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To cite this article: José Maria Carrera-Chávez, Edson Eduardo Jiménez-Aguilar, Theisy Patricia Acosta-Pérez, José Alberto Núñez-Gastélum, Andrés Quezada-Casasola, Angélica María Escárcega-Ávila, Mateo Fabián Itza-Ortiz & Ernesto Orozco-Lucero (2020) Effect of *Moringa oleifera* seed extract on antioxidant activity and sperm characteristics in cryopreserved ram semen, Journal of Applied Animal Research, 48:1, 114-120, DOI: [10.1080/09712119.2020.1741374](https://doi.org/10.1080/09712119.2020.1741374)

To link to this article: <https://doi.org/10.1080/09712119.2020.1741374>



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Published online: 24 Mar 2020.



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Effect of *Moringa oleifera* seed extract on antioxidant activity and sperm characteristics in cryopreserved ram semen

José Maria Carrera-Chávez ^a, Edson Eduardo Jiménez-Aguilar ^a, Theisy Patricia Acosta-Pérez ^a,
José Alberto Núñez-Gastélum ^b, Andrés Quezada-Casasola ^a, Angélica María Escárcega-Ávila ^a,
Mateo Fabián Itza-Ortiz ^a and Ernesto Orozco-Lucero ^a

^aDepartamento de Ciencias Veterinarias, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Ciudad Juárez, México;

^bDepartamento de Ciencias Químico Biológicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Ciudad Juárez, México

ABSTRACT

Semen cryopreservation damages sperm due to oxidative stress. This study evaluated the antioxidant capacity of *Moringa oleifera* seed extract in cryopreserved ram semen and the impact of the extract on sperm characteristics. Semen from eight hair rams (four rams per sampling) was allocated into four groups, according to their treatment prior to cryopreservation: Control (no extract), 0.5 (M0.5), 5.0 (M5.0), and 10.0 (M10) mg/mL of *M. oleifera* extract. The antioxidant activity (ferric reducing antioxidant power, FRAP) and the spermatoc characteristics (sperm viability; progressive motility; fast motility; slow motility; acrosome damage; membrane damage; and mitochondrial activity) were assessed post-thawing. Variables were evaluated with analysis of variance followed by Tukey test. While no significant differences were detected in acrosomal damage, mitochondrial activity, fast-, or slow motility, the antioxidant activity was higher ($P < 0.05$) in M0.5 and M5 treatments. Viability and progressive motility increased in the M0.5 group ($P < 0.05$), whereas sperm membrane damage was lower ($P < 0.05$) in the same treatment. In conclusion, supplementation of ram semen with *M. oleifera* seed extract enhances antioxidant activity, sperm membrane integrity, viability, and progressive motility after thawing. This suggests that *M. oleifera* extract could be used as an antioxidant to improve the outcome of semen cryopreservation.

Highlights

- It is widely known that semen cryopreservation induces sublethal damage to sperm, deteriorating spermatoc characteristics, which is largely attributed to oxidative stress.
- There are scarce studies regarding the use of plant extracts as a replacement for conventional antioxidants to conserve sperm viability in cryopreserved ram semen.
- Addition of *Moringa oleifera* seed extract to a concentration of 0.5 and 5.0 mg/mL prior to ram semen freezing increased antioxidant activity after cryopreservation.
- *M. oleifera* seed extract at 0.5 mg/mL decreased post-thawing damage to the sperm membrane, increasing both viability and progressive motility.
- *M. oleifera* seed extract could be potentially used as a replacement for conventional antioxidants added to maintain sperm viability in cryopreserved ram semen.

ARTICLE HISTORY

Received 25 June 2019

Accepted 5 March 2020

KEYWORDS



Antioxidant; *Moringa oleifera*; semen cryopreservation; ram; sperm characteristics

1. Introduction

Semen cryopreservation, commercialization, and storage are widely used tools in livestock industry that promote genetic improvement. However, semen freezing induces sublethal damage to sperm, causing deterioration of cellular characteristics. This is attributed to thermal shock, ice crystal formation, osmotic changes, membrane lipids and proteins rearrangement, and high oxidation levels (Bucak et al. 2010). Oxidative stress occurs when there is an imbalance in the production of reactive oxygen species (ROS), in which ROS generation is greater than the biological ability to detoxify their reactive intermediates or easily repair the resulting damage (Agarwal

and Said 2003). This condition triggers toxic effects due to the production of peroxides and free radicals that damage cell components, including proteins, lipids, and DNA (Schafer and Buettner 2001). Thus, it is necessary to limit sperm damage generated by oxidative stress induced during cryopreservation.

