capacity (TAC); reactive oxygen species (ROS); and mitochondrial membrane potential (*Δ¥M*). Regression analysis and comparison of means using the Duncan test were performed. The ISO added had a slight impact on sperm motility, as evidenced by a reduction in TM at 24 h of cooling (but not prior) with the addition of 20 µM of ISO. Similarly, no effect of the ISO on the kinetics and functional integrity of the sperm membrane was observed at 96 h of cooling; however, the regression coefficients indicated that the ISO lowered the rate of decrease in sperm motility and the proportion of rapid spermatozoa relative to the concentration of ISO used. The ISO did not affect the TAC of the cooled semen; however, different concentrations of ISO lowered ROS production in the semen after 96 h of cooling. ISO also impacted the *Δ¥M* of the spermatozoa at 0 h of cooling, increasing the proportion of low *Δ¥M* cells and decreasing the proportion of high *Δ¥M* cells. In conclusion, ISO can reduce the loss of quality and oxidative stress occurring in boar semen during cooling and can modulate the mitochondrial activity of sperm.
Key words: antioxidant, boar, preservation, semen quality, spermatozoa.

Resfriamento de sêmen suíno em um diluente suplementado com isoespintanol

RESUMO: Durante a refrigeração, os espermatozoides sofrem estresse oxidativo, osmótico, químico e térmico, que diminuem sua qualidade e afetam sua capacidade de fertilização. A adição de antioxidantes ao diluente espermático é uma alternativa para mitigar essas alterações. O objetivo desta pesquisa foi avaliar o efeito do isospintanol (ISO) na refrigeração do sêmen suíno. Quinze ejaculados de cinco varrascos (*Sus scrofa domestica*) foram diluídos em BTS suplementado com ISO a 0 (controle), 5 (ISO5), 10 (ISO10), 15 (ISO15), 20 (ISO20), 25 (ISO25) e 30 (ISO30) µM e foram refrigerados por cinco dias a 16 °C. A motilidade total (MT), motilidade progressiva (MP) e cinética dos espermatozóides foram avaliadas a cada 24 h com um sistema CASA IVOS. Nos dias um e cinco de refrigeração, foram avaliadas a funcionalidade da membrana, a capacidade antioxidante total (CAT), as espécies reativas de oxigênio (ROS) e o potencial de membrana mitocondrial (*Δ¥M*), através do teste hiposmótico (HOS), espectrofluorimetría e citometria de fluxo. Foram realizadas análises de regressão e comparação de médias, pelo teste de Duncan. A adição de ISO teve pouca influência na motilidade espermática, apresentando apenas redução na MT em 24 h de refrigeração, devido à adição de 20 µM. Da mesma forma, não foi observada influência de ISO na cinética e integridade funcional da membrana em 96 horas de refrigeração; porém, os coeficientes de regressão mostraram que ISO produziu menor taxa de diminuição da motilidade e proporção de espermatozoides rápidos dependendo da concentração utilizada. ISO não influenciou significativamente na CAT do sêmen refrigerado; entretanto, diferentes concentrações de ISO reduziram a produção de EROs a partir do sêmen após de 96 h de refrigeração. ISO também influenciou o *Δ¥M* dos espermatozóides em 0 h de refrigeração, com aumento das células de baixo *Δ¥M* e diminuição das células de alto *Δ¥M*. Em conclusão, o isospintanol pode reduzir a perda da qualidade e o estresse oxidativo do sêmen suíno durante a refrigeração e pode modular a atividade mitocondrial do esperma.
Palavras-chave: Antioxidante, suíno, preservação, qualidade do sêmen, espermatozoides.

INTRODUCTION

The cryopreservation of boar semen is a critical biotechnology in the conservation and dissemination of genetic resources (YESTE et al., 2017). However, in swine reproduction, 99% of artificial inseminations are performed using refrigerated semen (PEZO et al., 2019), with no cryopreservation. This is because cryopreservation significantly reduces the fertilizing capacity of porcine spermatozoa (KNOX, 2015), inducing the death of 30% to 50% of sperm cells via cryogenic damage (WABERSKI et al., 2019).

However, when refrigerated at 5 ºC or 15 – 17 ºC, boar spermatozoa undergoes morphological and functional changes (HIDALGO, 2013) due to

Received 09.12.22 Approved 01.25.23 Returned by the author 03.26.23
CR-2022-0508.R1
Editors: Rudi Weiblen Bernardo Gasperin

heat shock and oxidative stress, which affect sperm quality (TIAN et al., 2019). This oxidative stress induces cell damage and lowers sperm motility and the integrity of the spermatozoa plasma membrane, significantly shortening the sperm life span (PEZO et al., 2020).

Hence, there is a need to find a way to counteract the stress produced during cold storage, such as using antioxidant molecules via supplementation in the extender to improve the fertilizing characteristics of semen at low temperatures and to enhance the preservation of desirable sperm characteristics (CÓRDOVA et al., 2009).

Isoespintanol (ISO) is a biosynthetic analog of thymol extracted from the leaves of *Oxandra cf. xylopioides* (Annonaceae), a naturally occurring substance with sufficient antioxidant capacity to neutralize free radicals and decrease the degree of membrane lipid peroxidation in sperm cells (ROJANO et al., 2008). Previous studies have demonstrated the ability of ISO to reduce alterations in equine and canine spermatozoa during cryopreservation (USUGA et al., 2021; RESTREPO et al., 2022). However, no studies have been conducted on the effects of this molecule (i.e., ISO) on refrigerated semen. This study evaluated the capacity of ISO as an alternative natural antioxidant for the conservation of boar semen *(Sus scrofa domestica*) subjected to cooling.

