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Evaluation of the quercetin and vitamin E addition to the cryopreservation medium of sheep semen on *in vivo* fertility

Evaluación de la adición de quercetina y vitamina E al medio de criopreservación de semen ovino sobre la fertilidad *in vivo*

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ABSTRACT

Cryopreservation induces oxidative stress that has adverse effects on post-thawed semen quality. The objective was to evaluate the effect of adding quercetin to sheep semen cryopreservation medium. Semen was collected from three sheep stallions by artificial vagina, and a commercial diluent was used. Treatments were: control; quercetin 200 µM; vitamin E 100 µM; and the quercetin and vitamin E combination. Vitality, motility, acrosome integrity and fertility were evaluated *in vivo*. Numerical variables were analyzed with ANOVA and gestation rate with chi-square. In the evaluation of sperm motility characteristics, no significant difference was found between treatments. The treatment with the highest percentage of live spermatozoa with intact acrosome was quercetin 200 µM (22.33±2.51%) compared to the other treatments (P<0.05). *In vivo* fertility was not statistically significant, but a numerical difference in the gestation percentage was found with the addition of 200 µM quercetin (51.92%) compared to other treatments. In conclusion, the addition of 200 µM quercetin to the ovine semen cryopreservation medium improved the vitality and integrity of the acrosome, but not the *in vivo* fertility percentage.

Keywords: antioxidant, freezing, gestation rate, sperm characteristics.

RESUMEN

La criopreservación induce estrés oxidativo que tiene efectos adversos en la calidad post-descongelado del semen. El objetivo fue evaluar el efecto de la adición de quercetina al medio de criopreservación de semen ovino. El semen se colectó de tres sementales ovinos mediante vagina artificial, y se utilizó un diluyente comercial. Los tratamientos fueron: control; quercetina 200 µM; vitamina E 100 µM; y la combinación de quercetina y vitamina E. Se evaluó la vitalidad, motilidad, integridad del acrosoma y fertilidad *in vivo*. Las variables numéricas se analizaron con un ANOVA y la tasa de gestación con ji cuadrado. En la evaluación de las características espermáticas de motilidad no se encontró diferencia significativa entre tratamientos. El tratamiento con mayor porcentaje de espermatozoides vivos con acrosoma intacto fue quercetina 200 µM (22.33±2.51%) comparado con los demás tratamientos (P<0.05). La fertilidad *in vivo* no fue estadísticamente significativa, pero se encontró una diferencia numérica en el porcentaje de gestación con la adición de 200 µM de quercetina (51.92%) en comparación a los demás tratamientos. En conclusión, la adición de 200 µM de quercetina al medio de criopreservación de semen ovino mejoró la vitalidad e integridad del acrosoma, pero no el porcentaje de fertilidad *in vivo*.

Palabras clave: antioxidante, congelación, tasa de gestación, características espermáticas.

INTRODUCTION

The objective of cryopreservation is to maintain the viability and functionality of spermatozoa at low temperatures; however, this causes damage and deficiencies in spermatozoa. At the time of cryopreservation, spermatozoa are exposed to physical and chemical impacts that hinder viability, decrease motility, damage the acrosome and decrease their fertility (Mata-Campuzano *et al*., 2015). This is partly due to the fact that cryopreservation induces oxidative stress, as a result of excessive formation of reactive oxygen species (ROS; El-Khawagah *et al*., 2020), since membrane polyunsaturated fatty acids are susceptible to ROS damage, and the sperm cytoplasm contains low concentrations of free radical scavenging enzymes (Karimfar *et al*., 2015).

