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Protective effects of *Opuntia ficus-indica* extract on ram sperm quality, lipid peroxidation and DNA fragmentation during liquid storage

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ABSTRACT

The present study aimed to assess the phenolic composition of the acetone extract from *Opuntia ficus indica* cladodes (ACTEX) and its effects on ram semen variables, lipid peroxidation and DNA fragmentation during liquid storage at 5 °C for up to 72 h in skim milk and Tris egg yolk extenders. Semen samples from five rams were pooled extended with Tris-egg yolk (TEY) or skim milk (SM) extenders containing ACTEX (0%, 1%, 2%, 4% and 8%) at a final concentration of 0.8×10^9 sperm/ml and stored for up to 72 h at 5 °C. The sperm variables were evaluated at different time periods (8, 24, 48 and 72 h). Sperm total motility and viability were superior in TEY than in SM whereas the progressive motility, membrane integrity, abnormality and spontaneous lipid peroxidation were greater in SM compared to TEY ($P < 0.05$). The results also indicated that the inclusion of 1% ACTEX in the SM or TEY extender increased the sperm motility, viability, membrane integrity, and decreased the abnormality, lipids peroxidation up to 72 h in storage compared to control group. Similarly, even at 72 h of storage, 1% ACTEX can efficiently decrease the negative effects of liquid storage on sperm DNA fragmentation ($P < 0.05$). In conclusion, SM and TEY supplemented with 1% of ACTEX can improve the quality of ram semen. Further studies are required to identify the active components in ACTEX involved in its effect on ram sperm preservation.

1. Introduction

Artificial insemination (AI) is the most practical available technology for rapid genetic improvement in livestock within a reasonable timeframe. However, for the success of this technique, sperm should be preserved for a long period without damages that greatly influence fertilizing capacity. No extender can be sued that preserves ram liquid semen longer than 2 to 3 days without a decrease in semen quality (Vishwanath and Shannon, 2000). Furthermore, ram sperm are extremely sensitive to oxidative stress due to the high content of unsaturated fatty acids present in the plasma membrane phospholipids in comparison to other species (Alvarez and Storey, 1983; Alvarez et al., 1987; Griveau et al., 1995). Many studies indicate that the qual-

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ity of semen deteriorates during liquid storage for a long period (Kasimanickam et al., 2007).

Seminal plasma confers some protection against ROS damage via glutathione (GSH), catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide-dismutase (SOD) (Weir and Robaire, 2007; Hammadeh et al., 2009). Nonetheless, the protective role of the seminal plasma is reduced after applying several dilutions for semen storage (Martínez-Páramo et al., 2009). To cope with such problems, different antioxidants have extensively been added to the extenders to improve ram sperm quality (Maxwell and Stojanov, 1996; Upreti et al., 1997; Sarlós et al., 2002; Stefanov et al., 2004; Bucak and Tekin, 2007). Different natural herbs have been studied for the several antioxidant properties. These herbs contain many phytochemicals (carotenoids, polyphenol, flavonoids) widely used in cosmetic, pharmaceutic and food industry with beneficial effects (Yanishlieva and Marinova 1996; Zheng and Wang, 2001).

Recently, there has been a global trend concerning natural antioxidants that are present in fruits, vegetables, plants, oil seeds, and herbs to preserve semen quality (Del Valle et al., 2013; Baghshahi et al., 2014; Motlagh et al., 2014). A research directed towards cladode (*Opuntia ficus-indica*) which is rich in different compounds with an antioxidant activity have shown its beneficial effect on sperm DNA fragmentation after semen cryopreservation in human (Meamar et al., 2012). The widely cited compounds are tocopherols, polyphenols, flavonoids, tannins, carbohydrates, phenolic acids, minerals and sulfur amino acids (Stintzing et al., 2001; Chavez-Santoscoy et al., 2009; Feugang et al., 2006; Cardador-Martínez et al., 2011; Morales et al., 2012). Therefore, with this background, the aims of the present study were to: (1) evaluate the efficacy of skim milk and Tris egg yolk for the preservation of ram semen of the Boujaâd breed at 5 °C for different time periods (8, 24, 48 and 72 h) and (2) assess the effect of different concentrations of ACTEX from *Opuntia ficus indica* cladodes supplementation in SM and TEY extenders on ram sperm variables, lipid peroxidation and DNA fragmentation during liquid storage for different time periods.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (Germany) and from Merck (Merck Schuchardt OHG, Germany).

2.2. Plant material and acetone extract preparation

Nopal *opuntia ficus indica* cladodes were collected from the experimental station of Regional Center-INRA Settat Morocco. These compounds were washed using distilled water, dried in an oven at 55 °C, and mechanically milled. The resulting powder was stored in a closed container at room temperature until use. The acetone extraction procedure was based on the report of Zhang et al. (2008) with some modifications. The dried powder (5 g) was mixed with 100 mL of acetone/water (70:30 v/v) mixture. The ACTEX was subsequently extracted by stirring the above mixture at room temperature for 4 days. The extracted solution was filtered through Whatman No, 4 filter paper. The solvent was evaporated at 38 °C under reduced pressure using a vacuum rotary evaporator (Buchi R-210, Switzerland). The residue was lyophilized (LABCONCO, Freezone −105 °C 4.5 L Cascade Benchtop Freeze Dry System, Kansas MO, USA) and stored at 4 °C until use.