The addition of antioxidants, such as alpha-tocopherol (Benhenia et al. 2016), Trolox (Sicherle et al. 2011), and taurine (Baghshahi et al. 2014) to ovine semen cryopreservation diluents has shown, to some extent, protective capacity against the adverse effects of ROS. This supplementation increases motility, viability, and post-thawing fertility (Sicherle et al. 2011; Baghshahi et al. 2014; Benhenia et al. 2016). Likewise,

CONTACT Ernesto Orozco-Lucero  ernesto.orozco@uacj.mx  Departamento de Ciencias Veterinarias, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Anillo Envoltente Pronaf y Estocolmo s/n. 32310 Cd. Juárez, Chihuahua, México

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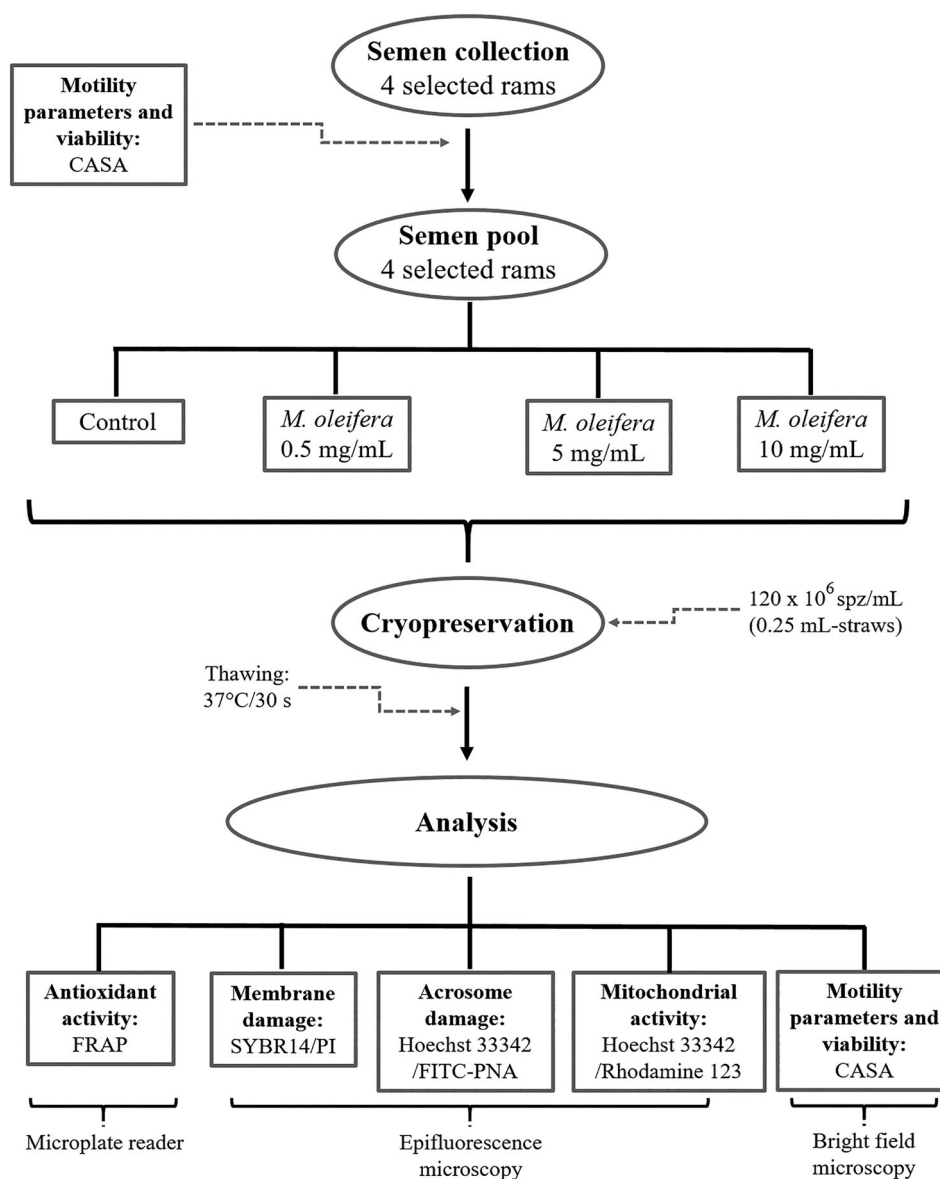