The addition of antioxidants in semen freezing diluents results in improved post-thaw characteristics, as they protect polyunsaturated fatty acids in spermatozoa, thus preventing oxidative stress damage. Numerous plants have been shown to have an antioxidant effect, as they contain flavonoids with antioxidant capacities such as quercetin, and antioxidant vitamins such as C, E and A (Berkovich *et al*., 2013). Quercetin is an antioxidant flavonoid commonly present in vegetables, capable of scavenging ROS (El-Khawagah *et al*., 2020) and has been reported to improve the quality of post-thawed semen (Silva *et al*., 2012; Gibb *et al.,* 2013). Vitamin E is a lipophilic antioxidant that protects unsaturated fatty acids against peroxidation as it is a potent scavenger of peroxyl radicals and a major inhibitor of the lipo-peroxidation chain reaction in animals (Allai *et al.,* 2018), and it is commonly used as an antioxidant in semen cryopreservation (Abdi-Benemar *et al*., 2015; Benhenia *et al*., 2016).

Compared to other insemination techniques, artificial insemination by laparoscopy is used as an alternative for the use of cryopreserved semen, as the damage that occurs to spermatozoa at the time of cryopreservation limits post-insemination fertility with vaginal or cervical techniques (Allai *et al.,* 2018). However, there is no report of *in vivo* fertility evaluation following the addition of antioxidants such as quercetin and vitamin E in sheep cryopreserved semen. Therefore, the aim of this study was to evaluate the effect of quercetin addition in sheep semen cryopreservation medium on sperm characteristics and *in vivo* fertility.

MATERIAL AND METHODS

Study area and animals

The study was conducted at the Animal Research and Reproduction Laboratory of the Autonomous University of Ciudad Juarez (UACJ). Artificial insemination was performed on a farm located in Juárez City at coordinates 31°43'12.9" N and 106°27'39.0" W, in the north of Chihuahua State. Semen processing and artificial insemination were performed during the reproductive season (July to February).

Semen was collected from three Katahdin stallions, approximately three years old, with a body condition of 4 (scale of 1-5). To evaluate the fertility of the cryopreserved semen, 201 hair breed females (Katahdin, Pelibuey, Blackbelly), aged between 2 and 4 years and with a body condition between 2 and 4 on the 1-5 scale, were inseminated. Animals were fed alfalfa, commercial concentrate and water *ad libitum*. All procedures in the present study were performed according to Mexican animal care and health techniques (NOM-051-ZOO-1995) and with the approval of the Institutional Ethics and Bioethics Committee of the UACJ (CIEB-2019-1-093).

Semen collection and pre-cryopreservation evaluation

Each stallion was worked individually for semen collection, using the artificial vagina technique. Once the samples were collected, they were kept in a water bath at 37.5 °C (Presicion Water 282, Thermo Scientific, USA) while the three samples were collected and evaluated. Semen collection was performed twice a week for six weeks. Twelve ejaculates per ewe were evaluated, for a total of 36 ejaculates evaluated.

Pre-cryopreservation evaluation was performed by taking 10 µL of each sample using a pipette (Magnetic-Assist Pipette, Rainin, USA), which was deposited in a 20 µm standard counting chamber (Leja Chamber, Leja Products, The Netherlands), to be evaluated by computer-assisted semen analysis (CASA; AndroVision, Minitube, Germany) with an upright microscope (AxioScope.A1, Zeiss, Germany). Progressive motility (%) and sperm concentration (cells/mL) were evaluated. The following were established as minimum levels required for freezing: ejaculate volume ≥ 0.5 mL, progressive motility ≥ 80%, and a concentration of $3x10^9$ spermatozoa/mL (Câmara *et al.,* 2011).

Dilution and processing of semen.

Ejaculates were processed individually, as they were used for artificial insemination. A dilution protocol was performed with a commercial diluent (Two Step; Continental Plastic Corp., USA) with 6% glycerol (v/v) and 10% egg yolk (v/v). The dilution was worked at a temperature of 37 °C. The ejaculate obtained was diluted to a concentration of $120x10^6$ motile spermatozoa per mL. Once diluted, the semen was packed in 0.25 mL straws, with a concentration of $30x10^6$ motile spermatozoa per straw, and stored at 5 °C for two hours.