2.3. Identification and quantification of phenolic compounds

The extract components were analyzed using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). For this analysis, twenty-four phenolic compounds (flavonoids, flavonoid glycosides, phenolic acids, phenolic aldehyde, coumarin) and three non-phenolic organic acids were used to identify the ACTEX samples of *Opuntia ficus-indica* cladodes. Crude extracts were extended to 250 mg/L in methanol and filtrated with 0.2 μm microfiber filter prior to analysis. LC–MS/MS analysis of the phenolic compounds were performed using a Nexera model Shimadzu UHPLC coupled with tandem mass spectrometry detection (Shimadzu LCMS 8040 triple quadruple mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes) (Ertas et al., 2015). LC–MS/MS data were collected and processed with LabSolutions software (Shimadzu, Kyoto, Japan).

2.4. Animals and semen collection

A total number of 50 ejaculates were collected from five mature Boujaâd rams (3–5 years of age) using an artificial vagina, during the breeding season (July to September 2013). Immediately after collection, the ejaculates were transferred to a water bath (37 °C). The quality assessment was performed immediately and only ejaculates with a volume ≥1 mL, subjective total motile sperm ≥70% and a concentration $\geq 2.5 \times 10^9$ sperm/mL were used. To eliminate individual differences, the ejaculates derived from the five rams were pooled and processed for extending.

2.5. Extender preparation

A skim milk (reconstituted with 11 g of skim milk powder in 100 mL of distilled water and heated to 95 °C for 10 min) and a Tris-based extender (2.666 g Tris, 0.44 g glucose, 1.398 g citric acid, egg yolk 12% (v/v): pH 6.8) were used as the base extenders. Penicillin and streptomycin (0.05 mg/mL) were added to prevent bacterial growth. In the present study, all diluents were prepared daily. The semen was extended using the base extender at 37 °C, containing 0% (control), 1%, 2%, 4% and 8% of ACTEX to reach the concentration of 0.8×10^9 sperm/mL. The sperm quality was assessed at 5 °C after 8, 24, 48 and 72 h of storage.

2.6. Experimental design

2.6.1. Experiment 1: effects of storage period, extender and ACTEX concentrations on ram sperm quality variables

This experiment was conducted to evaluate the effect of storage durations (8, 24, 48 and 72 h), extender (SM and TEY), and concentration of ACTEX (0%, 1%, 2%, 4% and 8%) on total motility, progressive motility, viability, membrane integrity, abnormality, and lipid peroxidation of ram sperm during liquid storage.

2.6.2. Experiment 2. effects of ACTEX on DNA fragmentation

Based upon on the results giving the best protective effects on sperm progressive motility in Experiment 1, the following experiment was designed to examine the influence of best ACTEX on DNA fragmentation at 0, 8, 24, 48 and 72 h using TUNEL technique.

2.7. Semen evaluation

2.7.1. Analysis of sperm motility

Sperm motility (proportion of total and progressive motile sperm) was assessed by means of a computer-assisted semen analysis (CASA) system with a warmed stage (37 °C) (ISAS, version 1.0.17, Proiser, Valencia, Spain). The preserved samples were further diluted using 0.9% NaCl to 20×10^6 sperm/mL at 37 °C. A semen sample (5 μL) was placed in a Makler counting chamber and four fields were examined at × 10 (negative phase contrast).

2.7.2. Sperm viability

The sperm viability was assessed using the eosin-nigrosin staining method (Evans and Maxwell, 1987). A semen sample was diluted 1:2 with the staining solution (eosin-Y 1.67 g, nigrosin 10 g, sodium citrate 2.9 g, dissolved in 100 mL distilled water) and placed as a smear on slides. After air drying, the smear was observed under a phase-contrast microscope (100×). Sperm cells ($n = 200$) were counted for unstained heads of sperm (live) and/or stained/partial stained heads of sperm (dead).

2.7.3. Sperm abnormality assessment

The abnormality of sperm was evaluated using the Diff-Quik kit (Diagnostic Systems S.L. Barcelona, Spain). Three μL of diluted semen was smeared on a glass slide and allowed to air dry. The slide was then fixed in the fixative solution for 1 min and first and second solutions seven to ten times. Between the fixing procedure and each of the staining procedures, the excess solutions were removed from the slides by placing them vertically on absorbent paper. At least 200 sperm were evaluated under light microscopy at 1000× magnification using UB203 microscope (UOP/Proiser, Paterna, Valencia, Spain).

2.7.4. Functional membrane integrity

The hypoosmotic swelling test (HOST) was performed to evaluate the functional integrity of the sperm membrane. A semen sample (30 μL) was mixed with 300 μL of a 100 mOsm hypoosmotic solution (9 g fructose, 4.9 g sodium citrate per liter of distilled water) and incubated at 37 °C for 60 min (Revell and Mrode, 1994). After incubation, the sample was gently mixed. A drop (15 μL) of the treated mixture was smeared on a pre-warmed slide and covered with a cover slip. A total of 200 sperm was counted in at least five different microscopic fields at 400×. The percentage of sperm cells with swollen and curved tails was then recorded (Buckett et al., 1997).

2.7.5. Measurement of lipid peroxidation

The concentrations of malondialdehyde (MDA) as indices of lipid peroxidation (LPO) in the semen samples were measured using the thiobarbituric acid reaction (Placer et al., 1966). The thiobarbituric acid reactive substances (TBARS) were measured in the semen (SLPO) or after incubation with 0.24 mM of $FeSO_4$ at 37 °C in a water bath for 60 min (ILPO). The TBARS concentration was determined by comparing the sample's absorbance at 532 nm with a standard curve created using MDA. The results were expressed in nmol TBARS/10^8 sperm.