MATERIALS AND METHODS

Collection, initial evaluation, and processing of the semen

A total of 15 porcine ejaculates were collected from five boars using the gloved hand method equipped with polyvinyl gloves and a solid *dummy*. After ejaculation, the first part of the ejaculate or gel fraction was filtered, and the seminal material was immediately incubated at 37 ºC in a water bath. For each ejaculate, volume, concentration, and sperm motility were evaluated using a graduated cylinder, an SDM1 photometer (Minitube GmbH, Tiefenbach, Germany), and the HTM-IVOS computer-assisted semen analysis (CASA) system, version 12.3 (Hamilton Thorne, Beverly, MA, USA). Then, seven 5 mL aliquots were prepared, diluting the semen in an MR-A extender (Kubus S.A, Madrid, Spain), until a concentration of 60 × 10^6 spermatozoa/mL was reached. These aliquots, with a final volume of 5 mL, were supplemented with ISO (5, 10, 15, 20, 25, and 30 µM), along with a control without an antioxidant, and then stored at 16 ºC for 96 h (5 days). Ejaculates with a minimum total motility (TM) of 80% and progressive motility (PM) of 60% were processed.

Assessment of semen quality parameters

During the five days of storage, the motility (TM and PM) and kinetics of the spermatozoa were evaluated using the HTM-IVOS CASA system. The functional integrity of the sperm plasma membrane was assessed at 0 h and 96 h of cooling using a modified hypoosmotic swelling (HOS) test methodology (NEILD et al., 2001), employing a 1.52% fructose-based solution. The resulting mixture of semen and hypoosmotic solution was then incubated at 37 ºC. A drop of this mixture was spread on a warm slide with a coverslip, and 200 spermatozoa were evaluated at 1000 magnification on an optical microscope (BUCKETT, 1997; FANG, 2017).

At the two cooling duration measurement junctures (0 h and 96 h), several complementary evaluations described subsequently were also performed via spectrofluorometry.

Total antioxidant capacity (TAC) was evaluated with the $ABTS^{\bullet+}$ test (ARTS et al., 2004), performed using 10 µl of semen and 990 µl of $ABTS^{\bullet+}$ radical solution. After 30 minutes of reaction at ambient temperature in the dark, TAC was measured in a Jenway 6405 UV/Vis spectrophotometer (Jenway Ltd., Essex, UK) as a function of the change in absorbance measured against a reference solution. The radical solution was generated via the oxidation of 3.5 mM ABTS with 1.25 mM potassium persulfate. After 24 h of reaction, the absorbance was adjusted with phosphate buffered saline (PBS) at pH 7.4 to 0.70 units at λ = 732 nm; then compared against a Trolox standard curve.

Reactive oxygen species (ROS) detection was performed using fluorescein diacetate (FDA) (GUTHRIE & WELCH, 2006). Every sample was prepared with 30 µl of 40 mM FDA (Thermo Fisher, Inc., Waltham, USA), 240 µl of PBS (pH 7.4), and 30 µl of semen. Controlled conditions were used, including keeping temperature at 37 ºC and pH at 7.4, with Trolox antioxidant (Merck KGaA, Darmstadt, Germany) as a reference. Readings were performed using an LS 55 spectrofluorometer (Perkin Elmer, Waltham, USA) at an excitation λ of 490 nm, with an excitation slit of 10, an emission λ of 530 nm, and an emission slit of 15. The results were expressed as relative fluorescence units (RFU).

Δ¥M was evaluated using flow cytometry. For this analysis, only the strongest ISO concentrations (ISO5 and ISO30) were used. *Δ¥M* was assessed using

a specific JC-1 probe. In this evaluation, mitochondria that exhibit high Δ¥M are expressed as dimers with emission at 590 nm when excited at 549 nm, while in mitochondria with low Δ¥M, JC-1 are presented as monomers emitting at 525 nm when excited at 488 nm (GUO et al., 2017). The protocol described by GUO et al. (2017) was followed for this evaluation. A semen sample was diluted in HANKS balanced salt solution at a concentration of 1 x 10^6/mL, and JC-1 (MitoProbe, Thermo Fisher, Inc., Waltham, USA) was then added to achieve a final concentration of 0.4 µM. The *Δ¥M* was measured using an LSRFortessaTM flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Specimen samples were excited using a 488 nm solid-phase laser, and JC-1 fluorescence was detected at 530/30 nm. The results were analyzed with FlowJo software, version 7.6.2 (FlowJo LLC, Ashland, OR, USA).

Statistical analysis

To evaluate the effect of the studied antioxidants on the quality of boar semen stored for 96 h, generalized linear models were fitted to determine the effect of the treatments on the dependent variables (TM, PM, TAC, ROS, *Δ¥M,* sperm kinetics, and the functional integrity of the plasma membrane). Comparison of means between treatments was performed using Duncan's multiple range test. Regression analyses were conducted for each semen quality parameter. The significance level considered for all evaluations was $P < 0.05$. All analyses were performed with SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Globally, a significant percentage of swine farms practice artificial insemination (AI) with cooled semen, using extenders that provide the necessary conditions to preserve the fertilizing capacity of the spermatozoa (PEZO et al., 2019). However, during preservation, spermatozoa are subjected to low temperatures, decreasing motility and other characteristics, which reduces sperm functionality (FANG et al., 2017).