Once the straws were refrigerated and equilibrated at 5 °C, they were placed in an ice chest 5 cm from the nitrogen mirror for 10 minutes, then dropped into nitrogen and allowed to stand for 5 minutes. The straws were then placed in a cryogenic thermos for preservation; they remained there until the time of analysis and later for use in artificial insemination by laparoscopy.

Treatments

The diluted samples were fractionated into four similar portions and four treatments were performed: control treatment was performed in the conventional manner; quercetin (Q) treatment, 200 µM quercetin (Q4951, Sigma Aldrich, USA) was added; vitamin E (VE) treatment, 100 µM vitamin E (47786, Sigma Aldrich, USA) was added; and Q + VE treatment, a combination of quercetin (200 µM) and vitamin E (100 µM) was added.

Thawing of semen samples

Thawing of the semen was carried out by placing each straw in a water bath (Presicion Water 282, Thermo Scientific, USA) at 37.5 °C for 40 seconds. To evaluate semen characteristics and acrosome integrity, a total of six straws per treatment per sheep (24 straws) were randomly selected and thawed.

Evaluation of post-thawing seminal characteristics

To evaluate seminal characteristics, 10 µL of semen were placed in the standard counting chamber to evaluate motility (%), progressive motility (%) and fast motility (%), using the CASA system. Table 1 shows the configuration of the CASA system used to evaluate sheep semen.

Evaluation of acrosomal membrane damage.

Ten µL of the semen sample was taken and deposited in a 1 mL microtube (Eppendorf, Germany) and mixed with 10 µL of trypan blue reagent (T8154, Sigma Chemical Co., USA), and 10 µL of the mixture was placed on a slide to make a smear. Subsequently, the smear was fixed with ethanol for two minutes, rinsed with distilled water and allowed to dry. Once fixation was completed, the slides were placed in a Giemsa-stained container (48900, Sigma Chemical Co., USA) where they remained for 18-20 hours. The slide was then rinsed to remove excess dye with distilled water and allowed to dry. Evaluation of sperm cell staining was performed by light microscopy at 100X (PrimoStar, Carl Zeiss, Germany). To obtain a representative mean of each straw, three smears per straw were performed making a count of 100 spermatozoa per smear. Spermatozoa were classified

into three types: live spermatozoa with intact acrosome (stained purple in the acrosomal region), live spermatozoa with damaged acrosome (no purple accumulation in the acrosomal region) and dead spermatozoa (cytoplasm stained blue).

Table 1. Configuration of the CASA* system used to evaluate sheep semen

Variable	Configuration
Horizontal/vertical resolution	1024 pixels x 1024 pixels
Horizontal/vertical pixel size	5.5 µm x 5.5 µm
Frames per second	Up to 101 frames per second
Area	10-100
Tail detection	5 µm
Camera depth	20 µm
Pixel ratio	0.54 µm
Reference volume	
Width	555.12 µm
Height	555.12 µm
In-motile spermatozoa	ALC < 20.00 µm s^{-1} and FBC < 10.00 µm s^{-1}
Motility	
Local motility	VCL < 20.00 µm s^{-1} and VLR < 10.00 µm s^{-1}
Progressive motility	
Circular motility	Radio > 10.00 µm s^{-1} a < 80.00 µm s^{-1} and rotation > 0.70 µm s^{-1}
Slow motility	VCL < 80.00 µm s^{-1}
Fast motility	VCL < 80.00 µm s^{-1}

* Androvision, Minitube, Germany.
ALC, head lateral displacement amplitude; FBC, tail beat frequency; VCL, curvilinear velocity; VLR, straight-line velocity.