2.7.6. DNA fragmentation

For Terminal Deoxynucleotidyl Tansferase-mediated dUTP nick-end labeling (TUNEL) technique, the *in situ* Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) was used to measure DNA fragmentation according to the manufacturer's protocol with slight modifications performed in ram by Nur et al. (2010). In brief, one drop of re-suspended sperma was smeared on a glass slide and fixed with 10% formaldehyde for 30 min at room temperature. The slides were washed in PBS (three times for 5 min each). After the slides were treated in a humidified chamber with proteinase K for 10 min at room temperature, slides were further washed using PBS. The slides were subsequently treated with 3% H_2O_2 for 10 min at room temperature and washed again using PBS. The slide was permeabilized with 0.1% Triton X-100 for 5 min on ice. The slides were incubated in the dark at 37 °C for 1 h with the TUNEL reaction mixture, which contained terminal deoxynucleotidyl transferase (TdT) plus dUTP label. After labeling, samples were washed using PBS and observed immediately under the fluorescence microscope (Zeiss eurostar, Germany 100×). At least 100 sperm were evaluated to determine the percentage of TUNEL positive sperm.

2.8. Statistical analysis

Data were checked for normality using the Shapiro–Wilk's test and all variables were found to fit the normal distribution and then analyzed using parametric statistical methods. Data were analyzed by a factorial design ANOVA. The statistical model included the fixed effect of base extender (SM *vs.* TEY), levels of ACTEX (0, 1, 2, 4 and 8%) and storage periods (8, 24, 48 and 72 h). All analyses were performed using the Statistical Analysis System software JMP SAS (version 10). Results were expressed as mean ± standard error of the mean (SEM). When statistically significant differences were detected, the Tukey's *post hoc* were used to compare the means and standard errors, considering the significance

Table 1
Identification and quantification of chemical components of cactus extracts by UPLC–MS/MS.[a]

Analyte no.	Analytes	RT[b]	Acetone Extract (μg analyte/g extract)[c]
1	Quinic acid	3.32	4369.69 ± 209.7
2	Malic acid	3.54	31,239.22 ± 1655
3	tr-Aconitic acid	4.13	77.15 ± 3.7
4	Gallic acid	4.29	D.
5	Protocatechuic acid	5.63	D.
6	Tannic acid	6.46	D.
7	tr- caffeic acid	7.37	D.
8	Vanillin	8.77	D.
9	p-Coumaric acid	9.53	D.
10	Rutin	10.18	110.5 ± 5.5
11	Hesperidin	9.69	174.59 ± 8.5
12	Hyperoside	10.43	62.09 ± 3.1
13	Quercetin	14.48	6.81 ± 0.5
14	Naringenin	14.66	D.
15	Kaempferol	15.43	D.
16	Apigenin	17.31	D.
17	Rhamnetin	18.94	D.
18	Chrysin	21.18	D.

D: peak observed, concentration is lower than the LOQ (Limit of quantification) but greater than the LOD (Limit of detection).
[a] Parent ion (m/z): Molecular ions of the standard compounds (mass to charge ratio).
[b] RT: Retention time.
[c] Values in μg/g (w/w) of plant extract.

level of $P < 0.05$. DNA Fragmentation data were analyzed using Student's t-test.

3. Results

3.1. Quantitative analysis of phenolic compounds presented in the cactus acetone extract by LC–MS/MS

According to the results of the LC–MS/MS analysis, 18 compounds were identified in ACTEX (Table 1). The major compounds are quercitin, hyperoside, rutin, hesperidin and high level of tr-Aconitic, malic and quinic acids.

3.2. Effect of ACTEX concentrations in SM and TEY on semen during different time points of storage

The effects of the storage period and extenders were assessed by the analysis of sperm variables in the TEY and SM supplemented by different concentrations of ACTEX after liquid storage for 8 to 72 h (Tables 2, 3 and 4).

The addition of 1% ACTEX in SM and TEY can significantly increase the total and progressive motility (Table 2) after storage from 8 to 72 h compared to the control groups ($P < 0.05$). Similarly, SM and TEY supplemented with 2% of ACTEX can also improve progressive motility from 8 to 48 h compared to the control groups while no effects have been recorded at 72 h of storage. Using the 4% and 8% concentrations, no differences have been observed comparing to the control groups.

Data for the viability and membrane functionality are in Table 3, the results provided evidence that ACTEX at 1% in TEY and SM extenders increase the viability from 24 h to 72 h of storage and HOST from 8 h to 72 h for storage.

Regarding the abnormality (Table 4), the results showed that SM supplemented with 1% ACTEX decrease the percentage of abnormal sperm compared to the control ($P < 0.05$) from 24 h to 72 h. In TEY, the inclusion of 1% ACTEX decreased the abnormality only at 72 h compared to the control ($P < 0.05$).

3.3. Effect of ACTEX concentrations on lipid peroxidation of sperm caused by liquid storage

The data about spontaneous (SPLO) and inducing lipid peroxidation (ILPO) in ram semen after storage in extenders containing different concentrations of ACTEX for different storage periods at 5 °C are included in Table 5. In the control group, the concentration of SLPO or ILPO was greater than that in the experimental groups (1% of ACTEX) ($P < 0.05$). The addition of 1% and 2% ACTEX to TEY or SM may have a positive effect compared to the other concentrations ($P < 0.05$). While no beneficial effects ($P > 0.05$) were observed when the concentration of ACTEX exceeds 2% compared to the control group.