Figure 1. Scheme summarizing the methodology used to assess the effects of *Moringa oleifera* seed extract supplementation on the antioxidant activity and spermatic characteristics of cryopreserved ram semen. *M. oleifera*: *Moringa oleifera*, mg/mL: milligrams per millilitre, spz/mL: spermatozoa per millilitre, mL: millilitre, °C: Celsius degrees, s: seconds, CASA: computer assisted semen analysis, PI: propidium iodide, FITC-PNA: fluorescein isothiocyanate-coupled peanut agglutinin, FRAP: ferric reducing antioxidant power.

plant extracts with antioxidant properties have been recently studied in order to improve the quality of cryopreserved semen. These extracts contain widely known antioxidant phytochemicals including carotenoids, polyphenols, and flavonoids (Allai et al. 2016). In this regard, tissues from the *Moringa oleifera* tree have been demonstrated to possess high antioxidant activity due to the presence of ascorbic acid, flavonoids, polyphenolics, and carotenes (Jayawardana et al. 2015). These components decrease both free radical levels and the risk of cell death (Wang et al. 2017). However, research suggesting the potential use of vegetal extracts to replace conventional antioxidants and preserve sperm viability is still scarce (Berkovich et al. 2013). Moreover, exploration of the impact of *M. oleifera* extracts addition on the antioxidant status of cryopreserved ram semen and spermatic characteristics has not yet been reported. Therefore, because of all the aforementioned, the

aims of the present study were to evaluate the effect of *M. oleifera* seed extract supplementation on the antioxidant capacity of frozen-thawed ram semen and identify the influence of different concentrations of this vegetal extract on sperm characteristics in cryopreserved ram semen.

2. Materials and methods

A schematic summary of the methods used in this work is shown in Figure 1.

2.1. Animals and semen collection

The authors assert that all procedures contributing to this work complied with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals in

Table 1. Settings of the CASA system* used to evaluate ram spermatozoa.

Variable	Setting
Resolution horizontal/vertical	1024 pixels × 1024 pixels
Pixel size horizontal/ vertical	5.5 µm × 5.5 µm
Frame rate	Up to 101 frames per second
Area	10–100
Tail detection	5 µm
Camera depth	20 µm
Pixel relation	0.54 µm
Reference volume	
Width	555.12 µm
High	555.12 µm
In-motile	ALH < 4.00 µm and BCF < 4.00 µm
Motility	
Local motility	VCL < 20.00 µm s ⁻¹ and VSL < 10.00 µm s ⁻¹
Progressive motility	
Circle motility	Radius > 10.00 µm s ⁻¹ to < 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.

ALH, Amplitude of lateral head displacement; BCF, Beat cross frequency; VCL, Curvilinear velocity; VSL, Straight-line velocity.

Mexico (NOM-051-ZOO-1995: Humanitarian care of animals during mobilization; NOM-033-ZOO-1995: Humanitarian slaughter of domestic and wild animals; and the institutional regulation of the Bioethics Committee of the Universidad Autónoma de Ciudad Juárez).

The present investigation was conducted at the Universidad Autónoma de Ciudad Juárez, Ciudad Juárez, Chihuahua, México (31° 44' 22" N, 106° 29' 13" W) during the breeding season (autumn and winter). Eight healthy hair breed rams (Katahdin, Black Belly, Pelibuey), two to four years old, kept in confinement with natural light and *ad libitum* access to forage and water, supplemented with 500 g/head/day of ground corn were employed for this study. Males were kept under similar management conditions during the entire study. Four out of the eight rams available were randomly selected and used per sampling session, which was carried out twice a week using the artificial vagina collection technique. The four samples were pooled to eliminate individual differences. 10 µL of the sample were placed in a standard 20 µm count chamber (Leja Camera, Leja Products, The Netherlands) to be evaluated by computer assisted semen analysis (CASA) system (AndroVision, Minitube, Germany) under a vertical microscope (AxioScope.A1, Zeiss, Germany). The minimum levels required to freeze were established as it follows: Volume of the ejaculate, ≥0.5 mL (Banday et al. 2017); progressive motility, ≥80%; and concentration, 3 × 10⁹ spermatozoa/mL (Cámara et al. 2011). Table 1 shows the CASA system settings used to evaluate ram sperm.