Artificial insemination by laparoscopy and pregnancy diagnosis

Ewes were synchronized in estrus using intravaginal sponges (Cronogest CR®, Intervet International, The Netherlands) impregnated with 40 mg fluorogestone acetate for 12 days. Upon sponge removal, 200 IU of equine chorionic gonadotropin (Novormon® 5000, Virbac, Argentina) was applied to each ewe. Estrus detection was performed by introducing a sire attached with a rope (without allowing the female to mount) to a pen with the previously synchronized females, 16 h prior to insemination (Alhamada *et al.*, 2017). Females in estrus were inseminated 55 h after sponge removal by laparoscopy technique. Prior to insemination, straws were thawed in a thawing thermos (Cito Products Incorporated, USA) at 37.5 °C for 40 sec. Semen was applied intrauterine with the aid of a laparoscope (250 W Halogen, Wolf, USA) and an aspic (Aspic, IVM, France). The insemination dose (0.25 mL) was equally distributed in both uterine horns. A total of 201 ewes were inseminated, distributed by treatment as follows: control, 50 ewes; quercetin (Q), 52 ewes; vitamin E (VE), 50 ewes; and Q + VE treatment, 49 ewes.

Gestation diagnosis was performed by ultrasound with a 6.5 MHz linear transducer (Kaixin, Xuzhou Kaixin Electronic Instrument CO., China), rectally, 35 days after insemination, regardless of the treatment performed on the extender used for semen cryopreservation. Fertility (gestation rate) was determined by obtaining the percentage of females that became pregnant out of the total number of females inseminated per treatment.

Statistical analysis

All statistical analyses were performed with the SAS 9.0 program (Inst. Cary, NC, United States of America). Percentage data were transformed into arcsine before statistical analysis. Analysis of variance was performed using the General Linear Model procedure for the numerical variables: motility, progressive motility, rapid motility, and acrosome integrity. Comparison of means between treatments was performed using Duncan's test. The chi-square test was used for gestation rate. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Effect results of different treatments on the sperm motility characteristics evaluated are shown in Table 2. In the study, no treatment presented a statistically significant difference ($P > 0.05$) in the motility, progressive motility and fast motility variables. Nevertheless, numerically, the treatment that presented the highest percentage of motility (71.57%) and progressive motility (68.13%) was the quercetin treatment compared to the other treatments. However, in the fast motility variable, the treatment with the highest percentage (numerically) was the vitamin E treatment with 31.48 %.

As mentioned, cryopreservation induces oxidative stress as a result of the formation of excess ROS, which affects the structure of the spermatozoa, and affects sperm characteristics, causing damage to DNA, lipids and proteins that protect sperm structure, as it alters enzyme systems and cell signaling pathways, which can lead to cell death. Sperm damage caused by ROS occurs during cryopreservation and thawing, and even during storage in liquid nitrogen. It is important to mention that sperm normally generate small amounts of ROS that are important for different sperm physiological processes such as capacitation, hyperactivation and oocyte fertilization (Mata-Campuzano *et al.,* 2015; Aitken, 2017); however, once these physiological levels exceed oxidative control mechanisms, oxidative stress is caused (El-Khawagah *et al*., 2020). Therefore, to reduce the damage caused by the negative effects of ROS, several authors have proposed the supplementation of antioxidants to freezing media, such as vitamin E and quercetin. For example, Sarlós *et al.* (2002), Silva *et al.* (2012), and Abdi-Benemar *et al.* (2015) have evaluated the addition of vitamin E to sheep semen cryopreservation media, and report favorable effects on acrosomal integrity, vitality, and motility. However, in the present study, no favorable effect of vitamin E addition as an antioxidant on the motility variables evaluated was found. These contradictory effects of the addition of vitamin E may be due to different components of the diluents, since Sarlós *et al.* (2002) mention that the effect of vitamin E varies in response to the sugar and buffer used in the diluents; this could also explain why in the treatment where vitamin E and quercetin were combined there was also no favorable effect on the variables evaluated. In the present study, the diluent used

contains fructose as an energy source and tris aminomethane (TRIS) as a buffer, and Sarlós *et al.* (2002) indicate that the addition of vitamin E is more effective when sucrose-based diluents contain glucose and TRIS.

