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Isoespintanol improves the post-thaw quality of stallion semen Isoespintanol improves stallion semen

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ABSTRACT

The study was conducted toassess the effect of thymol and isoespintanol when usedin stallion semen freezing.The semen of seven Colombian creole horses was collected using the artificial vagina method. Each sample was separated into three aliquots that were subjected to a freezing protocol using the treatments: Thymol (50 µM), Isoespintanol (40 µM) and Control. After thawing, sperm motility (MOT) was assessed via phase contrast microscopy; sperm vitality (SV) and normal morphology (NM) via supravital staining using eosin-nigrosin; cell membrane integrity was in turn evaluated (MI) with the hypo-osmotic swelling test (HOS), and the mitochondrial membrane potential (MMP) by means of a fluorometry in a microplate reader. The statistical assessment was performed by fitting mixed models and conducting a Pearson correlation analysis. The comparisons between the measurements were compared using Tukey's test. A statistically significant difference ($p<0.05$) was found between isoespintanol and the control group for MOV (47% vs. 36.3%), SV (57.1 vs. 51.8) and MI (44.4% vs. 36.5%). No effects were found for thymol and isoespintanol on the MMP of cryopreserved stallion spermatozoa. We conclude that isoespintanol improves the post-thaw quality of stallion semen.

Key words: Antioxidants, Cryopreservation, Horse, Isoespintanol, Thymol, Sperm.

Cryopreservation is a very important technique for the equine industry as it enables transportation and long-term storage of semen (Loomis and Graham 2008, Gibb *et al*. 2016). Likewise, it facilitates genetic enhancement of commercially valuable traits by means of selection or directed crossings (Neira*et al.* 2007). Unfortunately, the advancements in stallion semen cryopreservation are not on par with those of cryopreservation for other species, and artificial insemination has grown in popularity (Kirk *et al*. 2005, Rodríguez and Wallgren 2011).

Different types of stress have been described (osmotic, thermal and oxidative) during stallion semen cryopreservation (Ortega-Ferrusola*et al.* 2009; Lançoni*et al*. 2015). They have adverse effects on motility, vitality, mitochondrial membrane potential and permeability of the cell membrane of spermatozoa. Likewise, they cause cellular changes such as apoptosis (Brum *et al*. 2008). Similarly, cryopreservation may generate alterations in genetic components and interference in the fertilizing capacity of spermatozoa (Thomas *et al*. 2006, Wnuk*et al*. 2010).

The increase in the generation of reactive oxygen species (ROS), the decrease in antioxidant defense and the oxidation of lipids and proteins in stallion semen once it is cryopreserved (Morte*et al*. 2008, Macias-Garcia *et al.* 2011), have motivated a great deal of research on molecules or substances that strengthen its protection against oxidative stress (Barros *et al*. 2012, Gibb *et al*. 2013; Martins *et al*. 2015). In spite of this, in most cases results have been far from encouraging (Hussain *et al*. 2011; Martins *et al*. 2012). Two natural molecules, thymol (2-isopropyl-5–methylphenol) and isoespintanol (2-isopropyl-3,6-dimethoxy-5-methylphenol), are known for their antioxidant properties, which have been tested in emulsified systems because they can inhibit lipid peroxidation and strengthen antioxidant enzymatic action on mouse liver tissue (Rojano*et al*. 2008, Miguel *et al.* 2010, Al-Malki 2010). The antioxidant capabilities of thymol, a compound found in essential oils extracted from thyme (*Thymus vulgare*) and oregano (*Origanum vulgare*), have been demonstrated when used for protecting animal cell cultures from the cytotoxic effects of hydrogen peroxide. Moreover, thymol has shown high cell membrane protection capacity (Özkan and Erdoðan 2011, Aman *et al*. 2013). On the other hand, isoespintanol, which is extracted from the leaves of *Oxandracfxylopioides* (*Annonaceae*), has been shown to be highly successful at inhibiting inflammations (Rojano*et al*. 2007)) and is a better free radical scavenger than thymol (Rojano*et al*. 2008). For this reason these molecules may be alternatives to be used in stallion semen cryopreservation given its well-known issues regarding oxidative stress. The objective of this study was to assess the performance of

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thymol and isoespintanol in regards to stallion semen cryopreservation.