2.2. Production and characterization of *Moringa oleifera* seed extract

Seeds of *M. oleifera* were collected in the state of Sonora, in Northwestern Mexico. Mature pods were manually collected in November 2017 and transported in plastic bags at room temperature. Seeds were obtained and oven-dried at 60°C for 8 h. Then, these structures were stored in plastic bags in a

dark room. All samples were ground and passed through a mesh sieve (60 meshes). The extract of *M. oleifera* was obtained from lyophilized defatted seeds processed following the method described by Núñez-Gastélum et al. (2019) with some modifications. Specifically, 100 mL of 80% (v/v) methanol were added to 10 mg of sample. The mixture was then sonicated at 40 kHz for 30 min in the dark. The extract was centrifuged at 1600 × g for 30 min at 4°C and the supernatant was recovered by filtration. The residue was re-extracted under the same conditions. Finally, supernatants were combined, rota-evaporated (Buchi R-3, Flawil, Switzerland), lyophilized (FreeZone 4.5, Labconco, Kansas City, MO), and stored at –80°C until use. To characterize the extracts, the total polyphenolics and flavonoids content was determined following the procedure described by Núñez-Gastélum et al. (2018) by using the method of Folin–Ciocalteu and complexation with aluminum chloride, respectively.

2.3. Semen processing and treatments

A diluent based on Tris (Two Step, Continental, Delavan, WI) was used with 6% (v/v) glycerol and 10% (v/v) egg yolk. Diluted samples were assigned into four treatments: Control, which included conventional antibiotic (55-, 275-, 330-, and 165 µg/mL of a mixture tylosin, gentamicin, spectinomycin, and lincosmycin, respectively), while the three remaining treatments contained no antibiotic but extract of *M. oleifera* seed at final concentrations (in the straw) of 0.5 (M0.5), 5.0 (M5), and 10.0 (M10) mg/mL. Semen concentration was adjusted to 120 × 10⁶ motile sperm/mL. Samples were manually aspirated into 0.25 mL straws (IMV Technologies, France) at room temperature, sealed with polyvinyl alcohol powder, and immediately cooled to 5°C at a rate of –0.2°C/min. Once semen was refrigerated and equilibrated at 5°C for one hour, straws were placed in an automatic freezer (Cryobath CL-8800, CryoLogic, Australia) previously stabilized at 5°C. Semen was frozen from 5 to –120°C at a rate of –12.5°C/min and then stored in a cryogenic container at –196°C until further analysis.

2.4. Evaluation of antioxidant activity in post-thawed semen

2.4.1. Sample preparation

For seminal plasma recovery it was followed the procedure described by Gallardo (2007) with some modifications. Briefly, straws were thawed 30 days after freezing at 37°C for 30 s in a water bath and centrifuged at 800 × g for 10 min at 4°C. The supernatant (seminal plasma and diluent) was removed and stored at –80°C until use.

2.4.2. Ferric reducing antioxidant power test

An acetate buffer was prepared for the ferric reducing antioxidant power (FRAP) assay. For that purpose, 1.3 g of sodium acetate (CH₃COONa) and 8 mL of glacial acetic acid were mixed with 300 mL of distilled water. The pH was adjusted to 3.6 and brought to a final volume of 500 mL. The reagent TPTZ was prepared with 31.2 mg of 2,4,6-Tri (2-pyridyl)-s-triazine (TPTZ) and 33 µL of 1.21 M hydrochloric acid, adjusted to 10 mL with distilled water, and stored in the absence of light.

The FRAP reagent was prepared using 1 mL of the TPTZ reagent and 1 mL of 20 mM FeCl₂. Afterwards, this mixture was adjusted to 10 mL with acetate buffer and then incubated at 37°C for 30 min. Immediately after FRAP reagent incubation, the sample (seminal plasma and diluent) was thawed at room temperature and 24 µL were placed per microplate well. 180 µL of the FRAP reagent were added to each well for subsequent reading at 595 nm in a microplate spectrophotometer (xMark, BioRad, Hercules, CA) at 37°C. The results were expressed as millimoles of Trolox equivalents (mmol TE) upon comparison with a Trolox standard curve (Núñez-Gastélum et al. 2018).

2.5. Evaluation of sperm characteristics in post-thawed semen

2.5.1. Viability and motility

Semen samples were thawed as previously explained and evaluated with the CASA system for the viability, progressive motility, fast motility, and slow motility variables.