Table 2. Effect of quercetin and vitamin E addition on sperm motility characteristics of cryopreserved sheep semen (Mean ± standard deviation)

Treatment	Motility (%)	Progressive motility (%)	Fast motility (%)
Control	67.20±20.82 [a]	63.52±21.31 [a]	26.65±16.68 [a]
Q	71.57±6.40 [a]	68.13±6.57 [a]	27.31±8.22 [a]
Vit E	69.36±11.41 [a]	65.92±12.62 [a]	31.48±13.15 [a]
Q + Vit E	60.07±13.16 [a]	53.95±14.50 [a]	16.93±8.72 [a]

[a] Different literals between rows indicate statistically significant difference ($P < 0.05$); Q = 200 µM quercetin; Vit E = 100 µM Vit E.; Q + Vit E = 200 µM quercetin +100 µM Vit E.

Similarly, it has been reported that the addition of quercetin in semen cryopreservation media of different species produces favorable effects on acrosomal motility and integrity (Gibb *et al.,* 2013; Seifi-Jamadi *et al.,* 2016; Seifi-Jamadi *et al.,* 2017; Ahmed *et al.,* 2019; El-Khawagah *et al*., 2020), although some authors have reported no positive effects of quercetin addition (Silva *et al.,* 2012; Banday *et al.,* 2017) or even report negative effects (Restrepo *et al.,* 2016). In the present study, although the addition of quercetin did not show a statistical difference in the motility variables evaluated, it showed a higher percentage (numerically) compared to the other treatments in the motility and progressive motility variables. The lack of a statistically different effect may perhaps be due to the excellent motility results obtained post-thawing of semen in all treatments, as normally a range of between 30 to 50% is reported for post-thawing motility (Motlagh *et al*., 2014; Abdi-Benemar *et al.,* 2015; Masoudi *et al.,* 2017), and in the present study in all treatments motility values were higher than 60 %.

In this study, by adding a concentration of 200 µM quercetin, higher percentage was obtained in motility and progressive motility (numerically) because it is a potent antioxidant that acts on spermatozoa, inhibiting free radicals, and has a more intense ROS scavenging activity than vitamin E (Stojanović *et al.,* 2001), prevents premature sperm capacitation before artificial insemination and acrosomal reaction during storage, which improves sperm lifespan (Restrepo *et al.,* 2016; Seifi-Jamadi *et al.,* 2016). In line with the above, El-Khawagah *et al*. (2020) report that the inclusion of quercetin reduces H_2O_2 and lipid peroxidation levels, which are indicators of its antioxidant effect. Also, the positive effect on motility variables may be related to the interaction with Ca^{2+}-ATPase, a key enzyme for motility regulation, since quercetin has an inhibitory effect on the Ca^{2+}-ATPase pump in the plasma membrane, and results in an elevation of Ca^{2+} levels, which has a

fundamental role in the production of cyclic adenosine monophosphate (cAMP), factors that control motility in spermatozoa (El-Khawagah *et al*., 2020).

Similar to what was found in the present study, Ardeshirnia *et al.* (2017) demonstrated that by using quercetin in sheep semen extracted from the epididymis, upon thawing spermatozoa showed better viability when using the concentrations of 5 and 10 µg/mL in the semen extender, but progressive motility and motility were not affected compared to the control treatment (0 µg/mL). On the other hand, Ahmed *et al.* (2019) used 150 and 200 µM quercetin in cryopreserved buffalo semen, and in post-thaw evaluation, these treatments were better for progressive motility and plasma membrane integrity compared to the other treatments (control, 50 and 100 µM quercetin).

Table 3 shows the percentages of live sperm with intact acrosome (LIA), live sperm with damaged acrosome (LDA) and dead sperm, obtained in the different treatments. The quercetin treatment showed a significant difference compared to the other treatments in LIA spermatozoa (P<0.05). With respect to LDA spermatozoa, a difference was found between the vitamin E (100 µM) and quercetin (200 µM) treatments, compared to the control (conventional) treatments and the combination of vitamin E (100 µM) and quercetin (200 µM) (P<0.05). The quercetin treatment (200 µM) showed a significant difference, having fewer dead spermatozoa compared to the other treatments (P<0.05), indicating higher viability.