3.4. Effects of ACTEX on DNA fragmentation of ram sperm after liquid storage

The results about effects of ACTEX on DNA fragmentation on ram semen after liquid storage are depicted in Fig. 1. The TEY and SM supplemented with selected concentration of ACTEX can reduce DNA damage at 24, 48 and 72 h of storage compared to the control ($P < 0.05$). No effects have been observed after liquid storage for 8 h while using TEY supplemented with 1% ACTEX.

4. Discussion

Artificial insemination in sheep using fresh or cooled extended semen has had acceptable success for many countries. Cooled, diluted semen is a good alternative to frozen semen, when it is used within a short period of time. The quality of ram sperm after liquid storage needs to be evaluated before practical application in the field. The decrease in the percentage of motile sperm during refrigeration in the present study was similar to that reported in existing literature (Paulenz et al., 2002; Bucak and Tekin, 2007). The extender is another important variable when designing a protocol for storing ram semen. In the present work, the extender nature clearly affected sperm quality. Lipid or protein supplements can help to maintain sperm quality, possibly by interacting with the plasma membrane and by modulating the activity of seminal plasma proteins (Bergeron and Manjunath, 2006; Beirão et al., 2012). The protective effects of egg yolk on spermatozoa membrane may correlate with its antioxidant properties. Egg-yolk is a complex biological compound containing mainly phospholipids and also proteins, vitamins, glucose and antioxidants, which are useful to protect sperm, because egg yolk protect ram semen from cold shock and can, prevent the oxidation of polyunsaturated fatty acids (White, 1993; Bucak and Tekin, 2007). In skim milk, the casein micelles can protect ram spermatozoa during storage at low temperature (Martin, 1966), reduce sperm lipid loss, while

Table 2
Effects of ACTEX concentration added to SM and TEY extenders on ram sperm motility after 8, 24, 48 and 72 h of storage at 5 °C.

Extenders	Groups	TM (%)				PM (%)			
		8 h	24 h	48 h	72 h	8 h	24 h	48 h	72 h
SM	CONTROL	84.8 ± 0.6^{bA}	77.6 ± 0.9^{bcB}	75.8 ± 1.2^{bB}	62.7 ± 0.8^{bC}	54.4 ± 1.1^{bA}	48.1 ± 0.7^{bB}	37.3 ± 1.7^{bC}	31.3 ± 0.9^{bD}
	ACTEX1%	87.9 ± 0.6^{aA}	82.8 ± 0.8^{aB}	81.3 ± 1.6^{aB}	72.6 ± 1.0^{aC}	63.8 ± 0.8^{aA}	56.8 ± 1.3^{aB}	54.2 ± 1.5^{aC}	38.4 ± 1.7^{aD}
	ACTEX2%	84.4 ± 0.8^{bcA}	80.2 ± 1.0^{abB}	78.0 ± 1.7^{abB}	69.3 ± 0.9^{aC}	61.8 ± 1.0^{aA}	57.1 ± 1.4^{aB}	55.3 ± 2.0^{aB}	32.1 ± 1.3^{bC}
	ACTEX4%	83.5 ± 0.7^{bcA}	75.4 ± 1.4^{cdB}	69.3 ± 2.0^{cC}	58.0 ± 1.7^{bD}	57.3 ± 1.5^{bA}	48.5 ± 1.6^{bB}	38.7 ± 0.5^{bC}	24.5 ± 1.9^{cD}
	ACTEX8%	82.4 ± 0.8^{cA}	73.4 ± 1.4^{dB}	69.8 ± 2.1^{cC}	58.9 ± 1.6^{cD}	51.0 ± 1.0^{cA}	44.7 ± 1.1^{cB}	35.0 ± 2.3^{bC}	21.8 ± 1.2^{cD}
TEY	CONTROL	85.5 ± 0.6^{bcA}	80.9 ± 0.6^{bB}	76.1 ± 1.9^{bcC}	71.7 ± 1.0^{bD}	44.8 ± 1.1^{cA}	40.0 ± 0.6^{bB}	32.5 ± 1.3^{cC}	24.6 ± 0.8^{bD}
	ACTEX1%	89.1 ± 0.6^{aA}	85.8 ± 0.9^{aB}	83.0 ± 1.1^{aB}	77.8 ± 1.2^{aC}	52.4 ± 1.1^{aA}	48.6 ± 1.0^{aB}	43.6 ± 1.6^{aC}	35.8 ± 0.8^{aD}
	ACTEX2%	87.7 ± 0.6^{abA}	85.4 ± 0.8^{aA}	79.6 ± 2.4^{bB}	69.9 ± 1.8^{bcC}	48.7 ± 0.7^{bA}	46.6 ± 1.2^{aA}	38.7 ± 1.3^{bB}	25.6 ± 1.6^{bC}
	ACTEX4%	86.9 ± 0.7^{abcA}	81.0 ± 1.2^{bB}	75.1 ± 1.5^{bcC}	67.0 ± 2.0^{cD}	46.3 ± 1.0^{bcA}	42.2 ± 0.9^{bB}	32.2 ± 2.7^{cC}	25.0 ± 1.1^{bD}
	ACTEX8%	84.2 ± 0.8^{cA}	81.1 ± 0.7^{bB}	71.3 ± 1.8^{cC}	61.0 ± 1.9^{dD}	45.0 ± 1.0^{cA}	41.4 ± 0.7^{bB}	30.8 ± 1.9^{cC}	24.5 ± 1.3^{bD}

TM: Total motility; PM: Progressive motility; SM: Skim milk; TEY: Tris egg yolk; ACTEX: Cactus acetone extract.Values are expressed as mean ± SEM. ^{a,b,c,d}Different superscripts within each column indicate an effect of ACTEX within each duration of storage ($P < 0.05$). ^{A,B,C,D}Different superscripts with in rows indicate an effect of duration for each concentration of ACTEX ($P < 0.05$).

