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Effect of Adding α-tocopherol on Fertility Parameters in Bovine Semen Cryopreservation

L.N. Espinosa-García[1], F.G. Véliz-Deras[1], R.A. Delgado-González[1], L.R. Gaytán-Alemán[1], J.L. Morales-Cruz[1], D.I. Carrillo-Moreno[1], J. Moran-Martínez[1]

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ABSTRACT

Background: Cryopreservation of bovine semen affects survival of spermatozoa. It is necessary to improve semen quality to increase fertility. The aim was to determine the effect of α-tocopherol added to the bovine semen diluent at different time of freezing, upon sperm quality post thawing.

Methods: Semen was collected from Charolais breed bulls. The semen was divided into two portions and one portion was added with α-tocopherol and the other was used as control. These portions were further divided into three parts and frozen for 8, 11 and 15 minutes, respectively. After 60 days of cryopreservation, the progressive motility, non-progressive motility, absence of motility, vitality and DNA fragmentation were evaluated.

Result: The lower percentages of DNA fragmentation were treated with antioxidants (15.52, 14.83 and 14.71%). The highest vitality and progressive motility were found in the sample with α-tocopherol and frozen for 11 min with 72.91 and 58.61%, respectively. The addition of α-tocopherol to the semen diluent decreased damages to spermatozoa and enhanced the semen quality.

Key words: Antioxidant, Bovine, Freezing, Semen, Spermatozoa, Vitamin E.

INTRODUCTION

The use of frozen semen in bovine artificial insemination has allowed for great advances to animal improvement. Nonetheless, it has a lower fertility when compared to fresh semen due to the cryoinjury which damages 50% of the spermatozoids (Al Naib *et al.,* 2011). These damages are attributed to oxidative stress and cold shock among others (Bailey *et al.,* 2000; Campanholi *et al*., 2017; Pathak *et al*., 2020). The oxidative stress mainly occurs at the time of temperature reduction, were polyunsaturated fatty acids (PUFAs) of the plasmatic membrane are united with oxygen, which leads to the production of reactive oxygen species (ROS) that damage the spermatozoa (Bailey *et al.,* 2000; Gadea *et al*., 2013). The excessive production of ROS negatively alters motility, membrane function, viability and DNA fragmentation (Batool *et al*., 2012). Different antioxidants have been tested to reduce these effects (Kumar *et al*., 2019; Chaturvedi *et al*., 2022). One of them is α-tocopherol, this is the active form of vitamin E and can eliminate radicals such as peroxyl and alkoxyl, reduce malondialdehyde and increase reduced glutathione levels, preventing the propagation of free radicals in the membrane (Xiang-Rong *et al*., 2017; Bhardwaj *et al*., 2022).

The aim was to determine the effect of α-tocopherol added to the bovine semen diluent at different time of freezing, upon sperm quality post thawing.

MATERIALS AND METHODS

Experimental design

The research was performed from April to July of 2020 at the Universidad Autónoma Agraria Antonio Narro premises. Three Charoláis breed bulls were used. A semen collection from each bull was evaluated and divided into six portions. Three of these portions received α-tocopherol and the rest were used as control. For freezing, all six portions from each bull were divided into three groups, each with two samples: one with α-tocopherol and the other without. Afterwards, they were frozen by exposure to nitrogen vapor during 8, 11 and 15 min respectively. At the end, half the portions had α-tocopherol (E+8 min, E+11 min, E+15 min) and the rest without antioxidant (8 min, 11 min, 15 min). The straws were kept submerged in liquid nitrogen until their analysis 60 days later.

Experimental animals and semen collection

Three bulls in the age of 4 years were used for semen collection. They were kept under the same housing conditions and nutrition with water ad libitum. Semen collection were performed using artificial vagina and the initial evaluation was performed immediately using a Zeiss® optic microscope (Carl Zeiss MicroImaging GmbH, series No. 3108027112). Motility was microscopically (400×)

[1]Department of Cellular Biology and Ultrastructure, Autonomous University of Coahuila, UAdeC, Torreón, Coahuila, México.