2.5.2. Sperm membrane damage

SYBR14/PI staining (15407/0001, Minitube, Germany) was used for sperm membrane damage evaluation. Propidium iodide only penetrates permeated membranes. Therefore, damaged sperm nuclei were stained red. 50 µL of semen and 2.5 µL of dye were mixed and incubated at 37°C during 15 min protected from light. A 10 µL-drop of this preparation was placed on a slide and covered with a coverslip. The sample was then analyzed with the membrane integrity module of the CASA system under an epifluorescence microscope (200X; AxioScope.A1, Zeiss, Germany). The fluorescence filter (AHF Analysentechnik, Germany) characteristics were: 481–501 nm and 560–589 nm excitation; 503 and 589 nm beam splitters (BS); 512–550 nm and 600–660 nm emission.

2.5.3. Acrosome damage

A double fluorescence stain with Hoechst 33342/FITC-PNA (15407/0011, Minitube) was used to determine the grade of acrosome damage. 50 µL of thawed semen and 27 µL of dye were mixed and incubated for 20 min at 37°C in the dark. 10 µL of this mixture were loaded on a slide and covered with glass. This preparation was evaluated with the acrosome integrity module of the CASA system with an epifluorescence microscope (200X). The fluorescence filter (AHF Analysentechnik) characteristics were: 392–414 nm and 481–501 nm excitation; 419 and 503 nm BS; 440–470 nm and 512–550 nm emission. Heads of all spermatozoa showed blue colouration. Meanwhile, damaged acrosomes were stained green.

2.5.4. Mitochondrial activity

A double fluorescent stain was performed with Hoechst 33342/Rhodamine 123 (15407/0012, Minitube) to evaluate mitochondrial function. 50 µL of thawed semen and 4 µL of dye were mixed and incubated for 20 min at 38°C protected from light. The sample was then centrifuged at 800 × g for 4 min and the supernatant was removed. 50 µL of BTS diluent (13525/0001, Minitube) was added and incubated for 5 min at 38°C. A 10 µL-drop was placed on a slide, covered with glass, and

analyzed with the mitochondrial activity module of the CASA system with a microscope of epifluorescence (200X). The fluorescence filter (AHF Analysentechnik) characteristics were: 392–414 nm and 481–501 nm excitation; 419 and 503 nm BS; 440–470 nm and 512–550 nm emission. Heads of all spermatozoa were stained blue due to the action of Hoechst 33342, whereas the intermediate part of the spermatozoa with functional mitochondria showed green colour due to the Rhodamine 123 stain.

2.6. Statistical analysis

Six replicates for each test were carried out. The experimental unit was each of the six mixtures formed by the ejaculate of the four rams used in each collection. Data analysis was performed with the statistical package SAS (SAS, 2009/STAT v. 9.3, SAS Institute, Cary, NC). The variables were assessed with one-way ANOVA and compared with a Tukey test. A $P < 0.05$ was considered as significant. Percent values of viability, progressive motility, fast motility, slow motility, sperm membrane damage, acrosomal damage, and mitochondrial activity were transformed to arcsine before analysis, although all data were expressed as non-transformed means ± SEM.

3. Results

Data of the total polyphenolic and flavonoid composition of the *M. oleifera* seed extract supplemented prior to ram semen freezing are shown in Table 2. Upon such treatment ram semen was cryopreserved. Subsequently, both antioxidant capacity and spermatoc characteristics were evaluated, rendering the following findings:

First, it was observed that M0.5 (0.205 ± 0.028 mmol TE) and M5 (0.205 ± 0.017 mmol TE) groups had similar results ($P > 0.05$) in relation to the antioxidant activity in seminal plasma, as indicated in Figure 2. Nevertheless, either M0.5 or M5 treatments showed significantly higher antioxidant activity ($P < 0.05$) when compared to M10 (0.129 ± 0.005 mmol TE) and Control groups (0.119 ± 0.004 mmol TE).

Concerning integrity and activity of spermatoc structures, the percentage of sperm membrane damage was significantly lower ($P < 0.05$) in M0.5 (73.88 ± 2.64%) when compared to the Control group (80.75 ± 1.06%). However, the membrane damage rate of the M0.5 group did not differ ($P > 0.05$) from that of M5 (78.38 ± 1.91%) and M10 (79.50 ± 1.79%) treatments (Figure 3). On the other hand, the results of both acrosomal damage and mitochondrial activity were similar ($P > 0.05$) among Control (47.63 ± 3.80% and 52.75 ± 1.73%, respectively), M0.5 (51.38 ± 3.2% and 55.38 ± 2.05%), M5 (47.88 ± 4.50% and 55.38 ± 2.16%), and M10 (54.87 ± 3.50% and 58.00 ± 1.84%) treatments, as shown in Figure 3.