In this study, sperm quality was evaluated during cooling. Results showed that TM and PM values decreased with increasing cooling time. This result pattern is consistent with the effect produced in two previous studies in which rosmarinic acid (FENG et al., 2020) and salvianic acid A (TIAN et al., 2019), both natural antioxidants, were added to cooled boar semen. In this study, however, there was no significant difference between the control and the ISO supplementation specimens ($P > 0.05$), except for a reduction in TM at 24 h of cooling due to supplementation with 20 µM of ISO (Figures 1 and 2). There were also no significant differences in sperm motility, sperm kinetics (Table 1), or sperm membrane functional integrity (Table 2) at 96 hours of cooling.

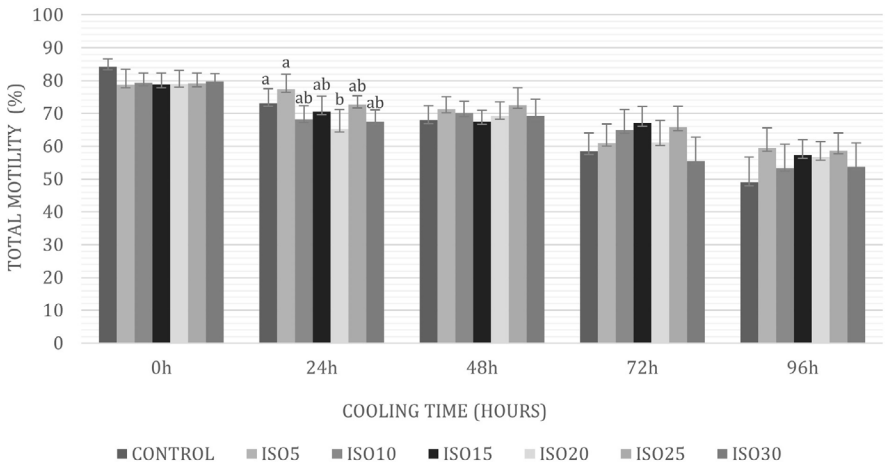

Figure 1 - Total motility of cooled semen.

Total motility values are expressed as percentage ± standard error of the mean. a-b: Different superscripts indicate differences between treatments within the same evaluation time ($P < 0.05$). 0h, 24h, 48h, 72h and 96h refer to hours of cooling at 16 °C.

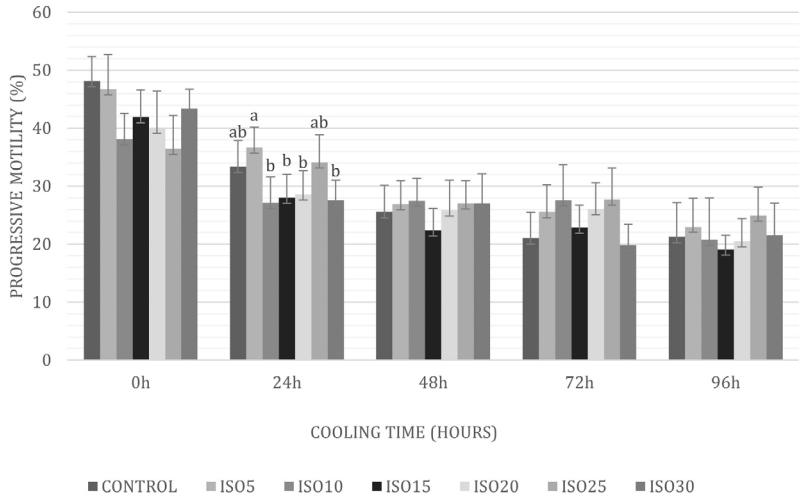

Figure 2 - Progressive motility of cooled semen.

Progressive motility values are expressed as percentage ± standard error of the mean. a-b: Different superscripts indicate differences between treatments within the same evaluation time ($P < 0.05$). 0h, 24h, 48h, 72h and 96h refer to hours of cooling at 16 °C.

Regression coefficients were obtained from the regression analysis performed, which express the degree of change in sperm motility, sperm kinetics, and sperm membrane functionality as a function of the cooling time (Table 3). These regression coefficients indicate that ISO reduced the rate of decrease in various motility parameters and the proportion of rapid spermatozoa, depending on the concentration of ISO used. ISO25 was the treatment that yielded the strongest protective capacity during cooling, generating lower losses per day of storage in TM, PM, VAP, VSL, VCL, ALH, RAP, and HOS.

Several studies have shown that oxidative stress is one of the primary factors impacting the quality and fertilizing capacity of spermatozoa during preservation (FANG et al., 2017). This condition is induced by an excessive accumulation of ROS, which damages sperm cell membranes (PEREIRA et al., 2019), produces peroxidized lipids (BOLLWEIN & BITTNER, 2018), alters cell structure and function, damages DNA (SCHULTE et al., 2010), and reduces intracellular ATP levels (AITKEN et al., 2012), all of which explain the decrease in sperm motility that was observed in this study. Storing semen with the addition of antioxidants in the preservation extender is an alternative approach that reduces the adverse effects of oxidative stress and preserves sperm functionality for a longer period (TIAN et al., 2019).

Therefore, TAC (Figure 3), ROS (Figure 4), and Δ¥M (Figure 5) were monitored at 0 h and 96 h of cooling. Figure 3 shows that the TAC evaluated using ABTS did not decrease during cooling, except for ISO25, contrary to that reported by TIAN et al. (2019) with salvianic acid A, in which TAC decreased with storage time. In addition, that the TAC decreased with storage time shows that at 0 h of cooling, TAC evaluated using an ABTS test evidences significant differences ($P < 0.05$) between the control and ISO20, while at 96 h, control did not differ for either treatment. In this study, the antioxidant contribution of ISO in a porcine sperm extender was not clearly evidenced, which can be attributed to the interaction of this molecule with antioxidant enzymes (such as superoxide dismutase and glutathione peroxidase) responsible for counteracting the damage produced by ROS. In this sense, the molecules of ISO can reduce the activity of these enzymes, possibly generating alterations in TAC results. This situation has been previously reported in equine cryopreserved semen with ISO (RESTREPO & ROJANO, 2017).