Table 3
Effects of ACTEX concentration added to SM and TEY extenders on ram sperm viability and membrane integrity after 8, 24, 48 and 72 h of storage at 5 °C.

Extenders	Groups	VIAB (%)				HOST (%)			
		8 h	24 h	48 h	72 h	8 h	24 h	48 h	72 h
SM	CONTROL	88.0 ± 0.5^{abA}	81.4 ± 0.5^{bB}	76.1 ± 0.7^{bcBC}	72.0 ± 1.4^{cC}	70.3 ± 0.6^{bA}	67.1 ± 1.7^{abA}	60.5 ± 1.8^{bB}	50.0 ± 1.0^{bC}
	ACTEX1%	90.7 ± 0.6^{aA}	85.0 ± 1.2^{aAB}	83.5 ± 1.4^{aBC}	80.6 ± 0.7^{aC}	74.1 ± 1.4^{aA}	70.1 ± 0.6^{aAB}	66.8 ± 1.8^{aB}	60.8 ± 0.9^{aC}
	ACTEX2%	87.3 ± 0.8^{aA}	82.5 ± 1.1^{bAB}	78.0 ± 1.3^{bBC}	74.5 ± 2.3^{bcC}	69.7 ± 0.8^{bcA}	65.1 ± 1.7^{bA}	54.4 ± 1.3^{cB}	43.3 ± 1.8^{cC}
	ACTEX4%	86.2 ± 0.9^{bA}	82.3 ± 0.7^{abA}	75.4 ± 1.1^{cB}	69.4 ± 0.8^{dC}	66.7 ± 1.6^{bcA}	63.4 ± 1.4^{bA}	53.1 ± 1.3^{cB}	41.7 ± 1.1^{cC}
	ACTEX8%	85.6 ± 1.0^{bA}	81.1 ± 0.9^{abA}	75.12 ± 1.9^{cB}	66.1 ± 1.4^{dC}	64.3 ± 1.1^{cA}	58.7 ± 1.6^{cB}	48.1 ± 1.3^{dC}	35.0 ± 1.5^{dD}
TEY	CONTROL	88.8 ± 0.8^{abA}	83.1 ± 1.6^{bcB}	77.8 ± 1.2^{bB}	76.1 ± 1.5^{bC}	65.8 ± 1.1^{bA}	60.8 ± 1.6^{cB}	57.6 ± 0.9^{bB}	43.8 ± 0.8^{bC}
	ACTEX1%	92.0 ± 0.8^{aA}	89.0 ± 0.7^{aAB}	86.3 ± 1.2^{aBC}	83.2 ± 1.3^{aC}	72.0 ± 1.0^{aA}	70.6 ± 0.5^{aAB}	66.0 ± 1.2^{aB}	53.6 ± 1.9^{aC}
	ACTEX2%	88.3 ± 1.2^{abA}	86.0 ± 1.2^{abAB}	83.0 ± 1.1^{abB}	75.8 ± 0.8^{bC}	69.1 ± 0.9^{abA}	66.3 ± 1.5^{bA}	58.1 ± 2.7^{bB}	44.8 ± 1.5^{bC}
	ACTEX4%	87.7 ± 1.1^{bA}	83.4 ± 0.6^{bcB}	79.7 ± 0.6^{bcC}	74.0 ± 1.0^{bD}	65.8 ± 1.6^{bA}	63.1 ± 1.3^{bcA}	54.2 ± 1.5^{bB}	35.5 ± 1.2^{cC}
	ACTEX8%	87.0 ± 0.8^{bA}	81.9 ± 1.0^{cB}	76.8 ± 1.5^{cC}	69.0 ± 0.6^{cD}	65.3 ± 1.1^{bA}	61.3 ± 0.5^{cA}	55.5 ± 1.7^{bB}	34.5 ± 1.7^{cC}

VIAB: viability; HOST: Hyperosmotic swelling test; SM: Skim milk; TEY: Tris egg yolk; ACTEX: Cactus acetone extract; Values are expressed as mean ± SEM. ^{a,b,c,d}Different superscripts within each column indicate an effect of ACTEX within each duration of storage ($P < 0.05$). ^{A,B,C,D}Different superscripts with in rows indicate an effect of duration for each concentration of ACTEX ($P < 0.05$).

maintaining sperm motility and viability (Bergeron et al., 2007). However, if the sperm are diluted with milk, excess of BSP protein is sequestered by the casein milk. This protein–protein interaction prevents continual extraction of membrane lipids of spermatozoa caused by BSP proteins, this process ensures better conservation of sperm (Quan et al., 2016). Furthermore, the SM contains the lactoferrin. It's considered as a source of free radical-scavenging peptides. Its advantage as an antioxidant on semen and it gives the possibility to improve the quality of stored ram sperm (Ollero et al., 1998). The difference recorded between TEY and SM in the present study is probably due to the presence of casein in skim milk, supposing that casein could be more efficient to protect the semen during liquid storage than Tris egg yolk.