In relation to cryopreserved semen quality parameters (drawn from motility analysis), the percentage of both post-

Table 2. Total polyphenolics and flavonoids in *Moringa oleifera* seed extract.

Polyphenolics (mg GAE/g)	Flavonoids (mg CE/g)
3.11 ± 0.11	2.43 ± 0.11

mg GAE/g: milligrams of gallic acid equivalents per gram, mg CE/g: milligrams of catechin equivalents per gram. Data are presented as mean ± SEM.

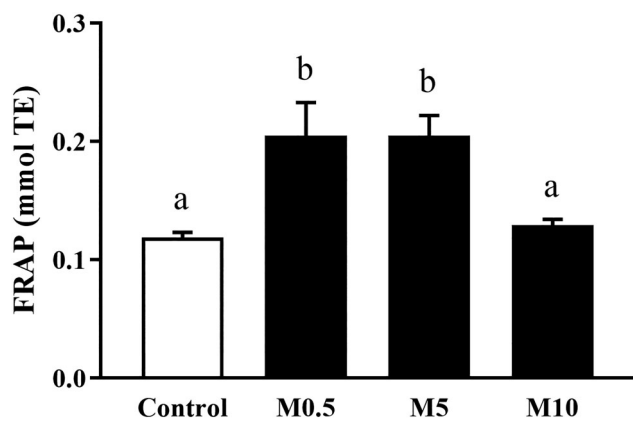


Figure 2. Antioxidant activity in ram semen following cryopreservation. FRAP: ferric reducing antioxidant power, mmol TE: millimoles of Trolox equivalents, Control: control group, M0.5: 0.5 mg/mL of *Moringa oleifera* seed extract, M5: 5 mg/mL of *M. oleifera* seed extract, M10: 10 mg/mL of *M. oleifera* seed extract. Data are presented as mean \pm SEM. ^{a,b} Different literals across bars indicate significant differences ($P < 0.05$).

thaw sperm viability and progressive motility were higher ($P < 0.05$) in M0.5 in comparison to the Control treatment (Table 3). Nevertheless, M0.5 resulted similar to M5 and M10 groups in terms of viability and progressive motility ($P > 0.05$). In contrast to sperm viability and progressive motility, there were no significant differences among all treatments regarding the variables of fast- and slow motility in post-thawed semen ($P > 0.05$).

4. Discussion

The polyphenolic and flavonoid content of *M. oleifera* seed extracts used in the present study is in the range reported for defatted seed extracts in other studies (Singh et al. 2013; Nascimento et al. 2017; de la Mora-López et al. 2018). This reflects the antioxidant capacity of the extract since it is widely known that both phenolic and flavonoid compounds possess high antioxidant activity (Covas et al. 2010; Castaldo et al. 2019; Olszowy 2019).

Although other studies have reported a protective effect of the addition of antioxidants, such as hypotaurine, on acrosome

Table 3. Quality of cryopreserved ram semen supplemented with *Moringa oleifera* extract at 0.5, 5.0, and 10.0 mg per mL.

	Viability (%)	Progressive motility (%)	Fast motility (%)	Slow motility (%)
Control	41.44 \pm 2.17 ^b	35.84 \pm 2.15 ^b	7.75 \pm 0.92 ^a	28.06 \pm 1.40 ^a
M0.5	51.33 \pm 3.31 ^a	45.61 \pm 3.47 ^a	11.69 \pm 1.40 ^a	33.89 \pm 2.16 ^a
M5	48.76 \pm 3.77 ^{ab}	43.31 \pm 3.82 ^{ab}	11.16 \pm 1.79 ^a	32.08 \pm 2.16 ^a
M10	44.71 \pm 2.96 ^{ab}	38.71 \pm 2.88 ^{ab}	8.81 \pm 0.83 ^a	29.90 \pm 2.10 ^a

M0.5: 0.5 mg/mL of *Moringa oleifera* seed extract, M5: 5.0 mg/mL of *M. oleifera* seed extract, M10: 10.0 mg/mL of *M. oleifera* seed extract. Data are presented as mean \pm SEM. ^{a,b} Different literals within columns indicate significant differences ($P < 0.05$).