MATERIALS AND METHODS

The semen used in this study was obtained from sevenColombian creole horses (*Equus caballus*), located in the municipality of Girardota (Antioquia, Colombia); latitude: 6°23'41.50"N, longitude: 75°25'27.95"O, average elevation: 1.475 masl, average temperature: 22°C and a rainfall close to 2,200 mm / year. The age of the animals ranged from 2 to 8. Samples were collected from them at least once a week, and their fertility was confirmed by their living offspring.The body condition score of the horses ranged from 6 to 7 (scale ranges from 1 to 9). They were subjected to the same animal husbandry conditions during stabling and their diet was based on hay composed of star grass (*Cynodonnlemfuensis*) administered at will. Animals were cared for under the institutional ethics committee guidelines.

Semen was collected three to six times from each animal using the artificial vagina method for a total of 32 ejaculates. For this purpose, a Missouri model (Minitube, Tiefenbach,Germany) artificial vagina lubricated with non-spermicidal gel and a real mare were used. The gel fraction of the ejaculate was removed by filtration, andthe semen was diluted 1:1 with anextender composed of semi-skimmed milk, sodium caseinate and sugars. Finally, it was taken to the laboratory at 5°C in an insulating transportation box.

Spermatozoa concentration was assessed from a drop of fresh semen using a photometer (Spermacue®, Minitube,Tiefenbach, Germany). Sperm motility (MOT) was assessed through phase contrast microscopy (Eclipse E200®, Nikon Inc., Tokyo, Japan) in at last five fields of observation(Gamboa*et* al. 2005). Normal morphology (NM) and sperm vitality (SV) were assessed using the modifiedeosin-nigrosin test (Brito *et al.* 2011).A droplet of semen and a droplet of eosin-nigrosin(Sigma-Aldrich, St. Louis, USA) were placed on a microscope slide, mixed, smearedand placed on a warming plate at 37°C. Subsequently, 200 spermatozoa were assessed individually in an Eclipse E200 (Nikon Inc., Tokyo, Japan) phase contrast microscope. The functional integrity of the cell membrane (MI) of the spermatozoa was assessed using the hypo-osmotic swelling test (HOS) (Neild *et al*. 1999). To achieve this, 100 µL of semen were added to a tube with 500 µL of a hypo-osmotic sucrose solution 5.4% (100 mOsmol / L). This mixture was incubated at 38.5ºC for 30 minutes. Then the reaction (tail curling) of 200 spermatozoa was assessed in at least 5 fields of observation using an Eclipse E200 (Nikon Inc., Tokyo, Japan) phase contrast microscope. Regarding selection criteria, the only ejaculates processed were those with the following minimal parameters:a concentration of 100 x 10^6 spermatozoa / mL, a MOT of 60% and a NM of 70% (Pérez-Osorio *et al*. 2008).

Semen cryopreservation was carried out using a modified freezing protocol (Bustamante *et al.* 2009). The semen was centrifuged for 15 min at 1,200 x g, then the pellet was resuspended in anextender composed of semi-skimmed milk, sodium caseinate and sugars, supplemented with 4% egg yolk and 5% *N,N*-dimethylformamide (Sigma- Aldrich, USA); the quantity was sufficient for reaching a final concentration of 100 x 10^6 spermatozoa / mL.Extended semen was divided into three aliquots which were then randomly assigned to one of three treatments: thymol 50 µM(Sigma-Aldrich, St. Louis, USA), isoespintanol 40 µM or control (no antioxidant). Isoespintanol was extracted from the leaves of *Oxandracfxylopioides*(*Annonaceae*) after drying and grinding them (Rojano*et al*. 2008). Subsequently, the semen was cooled at 5°C for 15 min and then packed in 0.5 mL straws. Straws were then subjected to liquid nitrogen vapor for 15 min. Finally, they were stored in liquid nitrogen. After a storage period of at least two weeks, semen straws were thawed in a water bath at 37°C for 1 min for post-thaw assessment.

Post-thaw MOT, SV, NM and MI were assessed using the previously described procedures. Additionally, the internal mitochondrial membrane potential (MMP) of spermatozoa was assessed using a fluorescence microplate reader and the JC-1 cationic fluorescent probe(Molecular Probes™, Waltham, USA) (Gravance*et al*. 2000). Semen was diluted until reaching a concentration of 20 x 10^6 spermatozoa / mL and then incubated for 20 minutes at 35°C with JC-1 in DMSO (2 mM). Readings were performed using an LS 55 fluorescence spectrometer (Perkin Elmer, Waltham, USA*)* at the excitation/emission wavelengths for low and high internal mitochondrial membrane potentials (loMMP and hiMMP) of514 / 529 nm and 585 / 590 nm, respectively.