Corresponding Author: J. Moran-Martínez, Department of Cellular Biology and Ultrastructure, Autonomous University of Coahuila, UAdeC, Torreón, Coahuila, México. Email: javmoran@yahoo.com

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evaluated using a preheated (37°C) slide. Sperm concentration was determined with a Spermacue® digital counter (Minitube of America, Inc. Series No. 1020204133. EE. UU.). Samples with a motility ≥85 %, volume of ≥10 mL and a concentration of ≥900X10^6 SPZ • mL^{-1} were processed. Semen collections that complied with the stablished parameters, were mixed with a diluent 1:1 (v/v) proportion until an 80×10^6 SPZ • mL^{-1} concentration was achieved.

Diluent preparation with or without α-tocopherol

The diluent used was Optidyl® (Biovet, Francia) brand. It was diluted at a 1:1.5 mL proportion with distilled water. The resulting dilution was divided into two parts. The first portion was used as an antioxidant group to which 5 mg • mL^{-1} of α-tocopherol (Membrillo-Ortega *et al*., 2011) were added, the other portion only contained the diluent and distilled water. Once the dilutions were performed, they were heated to 37°C for mixing with the semen. Afterwards, both portions (with and without antioxidant) were cooled to 4°C for 4 h and, immediately placed in 0.5 mL straws with a sperm concentration of 40×10^6 SPZ • mL^{-1} per straw and sealed with polyvinyl alcohol. Subsequently, they proceeded to freezing.

Freezing and thawing of straws

Vapor freezing was performed by the polystyrene foam box method using liquid nitrogen (Nasiri *et al.,* 2012). The freezing process was the same for all samples, with the difference that, for each group, the exposure time to nitrogen vapors changed (8, 11,15 min). All the straws were pooled and frozen in separate groups (8, 11 and 15 min). For thawing, semen straws were immersed in a water bath at 37°C for 40 seconds.

DNA fragmentation

This was determined with the sperm chromatin dispersion test (SCD) given by Fernández *et al.* (2003). The adaptation of the technique to bovine semen was in the control sample, in which, to cause damage to the chromatin, the use of peroxidation by DNase was changed. For this, 100 µL of the semen sample was placed in an Eppendorf tube and centrifuged at 12,800 G for 10 min. The excess liquid was then removed, replacing it with 100 µL of the enzymatic solution, then it was stirred and allowed to stand for 30 min, at which time the steps described for the technique were followed.

Evaluation of motility and mobility

Motility was evaluated on slides preheated to 37°C. To avoid ambiguities in the subjective evaluation, the evaluation criteria were categorized according to the WHO (2010) manual. Three types of movements were recorded: progressive motility (PM), non-progressive (NP) and no motility (NM). With this procedure a confidence interval of at least 95% was reached. The same counting method was used for percentage viability and DNA fragmentation. Viability was evaluated with the eosin-nigrosine (E/N) staining technique (WHO, 2010; Nasiri *et al*., 2012).

Statistical analysis

The statistical package SPSS (IBM Corp. Released, 2017) was used. For the time and antioxidant variables effect, a Kolmogorov-smirnov normality test was performed. Variables that presented a normal distribution were analyzed by and ANOVA and Tukey tests. For data that did not have a normal distribution, a Kruskal-Wallis tests was used. For the α-tocopherol effect upon each variable a Student-t and Mann-Whitney U (non-normally distributed data) tests were used. Results are presented as mean ± standard error. A $p \leq 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

DNA fragmentation

Oxidative stress has a detrimental effect on DNA, being able to fragment chromatin and trigger apoptosis (Aitken *et al.,* 2015). In this study, the lowest percentages of damage were in the three treatments that were added with antioxidant (E+8 min, E+11 min y E+15 min) (Table 1). The effect obtained by the addition of α-tocopherol on DNA, vitality and motility are shown in Fig 1. When analyzing the staining of the halos of the sperm cells that did not contain α-tocopherol, they showed a higher proportion of large halos, which evidenced fragmented DNA (Fig 2). These findings indicate that α-tocopherol added to the diluent decreases DNA fragmentation. DNA fragmentation occurs due to the exposure of the nucleus to high ionic strength during cryopreservation inducing deterioration of the chromatin assembly. This deterioration causes the DNA to be exposed

Table 1: Effect of adding α-tocopherol to the Optidyl® diluent and different times of exposure of bovine semen to nitrogen vapors upon motility percentage, viability and DNA fragmentation post-thawing.