integrity and mitochondrial activity (Bucak et al. 2013), this investigation did not find any significant effect of the *M. oleifera* extract on either the acrosomal status or mitochondrial activity in cryopreserved ram sperm. In agreement, Câmara et al. (2011) also found no differences neither in the percentage of ram sperm with intact acrosome nor in mitochondrial membrane potential in samples added with antioxidants. However, these authors mention that there might be latent damage, in which the lesion degree manifests during post-thaw incubation, reducing sperm longevity in the female genital tract after insemination. Similarly, Silva et al. (2011) also found no difference in the results of fluorescent tests of acrosome integrity and mitochondrial membrane potential when they added superoxide dismutase or reduced glutathione to cryopreserved semen. However, ultrastructural analysis by transmission electron microscopy showed that the addition of these antioxidants helped to conserve acrosome and mitochondria structure (Silva et al. 2011). This might suggest that in the present study, although an effect of the seed extract of *M. oleifera* on the variables of acrosomal integrity and mitochondrial activity was not found, there could be a protective effect that was reflected in the increase in progressive motility. Nevertheless, clarification of these possibilities deserves further exploration.

In the present study, it was observed that a low concentration of *M. oleifera* seed extract (0.5 mg/mL) protected the sperm membrane, apparently due to the antioxidant properties of this vegetal extract. It can be inferred that conservation of

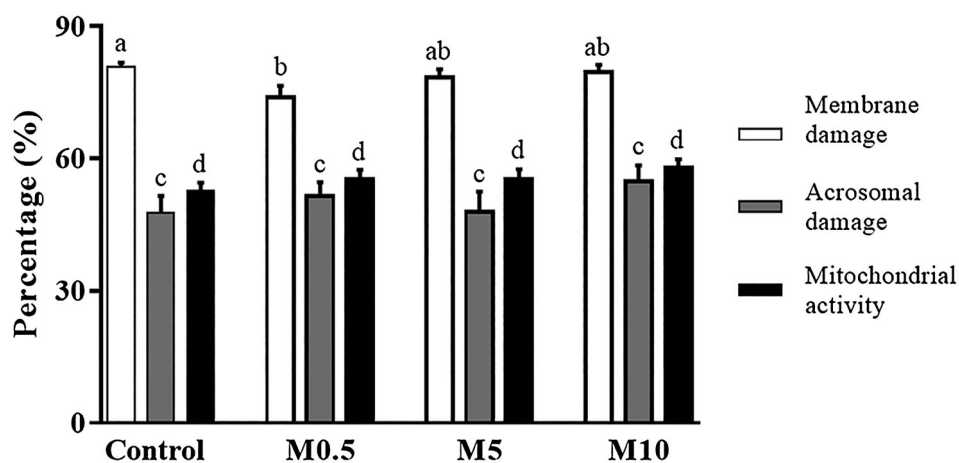


Figure 3. Sperm membrane damage, acrosome damage, and mitochondrial activity in ram semen following cryopreservation. Control: control group, M0.5: 0.5 mg/mL of *Moringa oleifera* seed extract, M5: 5 mg/mL of *M. oleifera* seed extract, M10: 10 mg/mL of *M. oleifera* seed extract. Data are presented as mean \pm SEM. ^{a,b} Different literals across bars of the same colour indicate significant differences ($P < 0.05$).