The plasma membrane of ram sperm is rich in polyunsaturated fatty acids, which make ram spermatozoa sensitive to peroxidative damage (Alvarez and Storey, 1983; Alvarez et al., 1987; Griveau et al., 1995). Some chemicals have been used to increase the tolerance to oxidative stress of sperm preserved at low temperature. For example, a protective effect of alpha-tocopherol on boar (Cerolini et al., 2000), stallion (Ball and Vo, 2002) and ram semen (Upreti et al., 1997) was reported. Reports with *in vitro* studies demonstrated that other exogenous antioxidants, such as taurine (Bucak et al., 2007), methionine (Çoyan et al., 2010), reduced glutathione and catalase (Câmara et al., 2011), ascorbic acid (Thuwanut et al., 2011), vitamin B12 (Asadpour, 2012) and lycopene (Akalin et al., 2016) can prevent oxidative damage in ram sperm during liquid storage.

Currently, there is no relevant research investigating the protective effect of ACTEX on ram sperm. Furthermore, according to many investigations, the addition of other natural herbs such as clove bud *(Syzygium aromaticum)* and rosemary (*Rosmarinus officinalis* L.) lead to improve ram sperm quality (Baghshahi et al., 2014; Motlagh et al., 2014). In the present study, the effects of ACTEX extract from *opuntia ficus indica* on sperm motility, viability, membrane integrity, abnormality, lipid peroxidation, and DNA fragmentation were investigated after liquid storage for up to 72 h. The results showed that the protective effects of ACTEX might be dependent on the concentration used.

More precisely, the inclusion of 1% ACTEX in TEY and SM can maintain the total motility, progressive motility, viability and membrane integrity of sperm cells compared to the control groups. In contrast, all semen variables assessed in the present research were affected when the ACTEX concentrations were greater than 2%. Indeed, it has been highlighted that an appropriate antioxidant concentration is required (Roca et al., 2004). The beneficial effects of ACTEX may be due to the protective properties against lipid

Table 4
Effects of ACTEX concentration added to SM and TEY extenders on ram sperm abnormality after 8, 24, 48 and 72 h of storage at 5 °C.

Extenders	Groups	ABN (%)			
		8 h	24 h	48 h	72 h
SM	CONTROL	8.8 ± 1.3^{bcD}	14.8 ± 0.8^{bC}	18.6 ± 0.6^{aB}	23.5 ± 1.4^{aA}
	ACTEX1%	7.8 ± 0.6^{cC}	10.3 ± 0.4^{cBC}	12.1 ± 1.0^{bAB}	15.5 ± 1.4^{bA}
	ACTEX2%	11.5 ± 1.2^{abB}	14.3 ± 1.2^{abB}	15.8 ± 1.6^{aB}	21.8 ± 1.0^{aA}
	ACTEX4%	13.0 ± 1.0^{aC}	16.0 ± 1.0^{abBC}	18.7 ± 0.5^{aB}	22.4 ± 1.1^{aA}
	ACTEX8%	14.0 ± 0.5^{aC}	16.6 ± 1.0^{aBC}	18.5 ± 1.1^{aAB}	22.3 ± 1.4^{aA}
TEY	CONTROL	7.3 ± 0.8^{bC}	10.5 ± 0.9^{bcBC}	13.5 ± 1.1^{bcB}	21.5 ± 1.9^{aA}
	ACTEX1%	6.5 ± 0.8^{bC}	8.0 ± 0.7^{cBC}	11.1 ± 0.8^{Cb}	14.8 ± 0.8^{bA}
	ACTEX2%	8.5 ± 0.7^{bC}	10.0 ± 0.8^{bcC}	13.4 ± 1.5^{bcB}	20.9 ± 1.1^{aA}
	ACTEX4%	7.6 ± 0.5^{abC}	11.5 ± 1.0^{abC}	15.9 ± 1.1^{abB}	22.4 ± 1.1^{aA}
	ACTEX8%	10.0 ± 0.9^{aC}	13.6 ± 0.9^{aC}	19.0 ± 0.7^{aB}	24.3 ± 1.4^{aA}

SM: Skim milk; TEY: Tris egg yolk; ACTEX: Cactus acetone extract; Values are expressed as mean ± SEM. ^{a,b,c,d}Different superscripts within each column indicate an effect of ACTEX within each duration of storage ($P < 0.05$). ^{A,B,C,D}Different superscripts within rows indicate an effect of duration within each concentration of ACTEX ($P < 0.05$).

Table 5
Effects of ACTEX concentration added to SM and TEY extenders on ram sperm lipid peroxidation (TBARS, nmol/10^8 sperm) after 8, 24, 48 and 72 h of storage at 5 °C.