α-tocopherol and/or time	PM	NP	NM	Viability	DNA fragmentation
E+8 min	46.26±3.71^{bc}	10.9±1.97	42.83±2.64^{abc}	63.28±3.14^{b}	15.52±0.69^{bc}
E+11 min	58.61±1.24^{a}	10.11±0.79	31.27±1.09^{d}	72.91±1.39^{a}	14.83±0.06^{c}
E+15 min	49.12±4.16^{b}	10.16±0.13	40.7±4.05^{bc}	65.25±4.87^{ab}	14.71±0.20^{c}
8 min	37.47±1.42^{c}	13.33±1.38	49.32±0.78^{a}	47.08±1.96^{d}	17.19±0.11^{a}
11 min	48.70±0.7^{b}	13.96±1.53	37.32±1.32^{cd}	61.94±0.45^{bc}	16.53±0.20^{a}
15 min	40.73±6.71^{bc}	11.18±1.37	48.07±7.37^{ab}	53.59±5.98^{cd}	16.33±0.20^{ab}

Means with different letters between columns are statistically different (Tukey test P≤0.05) ± Mean standard error. Letter E on the first column refers to the use of α-tocopherol (PM= Progressive motility, NP= Non progressive motility, NM = Non-motile).

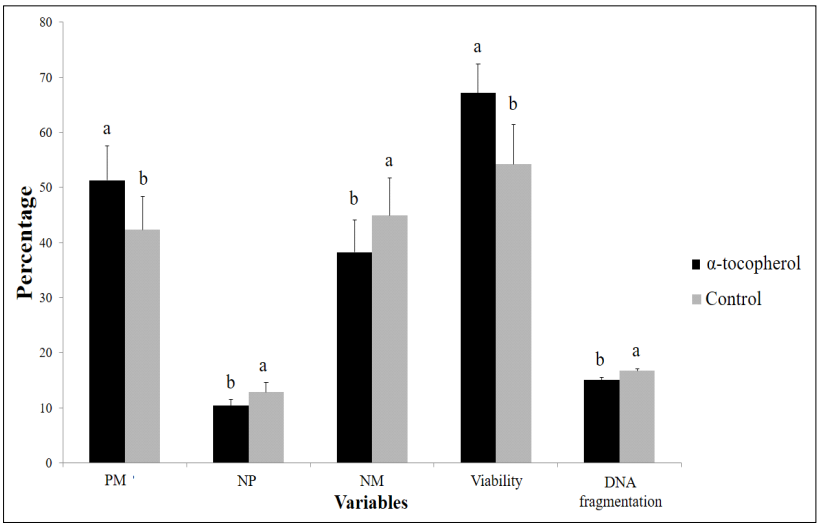


Fig 1: Effect of adding α-tocopherol to the Optidyl® diluent and different times of exposure of bovine semen to nitrogen vapors upon motility percentage, viability and DNA fragmentation post-thawing.

Means with different letters between columns are statistically different (Tukey test P≤0.05) ± Mean Standard Error. Letter E on the first column refers to the use of α-tocopherol. (PM= Progressive motility, NP= Non progressive motility, NM = Non-motile).

to oxidation through additional or intracellular ROS (Yeon-Ji *et al.,* 2008; Simões *et al*., 2013). Likewise, the influx of calcium ions after thawing, possibly promotes further division of nucleoprotein and DNA through endogenous protease and nuclease stimulation (Métayer *et al.,* 2002). Therefore, if ROS are decreased with the addition of α-tocopherol, the damage caused to the genetic material by oxidation is less, as observed in this study.