Table 3. Effect of quercetin and vitamin E addition on sperm vitality and acrosome damage in cryopreserved sheep semen (Mean ± standard deviation)

Treatment	Live Intact Acrosome (%)	Live Damaged Acrosome (%)	Dead (%)
Control	8.66±4.50 ^{b}	48.33±4.50 ^{b}	43.00±4.00 ^{bc}
Q	22.33±2.51 ^{a}	58.66±1.52 ^{a}	19.00±2.64 ^{a}
Vit E	10.66±2.08 ^{b}	52.00±5.29 ^{a}	38.33±6.08 ^{b}
Q + Vit E	11.33±2.08 ^{b}	41.33±3.21 ^{b}	47.33±1.52 ^{c}

^{a,b,c} Different literals indicate statistically significant difference (P <0.05); Q = 200 µM quercetin; Vit E = 100 µM vitamin E.; Q + Vit E = 200 µM quercetin + 100 µM vitamin E.; Q + Vit E = 200 µM quercetin + 100 µM vitamin E.; Q + Vit E = 200 µM quercetin + 100 µM quercetin + 100 µM vitamin E.

These results may be due to the fact that, as mentioned above, antioxidants have a protective effect, since deficiencies in sperm motility and viability occur at the time of cryopreservation, and this is due to the damage caused by ROS (Karimfar *et al.,* 2015). Although ROS are normal products of cell metabolism, when there are high levels of these, ROS are harmful to spermatozoa, and are generated both at the time of

cryopreservation and thawing, damaging sperm morphology (Aitken, 2017), affecting lipids and proteins that protect spermatozoa, which can lead to cell death (Ardeshirnia *et al.*, 2017).

In this regard, , Ahmed *et al.* (2019), using 150 and 200 µM quercetin in cryopreserved buffalo semen, found that spermatozoa showed less damage in membrane integrity and acrosomal damage compared to the other treatments (control, 50 and 100 µM quercetin). In the present study, similar concentrations, 200 µM quercetin, were used in the cryopreservation medium for ovine semen and with this treatment a significant difference was obtained for the LIA spermatozoa variable, compared to the other treatments. This indicates a protective effect of quercetin on the acrosomal membrane.

Membrane integrity is important for maintaining sperm viability and it is precisely here where the main lesions occur at the time of cryopreservation-thawing, since the reduction and increase in temperature causes ultrastructural and functional damage. Membrane integrity is a fundamental requirement for sperm viability and successful fertilization (Martínez-Pastor *et al.*, 2004; El-Khawagah *et al*., 2020). Partial or total damage to the sperm acrosome results in an inability to fertilize, as seminal samples with a high proportion of altered or absent acrosomes tend to have low fertility. The use of quercetin in semen reduces lipid peroxidation of spermatozoa during freezing (El-Khawagah *et al*., 2020) and prevents their premature capacitation prior to artificial insemination (Restrepo *et al.*, 2016). This is because the presence and location of hydroxyl substitutions (-OH) and the catechol-type B-ring make quercetin an effective antioxidant, as it possesses a more intense ROS scavenging activity than vitamin E (Stojanovic *et al.*, 2001).

Gibb *et al.* (2013) report that quercetin at a concentration of 150 µM in cryopreserved equine semen improved sperm motility upon thawing, and also increased the percentage of sperm with intact acrosome compared to treatments with 200 U/mL catalase and 0.2 mg/mL cysteine. However, in the study by Seifi-Jamadi *et al.* (2016), by adding 100 µM quercetin to the cryopreservation medium for equine semen, they obtained a better result in motility, but in vitality and membrane integrity they did not find a significant effect.