Completely randomized mixed models were fitted for each quality parameter for post-thaw semen. Each model included the fixed effect for the treatment and the random effect for the ejaculate nested within the stallion. The covariables included in each model were defined through a Pearson correlation analysis. Given the use of parametric tests, data normality was assessed with the Shapiro-Wilk test. The comparison of the means between treatments was done with Tukey's test. The significance level used for all assessments was $p<0.05$. All analyzes were conducted using the SAS version 9.2software (SAS Inst. Inc., Cary, NC).

RESULTS AND DISCUSSION

A total of 32 semen samples were assessed and processed using different treatments. Table 1 shows the parameters considered in the assessment of fresh stallion semen.The results for semen volume and concentration had a high variability among the samples tested. In contrast, less variation was observed for the remaining quality parameters.

Table 2 shows the results of the statistical models for the various quality parameters considered when assessing cryopreserved semen after thawing. The random effect of the ejaculate nested within the stallion was statistically significant ($p<0.05$) for all the fitted models, shows that the variability existing in semen quality is largely

Table 1. Results of the assessment of fresh semen.

Variable	n	Mean	CV	SE
Volume (mL)	32	32.42	62.48	3.58
Concentration*	32	236.65	45.80	19.1
MOT (%)	32	75.62	13.40	1.79
SV (%)	32	80.65	9.80	1.39
NM (%)	32	82.40	8.39	1.22
MI (%)	32	74.68	14.3	2.13

*The results for concentration are expressed as spermatozoa x 10^6 / mL.CV: coefficient of variation (%).SE: standard error: MOT: sperm motility.SV: sperm vitality. NM: normal morphology. MI: functional integrity of the cell membrane.

Table 2. Results for the post-thaw semen assessment models.

Variable	n	Mean	CV	R^2	P value
MOT (%)	256	40.29	17.14	0.81	<0.0001
SV (%)	256	53.14	12.96	0.78	<0.0001
NM (%)	256	83.38	5.01	0.46	<0.0001
MI (%)	256	42.81	17.51	0.78	<0.0001
hiMMP (RFU)	118	207.59	7.16	0.97	<0.0001
loMMP (RFU)	118	25.78	8.48	0.96	<0.0001

n: number of straws assessed. CV: coefficient of variation (%).SE: Standard error. R^2: coefficient of determination. MOT: sperm motility. SV: sperm vitality. NM: normal morphology. MI: cell membrane integrity. hiMMP: mitochondrial membrane potential (high potentials). loMMP: mitochondrial membrane potential (low potentials).

attributable to the stallion and even to ejaculates from the same animal. The variability existing among males is one of the most important factors affecting the post-thaw viability of stallion semen (Hoffmann *et al*. 2011).

The fixed effect of the treatment was significant ($p<0.05$) for MOT, SV and MI. With the exception of the statistical model for normal morphology, all the fitted models for post-thaw semen assessment showed high coefficients of determination (R^2) (Table 2). Hence, we can infer that the fixed and covariate effects considered in this study greatly explain the variability of each parameter.

Cryopreservation is known to reduce various quality parameters of stallion semen (Brum *et al*. 2008;Waheed*et al*. 2013). In this study a strong decrease in the motility, vitality and integrity of the cell membrane of cryopreserved semen was observed with respect to fresh semen (Tables 1 and 2). The alterations caused by cryopreservation have been attributed to factors such as severe temperature changes, the formation and dissolution of ice crystals in the extracellular medium and oxidative stress. Furthermore, it is known that osmotic stress, both under hypotonic and hypertonic conditions, can cause oxidative stress due to the increased production of the superoxide anion (Burnaugh*et al.* 2007, Ortega-Ferrusola*et al.* 2008).

In order to reduce the oxidative stress caused when freezing stallion semen, different antioxidant molecules have been assessed; however, not only have most of them

Table 3. Post-thaw semen assessment by treatment.