In addition, for ROS production, ISO30 (Figure 4) showed significant differences ($P < 0.05$) vs. control at both cooling times (0 and 96 h), while this same concentration produced less ROS at 96 h. This decrease coincides with the results obtained in cryopreserved canine semen when ISO was added

Table 1 - Sperm kinetics of cooled semen.

	VAP	VSL	VCL	ALH	BCF	STR	LIN	RAP
----------0 h----------								
CONTROL	50.7 ± 3.2	42.9 ± 2.3	96.7 ± 4.0^{ab}	5.0 ± 0.2	42.4 ± 0.7^{a}	78.2 ± 1.7^{a}	46.0 ± 2.1^{a}	52.4 ± 4.6
ISO5	49.9 ± 3.9	39.7 ± 2.4	100.4 ± 5.0^{ab}	5.3 ± 0.2	42.3 ± 0.6^{ab}	75.4 ± 1.4^{ab}	40.6 ± 1.0^{b}	51.7 ± 6.8
ISO10	51.5 ± 2.6	38.4 ± 2.1	95.8 ± 4.3^{ab}	5.3 ± 0.2	40.2 ± 0.7^{b}	75.6 ± 1.5^{ab}	43.0 ± 1.4^{ab}	43.0 ± 4.7
ISO15	51.8 ± 2.3	39.7 ± 1.6	94.1 ± 3.8^{ab}	5.2 ± 0.2	40.3 ± 0.8^{ab}	77.2 ± 1.1^{ab}	44.5 ± 1.3^{ab}	46.1 ± 4.9
ISO20	51.4 ± 3.4	66.0 ± 27.0	95.0 ± 5.1^{ab}	5.3 ± 0.2	41.0 ± 0.8^{ab}	76.4 ± 1.3^{ab}	43.0 ± 1.5^{ab}	43.9 ± 6.7
ISO25	49.9 ± 3.6	38.4 ± 2.8	92.1 ± 5.5^{b}	5.1 ± 0.2	41.0 ± 0.5^{ab}	77.6 ± 1.4^{a}	43.4 ± 1.5^{ab}	40.5 ± 6.2
ISO30	53.6 ± 2.1	39.3 ± 1.4	103.2 ± 4.5^{a}	5.5 ± 0.2	41.4 ± 1.0^{ab}	74.0 ± 1.3^{b}	40.2 ± 1.3^{b}	48.8 ± 3.8
----------24 h----------								
CONTROL	50.8 ± 2.8^{a}	36.4 ± 1.9^{a}	97.2 ± 5.6	5.7 ± 0.2	38.1 ± 1.1	73.0 ± 1.8	40.7 ± 1.8	39.0 ± 5.2^{a}
ISO5	52.8 ± 2.0^{a}	38.9 ± 1.3^{a}	100.3 ± 4.8	5.5 ± 0.3	39.4 ± 1.2	74.6 ± 1.8	42.1 ± 1.9	42.4 ± 4.2^{a}
ISO10	45.7 ± 2.5^{b}	33.9 ± 2.0^{b}	88.4 ± 3.7	5.5 ± 0.2	39.0 ± 1.3	74.8 ± 1.4	40.8 ± 1.6	31.0 ± 4.9^{b}
ISO15	46.1 ± 2.3^{b}	31.8 ± 1.6^{b}	91.8 ± 4.0	6.1 ± 0.2	37.9 ± 0.7	72.7 ± 1.6	39.0 ± 1.7	33.2 ± 4.8^{b}
ISO20	46.8 ± 2.5^{b}	33.5 ± 1.5^{b}	94.2 ± 4.5	6.0 ± 0.2	65.5 ± 26.9	73.1 ± 1.6	38.8 ± 1.6	34.4 ± 4.8^{a}
ISO25	49.2 ± 2.1^{a}	35.9 ± 1.6^{a}	96.7 ± 3.9	5.5 ± 0.3	63.4 ± 23.1	74.2 ± 2.0	40.4 ± 2.0	39.7 ± 5.2^{a}
ISO30	47.5 ± 2.2^{b}	33.7 ± 1.5^{b}	165.4 ± 2.4	5.8 ± 0.2	63.7 ± 24.3	72.6 ± 2.1	39.4 ± 2.4	33.0 ± 4.0^{b}
----------48 h----------								
CONTROL	45.1 ± 3.2	33.3 ± 2.5	91.5 ± 5.6	5.7 ± 0.3	38.7 ± 1.2^{b}	74.0 ± 1.7	38.8 ± 1.7	29.4 ± 5.1