Vitality

The best percentage of vitality was 72.91% (Table 1), similar to those of Batool *et al*. (2012) with 72.3%. The lowest values were observed in two treatments that did not add α-tocopherol. In our results, the best parameters were obtained in samples containing α-tocopherol. In general, upon any stress free radicals such as lipid peroxyl destabilize the plasma membrane due to their tendency to extract hydrogen atoms from PUFA to stabilize themselves. This process creates carbon-centered lipid radicals that combine with oxygen to generate more peroxyl radicals. In turn, they also extract hydrogen from adjacent PUFAs to stabilize again, generating a chain reaction of lipid peroxidation (Aitken *et al.,* 2015). This process affects the microarchitecture of the membrane and changes the functions of integral proteins. These are critical for motility, the functioning of ATP-dependent ion pumps and the regulation of ion channels. Then adding α-tocopherol donates electrons and neutralizes free hydroxyl radicals and superoxide anions. Thus, the lipid peroxidation chain reaction is reduced, increasing viability (Majzoub and Agarwal, 2018; Bhardwaj *et al*., 2022). Therefore, adding α-tocopherol decreases damage to sperm cell viability.

Motility

The results obtained in this study show a positive effect of

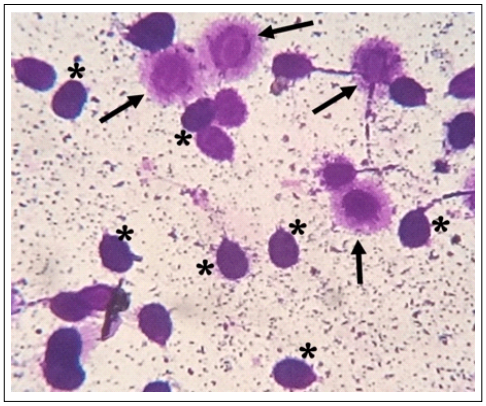


Fig 2: DNA fragmentation in a sample without adding α-tocopherol. Sperm with fragmented DNA (arrows) show chromatin scattering. Sperm with non-fragmented DNA do not show chromatin scattering (asterisks).

α-tocopherol on PM and NM (Table 1). In both, the best percentages were obtained when the semen was exposed to nitrogen vapor for 11 min with previous addition of α-tocopherol; which indicates that it is the time with the least deleterious effect. These results are the effect of the potentiation of α-tocopherol to superoxide dismutase, glutathione peroxidase and peroxidine isoforms located in the middle part of the sperm to protect against ROS damage (Nicholls and Ferguson, 2013). When ROS are high, they negatively affect sperm movement (Darr *et al*., 2016; Chaturvedi *et al*., 2022). In this way, by protecting the middle part from oxidation, mitochondria are also protected, improving sperm motility and viability since it is the organelle that provides ATP for sperm movement (Moraes and Meyers, 2018; Pathak *et al*., 2020). In addition, this protection provides structural support to the flagellum, increasing its

stability, defending it from hypoosmotic stress (Lindemann and Lesich, 2016). This positive effect of α-tocopherol in bovine semen has been reported in other investigations (Batool *et al*., 2012; Zhao *et al*., 2015) and similar results have been obtained in pigs (Yeon-Ji *et al.,* 2008) and equines (Nogueira *et al*., 2015). Therefore, adding α-tocopherol to the bovine semen diluent and exposing it to 11 min to nitrogen vapor provides the best percentage of motility. Further research is recommended to elucidate how α-tocopherol influences sperm movement discovered by Gadêlha *et al*. (2020).

CONCLUSION

Addition of α-tocopherol to the diluent of bovine semen decreases damage to motility, viability and DNA fragmentation post-thawing. In addition, the best results are found when exposing semen to nitrogen vapors for 11 min during cryopreservation. Based on our findings, we propose that sperm quality in terms of fertility parameters could be improved by adding α-tocopherol to semen.

Conflict of interest: None.

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