Finally, Table 4 shows the fertility results (gestation rate) in the ewes inseminated by laparoscopy with the cryopreserved semen of sheep added with the different antioxidants. Contrary to expectations, because quercetin treatment improved acrosome protection, no significant statistical differences were found between treatments (P>0.05). However, the treatment that showed the highest percentage result (numerically) compared to the other

treatments in the gestation rate was the quercetin 200 µM treatment with 51.92% fertility diagnosed at 35 days of gestation.

Damage to the cells causes destabilization and even rupture of the membranes, and loss of intracellular components, for example, metabolic enzymes and ATP, with the consequent loss of sperm viability. The membrane plays an important role in sperm viability and successful fertilization of the oocyte, so it is important that it is intact at the time of thawing (Martínez-Pastor *et al.,* 2004). Chromatin damage usually starts in the peri-acrosomal region, the basal regions of the sperm and subsequently expands to other regions of the nucleus during cryopreservation (Sousa *et al.,* 2016), significantly affects the acrosome status or even the sperm vitality, leading to an inability to fertilize. The low fertility reported in artificial insemination when using cryopreserved semen could be due to the damage caused to the spermatozoa during the cryopreservation-thawing process (Sousa *et al.,* 2016). Therefore, the addition of antioxidants in semen has been proposed to prevent damage to spermatozoa and improve semen fertility in artificial insemination.

Quercetin is an antioxidant that acts on the membrane, mitochondria and acrosome of spermatozoa protecting them upon temperature drop during cryopreservation (Seifi-Jamadi *et al.,* 2016). In a study by McNiven & Richardson (2006) determined that the inclusion of 50 to 300 µM quercetin in equine semen cryopreservation significantly reduced the percentage of sperm that underwent capacitation and acrosomal reaction upon thawing.

Table 4. Addition effect of quercetin and vitamin E in cryopreserved sheep semen on gestation rate in ewes inseminated by laparoscopic artificial insemination

Treatment	Gestation rate (%)
Control	23/50 (46.00%) [a]
Q	27/52 (51.92%) [a]
Vit E	17/50 (34.00%) [a]
Q + Vit E	21/49 (42.82%) [a]

[a] Different literals indicate statistically significant difference (P <0.05); Q = 200 µM quercetin; Vit E = 100 µM vitamin E.; Q + Vit E = 200 µM quercetin +100 µM vitamin E.

Ardeshirnia *et al.* (2017) report increased *in vitro* fertility of ovine semen, collected from epididymis, and cryopreserved with the addition of quercetin at concentrations of 5 and 10 µg/mL, compared to the other treatments (0, 15, 20 and 50 µg/mL). With these quercetin concentrations, the number of embryos at the zygote, morula and blastocyst stage increased. In the study by Ahmed *et al.* (2019), by adding quercetin at concentrations of 150 and 200 µM, in cryopreserved buffalo semen, *in vivo* fertility was higher (61.82 and 65.22%, respectively) compared to the control treatment (without any additive; 46.90%). In the present study, the numerical increase in fertility rate observed in

the quercetin-added treatment may perhaps be related to the increased vitality and protection of the acrosome, as this concentration of quercetin showed a significant difference in LIA spermatozoa compared to the other treatments, indicating that the fertility of cryopreserved semen could be increased by the addition of antioxidants such as quercetin in the extender, since post-thaw semen quality is one of the most important factors influencing the probability of gestation after insemination (Ahmed *et al.*, 2019). However, further studies are needed where different quercetin concentrations are evaluated and *in vivo* fertility evaluation is performed with a larger number of ewes.

CONCLUSIONS

The addition of 200 µM quercetin in cryopreserved ovine semen increased the percentage of live spermatozoa with intact acrosome and live sperm with damaged acrosome, which implies a reduction in the percentage of dead spermatozoa compared to the other treatments, indicating that it fulfilled its antioxidant functions by protecting the spermatozoa from damage caused by cryopreservation; however, this did not result in an increase in the gestation rate. It is recommended to continue studies with different concentrations of quercetin and to increase the studies with antioxidants in *in vivo* tests.

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