ISO5	47.1 ± 2.8	34.5 ± 1.9	91.7 ± 5.2	5.7 ± 0.3	82.5 ± 29.7^{a}	74.2 ± 1.5	67.2 ± 27.1	31.4 ± 4.4
ISO10	48.4 ± 2.7	31.7 ± 1.8	98.3 ± 4.5	6.2 ± 0.2	40.1 ± 1.2^{b}	72.4 ± 1.2	37.9 ± 1.3	32.9 ± 4.8
ISO15	43.4 ± 2.8	31.2 ± 1.8	89.4 ± 4.9	5.8 ± 0.2	40.3 ± 1.0^{b}	73.5 ± 1.5	37.8 ± 1.3	26.5 ± 4.7
ISO20	43.7 ± 3.0	32.0 ± 2.3	88.4 ± 5.7	5.4 ± 0.2	40.1 ± 1.0^{b}	74.8 ± 2.0	40.3 ± 2.5	29.9 ± 5.7
ISO25	46.9 ± 2.9	33.0 ± 1.8	94.7 ± 5.4	5.9 ± 0.2	40.1 ± 1.0^{b}	72.6 ± 2.2	38.2 ± 2.1	32.4 ± 4.4
ISO30	45.9 ± 2.8	32.4 ± 1.7	88.8 ± 5.8	5.4 ± 0.4	38.9 ± 1.1^{b}	74.5 ± 2.4	41.5 ± 2.4	32.1 ± 4.8
----------72 h----------								
CONTROL	46.7 ± 3.4	33.4 ± 1.9	91.2 ± 7.7	6.2 ± 0.3^{a}	36.0 ± 2.0	73.7 ± 2.8^{a}	44.0 ± 3.9	27.2 ± 5.7
ISO5	51.1 ± 3.7	36.3 ± 2.4	101.4 ± 7.4	6.0 ± 0.2^{a}	38.8 ± 0.9	73.0 ± 2.0^{a}	40.2 ± 2.7	30.7 ± 5.2
ISO10	49.3 ± 3.9	37.9 ± 3.3	91.3 ± 6.6	5.3 ± 0.3^{a}	38.5 ± 1.1	77.6 ± 1.9 ^{a}	45.3 ± 2.6	32.0 ± 6.8
ISO15	47.8 ± 3.7	34.3 ± 2.6	95.0 ± 7.0	6.0 ± 0.3^{a}	38.3 ± 0.9	73.7 ± 2.0^{a}	41.0 ± 2.5	29.2 ± 4.7
ISO20	51.6 ± 3.6	35.4 ± 2.4	100.0 ± 7.6	5.1 ± 0.5^{b}	40.1 ± 1.6	70.4 ± 3.6^{a}	39.8 ± 3.8	32.4 ± 5.1
ISO25	49.7 ± 3.5	36.2 ± 2.7	102.7 ± 8.3	5.9 ± 0.3^{a}	38.8 ± 1.1	73.5 ± 1.8^{a}	38.2 ± 2.0	33.0 ± 6.2
ISO30	44.4 ± 4.7	31.5 ± 3.4	89.0 ± 9.2	4.9 ± 0.6^{b}	36.2 ± 2.9	68.0 ± 5.7^{b}	37.3 ± 4.6	24.8 ± 4.4
----------96 h----------								
CONTROL	45.5 ± 3.3	34.0 ± 2.9	89.6 ± 4.6	5.6 ± 0.3^{a}	39.1 ± 1.1	76.1 ± 1.8	40.5 ± 1.7	24.0 ± 6.5
ISO5	49.3 ± 3.7	34.6 ± 2.6	95.8 ± 6.6	6.1 ± 0.3^{a}	36.9 ± 1.2	73.3 ± 1.8	40.6 ± 2.0	28.3 ± 5.8
ISO10	47.1 ± 3.3	33.3 ± 2.5	91.5 ± 6.1	5.2 ± 0.6^{a}	35.0 ± 2.2	74.8 ± 2.7	41.7 ± 2.7	25.7 ± 5.2
ISO15	46.5 ± 3.1	33.4 ± 2.4	94.3 ± 5.9	5.9 ± 0.3^{a}	39.6 ± 1.2	74.3 ± 2.3	38.9 ± 1.6	23.0 ± 2.9
ISO20	48.6 ± 3.7	33.5 ± 3.1	95.8 ± 6.6	5.3 ± 0.4^{a}	39.6 ± 1.8	70.7 ± 3.6	37.6 ± 2.5	25.8 ± 4.4
ISO25	50.0 ± 4.7	36.2 ± 3.5	98.0 ± 8.9	5.3 ± 0.5^{a}	37.7 ± 2.3	74.7 ± 2.92	40.3 ± 2.5	30.5 ± 5.5
ISO30	44.7 ± 5.2	32.1 ± 3.2	88.6 ± 9.4	4.5 ± 0.7^{b}	39.9 ± 1.8	75.8 ± 2.9	40.1 ± 3.0	26.7 ± 6.9