Extenders	Groups	Spontaneous lipid peroxidation				Induced LPO 0.24 nmol FeSO4/60 min			
		8 h	24 h	48 h	72 h	8 h	24 h	48 h	72 h
SM	CONTROL	0.40 ± 0.02^{bcD}	0.89 ± 0.04^{aC}	1.75 ± 0.03^{aB}	2.34 ± 0.03^{aA}	1.56 ± 0.12^{aD}	1.66 ± 0.06^{aC}	2.04 ± 0.03^{aB}	3.40 ± 0.10^{aA}
	ACTEX1%	0.26 ± 0.03^{cC}	0.45 ± 0.05^{bB}	0.60 ± 0.04^{bB}	1.31 ± 0.04^{cA}	0.48 ± 0.08^{bD}	0.71 ± 0.04^{cC}	1.23 ± 0.04^{bB}	1.82 ± 0.04^{bA}
	ACTEX2%	0.55 ± 0.05^{abD}	0.78 ± 0.04^{aB}	1.51 ± 0.07^{aC}	1.96 ± 0.14^{bD}	1.26 ± 0.03^{aC}	1.45 ± 0.03^{bC}	1.85 ± 0.05^{aB}	3.20 ± 0.11^{aA}
	ACTEX4%	0.68 ± 0.03^{aC}	0.80 ± 0.03^{aC}	1.53 ± 0.07^{aB}	2.10 ± 0.09^{aA}	1.36 ± 0.07^{aC}	1.46 ± 0.05^{abC}	1.84 ± 0.13^{aB}	3.18 ± 0.12^{aA}
	ACTEX8%	0.66 ± 0.03^{aC}	0.87 ± 0.03^{aC}	1.57 ± 0.08^{aB}	2.37 ± 0.15^{aA}	1.25 ± 0.08^{aD}	1.59 ± 0.05^{abC}	1.94 ± 0.05^{aB}	3.18 ± 0.08^{aA}
TEY	CONTROL	0.62 ± 0.07^{aD}	1.27 ± 0.04^{abC}	2.07 ± 0.05^{bB}	2.58 ± 0.15^{aA}	1.20 ± 0.08^{bD}	1.88 ± 0.04^{aC}	2.44 ± 0.05^{bcB}	3.27 ± 0.12^{aA}
	ACTEX1%	0.38 ± 0.04^{bB}	0.54 ± 0.05^{cB}	0.65 ± 0.04^{dB}	1.43 ± 0.11^{cA}	0.93 ± 0.04^{cA}	1.28 ± 0.02^{dB}	1.45 ± 0.10^{dB}	1.79 ± 0.06^{bC}
	ACTEX2%	0.59 ± 0.03^{abC}	1.21 ± 0.05^{bB}	1.56 ± 0.08^{cB}	2.29 ± 0.11^{bcA}	1.17 ± 0.05^{bD}	1.65 ± 0.06^{cC}	2.13 ± 0.13^{cB}	3.02 ± 0.13^{aA}
	ACTEX4%	0.72 ± 0.05^{aC}	1.41 ± 0.07^{aB}	2.08 ± 0.03^{bA}	2.38 ± 0.12^{aA}	1.42 ± 0.07^{aC}	1.71 ± 0.02^{bcC}	2.56 ± 0.13^{bB}	3.29 ± 0.10^{aA}
	ACTEX8%	0.70 ± 0.06^{aC}	1.45 ± 0.09^{aB}	2.36 ± 0.08^{aA}	2.27 ± 0.04^{abA}	1.39 ± 0.03^{aB}	1.82 ± 0.07^{abB}	3.07 ± 0.20^{aA}	3.28 ± 0.22^{aA}

SM: Skim milk; TEY: Tris egg yolk; ACTEX: Cactus acetone extract. Values are expressed as mean ± SEM. ^{a,b,c,d}Different superscripts within each column indicate an effect of ACTEX within each duration of storage ($P < 0.05$); ^{A,B,C,D}superscripts within rows indicate an effect of duration within each concentration of ACTEX ($P < 0.05$).

peroxidation and the powerful antioxidant properties of cactus cladodes extracts (Chougui et al., 2013; Ghazi et al., 2013).

According to many studies, the cactus cladode contains a large variety of compounds with antioxidant activities such as polyphenols (particularly some flavonoids and proanthocyanidins), vitamin C and E, β-carotene (provitamin A), glutathione, taurine, cysteine, methionine and arginine molecules (Tesoriere et al., 2005; Betancourt-Domínguez et al., 2006; Medina-Torres et al., 2011; Brahmi et al., 2012). The exact mechanism by which ACTEX extract enhance the ram sperm quality remains unclear and is of great interest. However, the positive effects of ACTEX in the present study may be due to the combined effects of many of its compounds. Data in Table 1 indicate acetone extract contains organic acids, flavonoids and polyphenols, such as quinic acid, malic acid, rutin, hyperoside and quercitin. To the best of our knowledge, the malic acid and quinic acid, which are the major compounds of our extract, have never been tested to improve ram semen quality. In fact, the organic acids mainly function in maintaining the ability to inhibit O_2^- accumulation, delaying H_2O_2 decrease (Ding et al., 2007; Huang et al., 2013), enhancing antioxidant enzyme activities of perixidase and polyphenol oxidase (Cao et al., 2010). The quinic acid has been previously reported to be an active compound with significant antioxidant properties (Farah and Donangelo, 2006; Pero et al., 2009). In addition, this compound is capable of reducing the oxidative stress in cells (Saltiel and Kahn, 2001). In addition to quinic acid, the quercitin is a flavonoid (Nass-Arden and Breitbart, 1990; McNiven and Richardson, 2002), which can suppress the formation of the superoxide ion, chelate iron, and inhibit lipid peroxy radical formation (Afanas'ev et al., 1989). In addition, quercitin can decrease DNA damage in sperm, protect the integrity of human sperm DNA, and affect intracellular calcium release (McNiven and Richardson, 2006; Silva et al., 2012). Likewise, quercitin modulates the capacitation of bull sperm, while decreasing the concentration of reactive oxygen species (ROS; Córdoba et al., 2006, 2007, 2008). Quercitin might be used to prevent adverse effects induced by hydrogen peroxide, reduce the concentrations of MDA, and maintain the mitochondrial membrane potential (Silva et al., 2012). In addition to quercitin, ACTEX contains rutin. Rutin is a glycoside of Q, Q 3 rutinoside, and it has been reported as one of the most common flavonol glycosides in the human diet (Hertog et al., 1993). Two studies reported the antioxidant effect of rutin against oxidative stress induced by Fe (2+)/ascor-