Treatment	n	MOT (%)	SV (%)	NM (%)	MI (%)
Control	84	36.36± 1.41^b	51.84± 1.35^b	84.59± 1.33^a	36.53± 1.15^b
Thymol	86	37.32± 1.13^b	50.65± 1.10^b	84.63± 1.33^a	38.05± 1.26^b
Isoespintanol	86	47.01± 1.06^a	57.09± 0.96^a	84.04± 1.24^a	44.43± 1.10^a

Results are presented as mean ± standard error. Different letters indicate statistically significant difference ($p<0.05$). n:number of straws assessed.MOT: sperm motility. SV: sperm vitality. NM: normal morphology.MI: cell membrane integrity.

lacked favorable effects, but they have also been deleterious to post-thaw semen quality (Hussain *et al*. 2011, Martins *et al*. 2012). Recent studies have identified molecules that improve some semen parameters. This has been attributed mainly to their antioxidant nature (Lisboa*et al.* 2012;Gibb *et al.* 2013, Martins *et al*. 2015).

The results of this study show that supplementing stallion semen with isoespintanol during the freezing process has a positive effect on the post-thaw motility, vitality and membrane integrity of spermatozoa ($p<0.05$). In contrast, no effect of thymol on post-thaw semen quality was found (Table 3). As a hypothesis, this result could be attributed to the decrease in ROS in stallion semen, which could have been mediated by the action of the antioxidant mechanism by transfer of a hydrogen atom (HAT) that is predominant in antioxidants such as thymol and isoespintanol. Hence, the antioxidant role of both molecules could lie in the interruption of the second stage of the propagation chain of lipid oxidation by neutralizing lipid radicals (ROO•) (Rojano*et al*. 2008). Another proposed mechanism is direct inhibition of hydrogen peroxide, since thymol has been observed to be capable of neutralizing this ROS (Özkan and Erdoðan 2011).

The differences between isoespintanol and thymol regarding the ability to protect stallion semen from the harmful effects of freezing could be explained by the former's properties as a better radical scavenger and reducing agent than thymol. Furthermore, the results obtained from evaluating the total antioxidant capacity (TAC) of both antioxidants proved that isoespintanol, as antioxidant, is twice as active as thymol. This was attributed to a highly stable antioxidant action of isoespintanol, since it forms intra- and intermolecular hydrogen bridges that make its reactive center (an O-H bond) available to give off electrons to other free radicals. Thymol, on the other hand, creates intermolecular bridges with solvents, thus reducing their antioxidant activity (Rojano*et al*. 2008). Likewise, it is possible to consider that there are differences in the interaction that these molecules could have with the antioxidant enzyme action of stallion semen. Al-Malki (2010) observed changes in the enzyme activity of the liver tissue of mice as a result of the effect of thymol.

Among the various apoptosis markers that can be used

Table 4. Post-thaw mitochondrial membrane potential by treatment.

Treatment	n	hiMMP(RFU)	loMMP(RFU)
Control	39	206.57 ± 5.21[a]	25.79 ± 0.47[a]
Thymol	40	208.03 ± 5.20[a]	25.92 ± 0.47[a]
Isoespintanol	40	208.90 ± 5.28[a]	25.63 ± 0.47[a]

Results are presented as mean ± standard error. Different letters indicate statistically significant difference ($p<0.05$). n: number of straws assessed. hiMMP: mitochondrial membrane potential (high potentials). loMMP: mitochondrial membrane potential (low potentials).

to predict the freezing resistance of equine spermatozoa, mitochondrial activity has the highest diagnostic power (Ortega-Ferrusola*et al.* 2009). Moreover, the reduction in mitochondrial activity of spermatozoa as a result of cryopreservation has been discussed extensively (Schober*et al*. 2007). In this study, none of the antioxidants assessed affected the MMP of the spermatozoa (Table 4). The inability of thymol and isoespintanol to affect MMP could be explained by: a low bioavailability of both molecules inside cells, an inability of both antioxidants to take part in the oxidative phenomena of mitochondria or a small amount of structural or functional damage to the mitochondria which prevents them from having any effect. Since there are no previous studies assessing the usage of thymol and isoespintanol on stallion semen, further research on its effects on the various structural levels and metabolic pathways of spermatozoa is required.

We conclude that isoespintanol improves the motility, vitality and integrity of the cell membrane of stallion spermatozoa subjected to freezing.

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