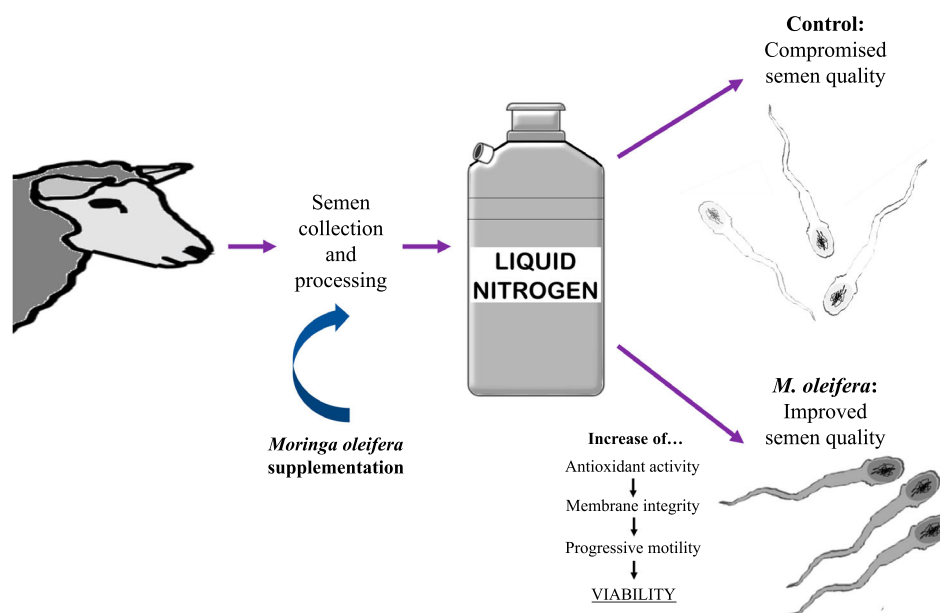


Figure 4. Improvement of cryopreserved ram semen quality (*in vitro* analysis) upon supplementation of *Moringa oleifera* seed extract prior to freezing.

sperm membrane structure was due to an antioxidant function given the significantly higher levels of antioxidant activity detected in semen after the addition of *M. oleifera* in this investigation. As mentioned before, this plant contains compounds encompassing polyphenols, flavonoids, vitamin C, vitamin E, β -carotene, zinc, selenium, vitamin A, riboflavin, nicotinic acid, folic acid, pyridoxine, cryptochlorogenic acid, isoquercetin, and astragaline. All these substances have strong antioxidant potential (Moyo et al. 2012; Vongsak et al. 2013; Jayawardana et al. 2015). In this way, it could be suggested that the higher antioxidant capacity detected here upon *M. oleifera* extract addition likely diminished formation of ROS in sperm plasma membrane. This is supported by the fact that antioxidant compounds decrease spermatic lipid peroxidation that results from the attack to the lipid matrix by ROS (Agarwal et al. 2008; Bucak et al. 2010; Allai et al. 2016). However, the physiology underlying sperm protection by the extract of *M. oleifera* is not completely clear since the positive effects may be a consequence of combined actions of its components (Cai et al. 2002). Therefore, future research is guaranteed to shed light on the exact basis of the mechanism of action that protected the sperm membrane, as well as the identification of the involved compound (or combination of substances). On the other hand, it is conceivable that the higher sperm membrane integrity observed in this work after supplementation of the vegetal extract in turn improved both viability and progressive motility of sperm. Accordingly, it is widely known that maintenance of spermatic plasma membrane is required for both vitality and motility, which are landmarks for male fertility throughout species, as indicated by Fraser et al. (2001), Ahmad et al. (2015), Reis et al. (2016). Thus, conservation of plasma membrane structure, as a consequence of increased antioxidant capacity upon *M. oleifera* extract addition here, also appears as a requirement for sperm viability and progressive motility in ram semen.

In conclusion, supplementation of the diluent used to freeze ram semen with *M. oleifera* seed extract increased antioxidant activity, viability, and progressive motility, while sperm

membrane damage was decreased. These results, arisen from *in vitro* analysis, suggest that *M. oleifera* extracts could be an option of antioxidant additive for ram semen freezing (Figure 4). Future studies should be conducted to better understand the mechanism of action, as well as to identify the specific active components in *M. oleifera* extracts involved in the improvement of antioxidant capacity and spermatic characteristics during ram semen cryopreservation.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by Consejo Nacional de Ciencia y Tecnología CONACYT-México under Grant INFR201501- 251729; and Universidad Autónoma de Ciudad Juárez under Grant UACJ- PIVA 312-17-15.

ORCID

José María Carrera-Chávez <http://orcid.org/0000-0002-6899-9485>
 Edson Eduardo Jiménez-Aguilar <http://orcid.org/0000-0003-0847-529X>
 Theisy Patricia Acosta-Pérez <http://orcid.org/0000-0002-5768-5050>
 José Alberto Núñez-Gastélum <http://orcid.org/0000-0002-7098-4485>
 Andrés Quezada-Casasola <http://orcid.org/0000-0003-0758-9762>
 Angélica María Escárcega-Ávila <http://orcid.org/0000-0002-4066-0586>
 Mateo Fabián Itza-Ortiz <http://orcid.org/0000-0003-0313-586X>
 Ernesto Orozco-Lucero <http://orcid.org/0000-0003-3238-2341>

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