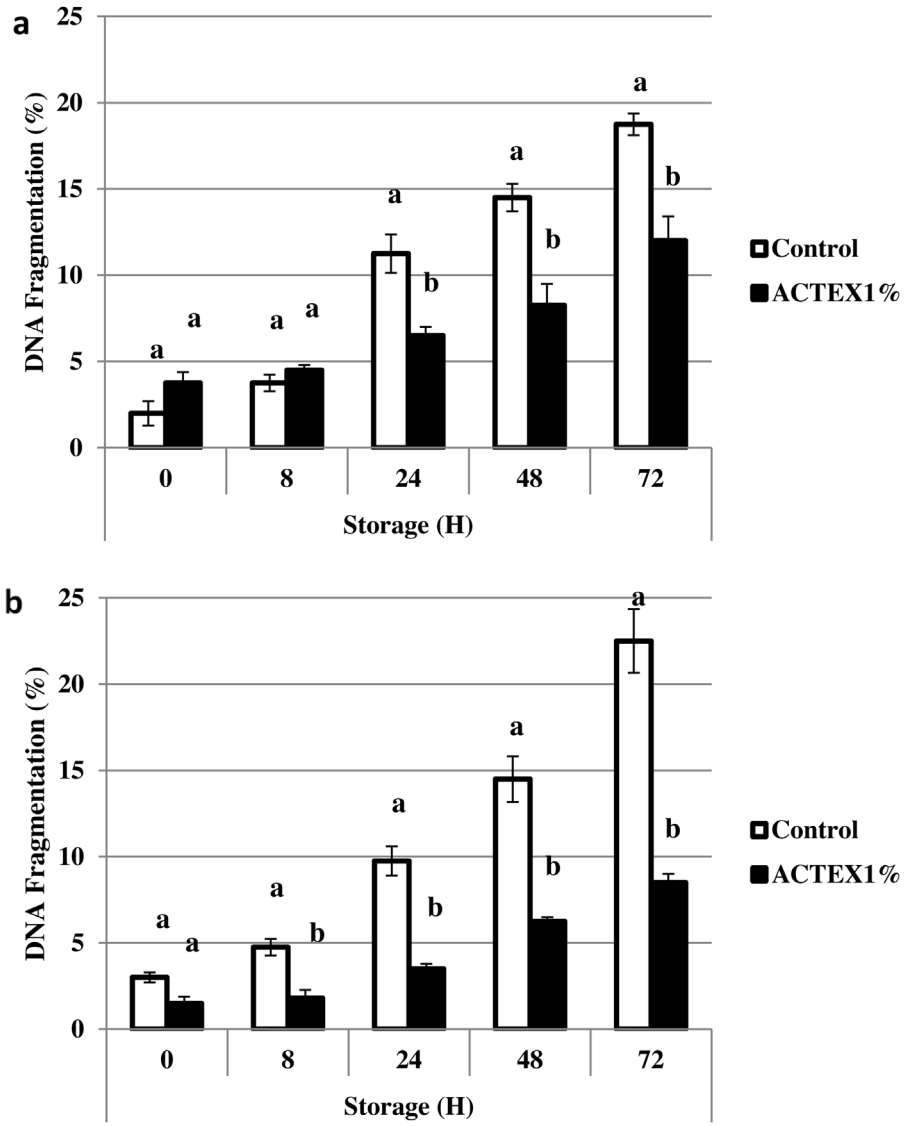

Fig. 1. Effect of ACTEX on sperm DNA fragmentation; Ram sperm were diluted with skim milk (a) and tris egg yolk (b) extenders supplemented with ACTEX and stored for 0, 8, 24, 48, 72 h at 5 °C; Different superscripts indicate a difference between ACTEX concentrations within each duration of storage ($P < 0.05$).

bate in red deer (Mata-Campuzano et al., 2012a) and ram (Mata-Campuzano et al., 2012b) thawed sperm.

5. Conclusion

From the present study, it was concluded that the ACTEX exacted from a natural plant has significant antioxidative effects on ram semen during liquid storage at 5 °C. The 1% concentration of ACTEX (1%) can improve sperm quality variables such as TM, PM, VIAB, HOST, ABN, oxidation and DNA fragmentation. However, greater concentrations of ACTEX cannot efficiently protect ram sperm preserved at 5 °C. In the future, more investigations are needed to identify the major chemicals compound present in ACTEX. Additionally, future research should focus on the mechanisms of the protective effect of the antioxidants ACTEX during liquid or cryopreservation. Moreover, a trial of AI is planned to predict the possible response of the female genital tract to ACTEX and to determine whether some components of the ACTEX could affect negatively the final fertilization outcome.

Conflicts of interest

none.

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