Results are expressed as the mean value ± standard error of the mean. a-b: Different superscripts in the same column by cooling time indicate differences between treatments ($P < 0.05$). VAP: mean velocity, VSL: linear velocity, VCL: curvilinear velocity, ALH: lateral head amplitude; BCF: beating frequency; STR: straightness index; LIN: linearity index; RAP: fast moving cells, HOS: functional integrity of plasma membrane.

to the sperm extender, which is a fundamental aspect when trying to reduce oxidative damage (USUGA et al., 2021).

Finally, for Δ¥M evaluation, ISO5 showed similar behavior to the control during 0 and 96 h of cooling, while ISO30 decreased the proportion of dimers and increased the population of monomers (Figure 5). This demonstrated that ISO30 was able to modify the mitochondrial membrane potential in cooled porcine semen, in contrast to cryopreserved

Table 2 - Functional membrane integrity (HOS) of cooled semen.

COOLING TIME (h)	----------TREATMENT----------	----------HOS----------
0	CONTROL	49.80 ± 2.06 [a]
	ISO5	48.30 ± 2.08 [ab]
	ISO10	43.90 ± 2.14 [b]
	ISO15	49.70 ± 2.77 [a]
	ISO20	50.10 ± 2.91 [a]
	ISO25	45.30 ± 2.45 [ab]
	ISO30	44.90 ± 1.97 [ab]
96	CONTROL	42.77 ± 2.91
	ISO5	41.33 ± 3.71
	ISO10	36.44 ± 3.73
	ISO15	42.44 ± 2.45
	ISO20	42.33 ± 4.12
	ISO25	39.78 ± 2.92
	ISO30	39.22 ± 3.88

Results are expressed as the mean value ± standard error of the mean. a-b: Different superscripts indicate differences between treatments within the same evaluation time ($P < 0.05$). 0h and 96h refers to the hours of cooling.

canine semen, where no effect on mitochondrial activity was evident (USUGA et al., 2021). The capacity to modify the mitochondrial membrane potential and reduce ROS production may be explained by the fact that ISO can regulate Ca^{2+} capture and ROS production in the mitochondria, which in turn could modify ATP synthesis and sperm motility (RESTREPO et al., 2022). Currently, there is little information about the evaluation of ISO as an antioxidant molecule. It has been studied in cryopreserved equine (RESTREPO & ROJANO, 2017; RESTREPO et al., 2022) and canine semen (USUGA et al., 2021), but no reports have been found on the addition of ISO to cooled semen and or in boar spermatozoa.

It has been reported that supplementation of ISO in doses higher than 80 µM generates cytotoxicity (MÁRQUEZ et al., 2018), while doses of 40 µM of this molecule in equine semen reduce TM (RESTREPO et al., 2022). In this study, ISO did not produce severe alterations in seminal quality; hence,

Table 3 - Regression coefficients of semen quality according to cooling time.

VARIABLE	CONTROL	ISO5	ISO10	ISO15	ISO20	ISO25	ISO30
TM	-0.372*	-0.175	-0.185*	-0.212*	-0.227*	-0.172*	-0.247*
PM	-0.314*	-0.266*	-0.144	-0.222*	-0.209	-0.082	-0.219*
VAP	-0.048	-0.007	-0.008	-0.044	-0.036	0.033	-0.057
VSL	-0.101	-0.055	-0.057	-0.068	-0.076	-0.008	-0.052
VCL	-0.115	-0.098	0.063	0.058	0.023	0.131	-0.113
ALH	0.001	0.004	0.006	0.006	0.001	0.006	-0.008
BCF	-0.046*	-0.056*	-0.036	-0.005	-0.014	-0.045	-0.024
STR	-0.009	-0.016	-0.063	-0.048	-0.091	-0.049	0.002
LIN	-0.043	0.018	-0.062	-0.090*	-0.083	-0.041	0.008
RAP	-0.328*	-0.266*	-0.109	-0.213*	-0.174	-0.047	-0.213*
HOS	-0.073	-0.076	-0.078	-0.076	-0.081	-0.058	-0.059

TM: total motility; PM: progressive motility; VAP: average path velocity; VSL: linear velocity; VCL: curvilinear velocity; ALH: lateral head amplitude; BCF: beating frequency; STR: straightness index; LIN: linearity index; RAP: fast moving cells; HOS: functional integrity of the plasma membrane. (*) indicate significant regression coefficients ($P < 0.05$).

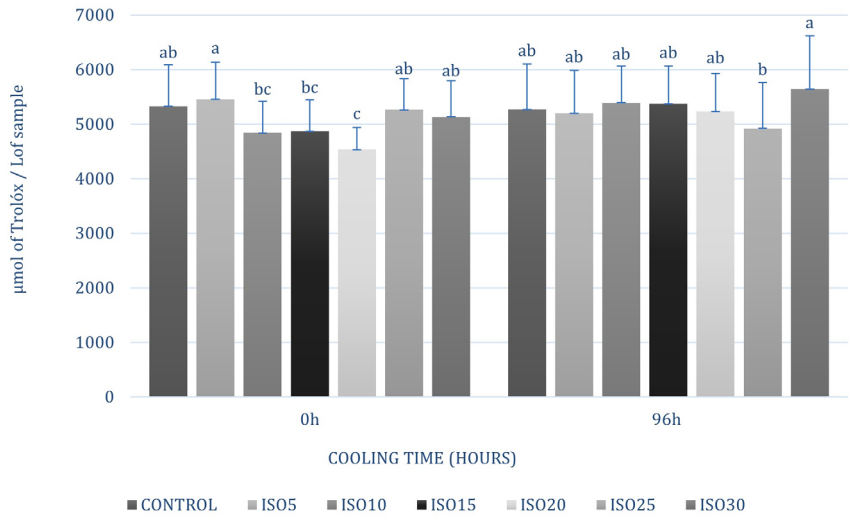

Figure 3 - Total antioxidant capacity (TAC) of cooled semen.

Total antioxidant capacity (TAC) values, evaluated by the ABTS assay are expressed in µmol Trolox / L of sample ± standard error of the mean. a-c: Different superscripts indicate differences between treatments within the same evaluation time ($P < 0.05$). 0h and 96h refers to hours of cooling at 16 °C.

it could be thought that it does not exert cytotoxic effects on boar sperm at the concentrations used in this study. Therefore, new studies are required to evaluate the cytotoxicity of ISO in sperm cells.

CONCLUSION

Isoespintanol can reduce the loss of sperm quality and damage induced by oxidative stress in boar

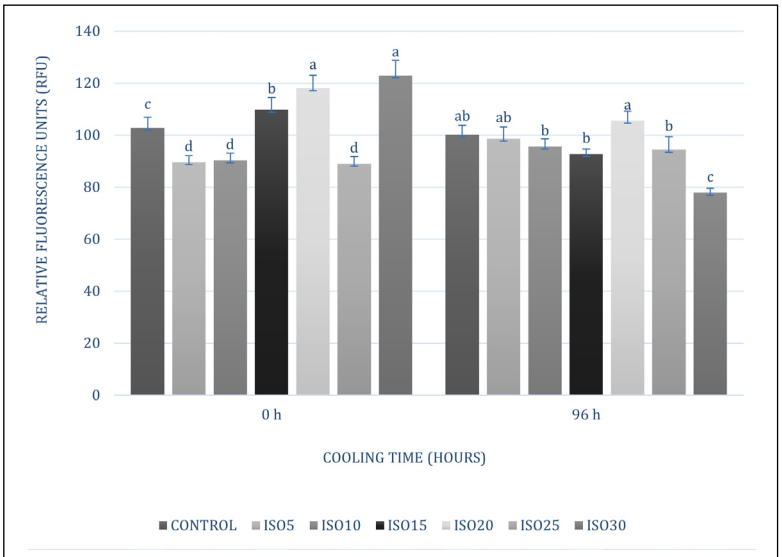

Figure 4 - Reactive oxygen species (ROS) production of cooled semen.

The production of reactive oxygen species is expressed in relative fluorescence units (RFU) ± standard error of the mean. a-d: Different superscripts indicate differences between treatments within the same evaluation time ($P < 0.05$). 0h and 96h refers to hours of cooling at 16 °C.

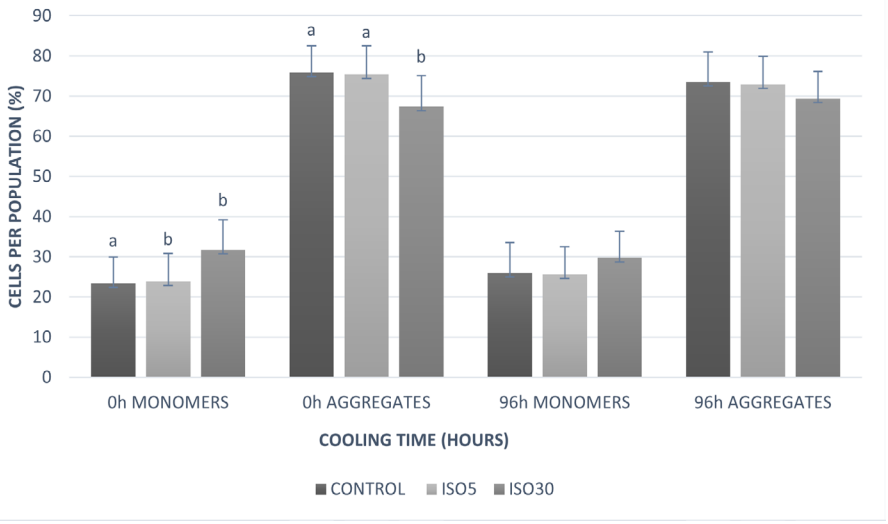

Figure 5 - Mitochondrial membrane potential of cooled semen.

Monomers: cells with low mitochondrial membrane potential (low - *Δ¥M*); Aggregates: cells with high mitochondrial membrane potential (*high-Δ¥M*). The number of cells in each population is expressed as a percentage (%) ± standard error of the mean. a-b: Different superscripts indicate differences between treatments within the same population at a given cooling time ($P < 0.05$).

semen during cooling. In addition, this antioxidant can modulate the mitochondrial activity of sperm.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors would like to thank to San Pablo Agricultural Station of Universidad Nacional de Colombia (UNAL).

ETHICAL STANDARDS

The study had the ethical endorsement number CICUA-054-20 of the Committee for the Care and Use of Animals (CICUA) of the Universidad Nacional de Colombia (UNAL), Medellín Headquarters.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the conception and writing of this manuscript. All authors critically reviewed the manuscript and approved the final version of the manuscript.

REFERENCES

AITKEN, R. J. et al. Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. **Biology of reproduction**, v.87, n.5, p.110–111, 2012. Available from: <https://academic.oup.com/biolreprod/article/87/5/110,%201-11/2513899>. Accessed: Feb. 20, 2020. doi: 10.1095/biolreprod.112.102020.

ARTS, M. et al. A new approach to assess the total antioxidant capacity using the TEAC assay. **Food Chemistry**, v.88, n.4, p.567–570, 2004. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0308814604001591>. Accessed: May, 15, 2021. doi: 10.1016/J.FOODCHEM.2004.02.008.

BOLLWEIN, H.; BITTNER, L. Impacts of oxidative stress on bovine sperm function and subsequent in vitro embryo development. **Animal Reproduction**, v.15, p.703–710, 2018. Available from: <https://www.animal-reproduction.org/article/doi/10.21451/1984-3143-AR2018-0041>. Accessed: Dec. 12, 2022. doi: 10.21451/1984-3143-AR2018-0041.

BUCKETT, W. et al. The hypo-osmotic swelling test in recurrent miscarriage. **Fertility and Sterility**, v.68, p.506–509, 1997. Available from: <https://pubmed.ncbi.nlm.nih.gov/9314923>. Accessed: Nov. 16, 2022. doi: 10.1016/s0015-0282(97)00241-0.

CÓRDOVA, A. et al. Estrés oxidativo y antioxidantes en la conservación espermática. **Revista Complutense de Ciencias Veterinarias**, v.3, n.1, p.1–38, 2009. Available from: <https://dialnet.unirioja.es/servlet/articulo?codigo=2961477>. Accessed: Sep. 1, 2022.

FANG, Y. et al. Effects of astaxanthin supplementation on the sperm quality and antioxidant capacity of ram semen during liquid storage. **Small Ruminant Research**, v.130, p.178–182, 2017. Available from: <https://dialnet.unirioja.es/servlet/

articulo?codigo=2961477>. Accessed: Sep. 1, 2022. doi: 10.1016/j.smallrumres.2015.05.016.

FENG, T. Y. et al. Rosmarinic acid improves boar sperm quality, antioxidant capacity and energy metabolism at 17°C via AMPK activation. **Reproduction in Domestic Animals**, v.55, n.12, p.1714–1724, 2020. Available from: <https://pubmed.ncbi.nlm.nih.gov/32969084>. Accessed: Sep. 9, 2022. doi: 10.1111/rda.13828.

GUO, H. et al. Relationships between mitochondrial DNA content, mitochondrial activity, and boar sperm motility. **Theriogenology**, v.87, p.276–283, 2017. Available from: <https://pubmed.ncbi.nlm.nih.gov/32969084>. Accessed: Aug. 13, 2022. doi: 10.1016/j.theriogenology.2016.09.005.

GUTHRIE, H. D.; WELCH, G. R. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. **Journal of Animal Science**, v.84, n.8, p.2089–2100, 2006. Available from: <https://pubmed.ncbi.nlm.nih.gov/16864869/>. Accessed: Dec. 11, 2022. doi: 10.2527/JAS.2005-766.

KNOX, R. V. The fertility of frozen boar sperm when used for artificial insemination. **Reproduction in Domestic Animals**, v.50, p.90–97, 2015. Available from: <https://onlinelibrary.wiley.com/doi/full/10.1111/rda.12552>. Accessed: Aug. 22, 2022. doi: 10.1111/rda.12552.

MÁRQUEZ, M. et al. Effect of isoespintanol isolated from Oxandra cf. Xylopioides against DNA damage of human lymphocytes**. Pakistan Journal of Pharmaceutical Sciences**, v.31, n.5, p.1777-1782, 2018. Available from: <https://pubmed.ncbi.nlm.nih.gov/30150170>. Accessed: Jul. 30, 2022.

NEILD, D. M. et al. The HOS test and its relationship to fertility in the stallion. **Andrologia**, v.32, n.6, p.351–355, 2001. Available from: <https://pubmed.ncbi.nlm.nih.gov/11131844>. Accessed: May, 2, 2021. doi: 10.1046/J.1439-0272.2000.00357.X.

PEREIRA, B. A. et al. Addition of chlorogenic acid and caffeine during the processing of cooled boar semen. **Arquivo Brasileiro de Medicina Veterinaria e Zootecnia**, v.71, n.2, p.489–499, 2019. Available from: <https://www.scielo.br/j/abmvz/a/YFYFpjjjgwjznrKVzhPLjpq/?lang=en>. Accessed: Jul. 18, 2022. doi: 10.1590/1678-4162-10415.

PEZO, F. et al. Preservation of boar semen: An update. **Reproduction in Domestic Animals**, v.54, n.3, p. 423–434, 2019. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1111/rda.13389>. Accessed: Sep. 8, 2022. doi: 10.1111/rda.13389.

PEZO, F. et al. Antioxidants and their effect on the oxidative/nitrosative stress of frozen-thawed boar sperm. **Cryobiology**, 2020. Available from: <https://pubmed.ncbi.nlm.nih.gov/33248047>. Accessed: Apr. 10, 2022. doi: 10.1016/j.cryobiol.2020.11.007.

RESTREPO, G. et al. Effect of supplementation of freezing media with isoespintanol on functional integrity of equine semen. **Annals of Animal Science**, v.22, n.3. p.953-960, 2022. Available from: <https://sciendo.com/es/article/10.2478/aoas-2022-0010>. Accessed: Dec. 2, 2022. doi: 10.2478/aoas-2022-0010.

RESTREPO, G.; ROJANO, B. A. Efecto del isoespintanol y el timol en la actividad antioxidante de semen equino diluido con fines de congelación. **Revista de Medicina Veterinaria**, v.35, p.149–158, 2017. Available from: <https://ciencia.lasalle.edu.co/mv/vol1/iss35/14>. Accessed: Dec. 5, 2021. doi: 10.19052/MV.4397.

ROJANO, B. et al. Actividad antioxidante del isoespintanol en diferentes medios. **Vitae**, v.15, n.1, p.173-181, 2008. Available from: <http://www.scielo.org.co/scielo.php?pid=S0121-40042008000100021&script=sci_abstract&tlng=es>. Accessed: Feb. 17, 2021.

SCHULTE, R. T. et al. Sperm DNA damage in male infertility: etiologies, assays, and outcomes. **Journal of Assisted Reproduction and Genetics**, v.27, n.1, p.3–12, 2010. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2826626>. Accessed: Mar. 5, 2022. doi: 10.1007/s10815-009-9359-x.

TIAN, X. et al. Supplementation of salvianic acid A to boar semen extender to improve seminal quality and antioxidant capacity. **Animal Science Journal**, v.90, n.9, p.1142–1148, 2019. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1111/asj.13263>. Accessed: Jun. 12, 2022. doi: 10.1111/ASJ.13263.

USUGA, A. et al. Cryoprotective effects of ergothioneine and isoespintanol on canine semen. **Animals**, v.11, p.2757, 2021. Available from: <https://pubmed.ncbi.nlm.nih.gov/34679779>. Accessed: Feb. 11, 2022. doi: 10.3390/ani11102757.

WABERSKI, D. et al. Application of preserved boar semen for artificial insemination: Past, present and future challenges. **Theriogenology**, v.137, p.2–7, 2019. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0093691X19301682>. Accessed: Apr. 28, 2022. doi: 10.1016/j.theriogenology.2019.05.030.

YESTE, M. et al. Artificial insemination with frozen-thawed boar sperm. **Molecular Reproduction Development**. v.84, p.802–813, 2017. Available from: <https://pubmed.ncbi.nlm.nih.gov/16866323>. Accessed: May, 1, 2022. doi: 10.1002/mrd.22840.