Protective effect of natural antioxidant Opuntia ficus-indica on ram semen quality stored at 5 °C for 72

Larbi ALLAI
laboratoire de recherche Management de l'agriculture durable (MAD), Ecole supérieure de technologie de Sidi Bennour, Université Chouaib Doukkali, El Jadida

Xavier Druart
INRA UMR 85: Physiologie de la reproduction et des comportements F 37380, Nouzzily

Pinar Terzioğlue
Faculty of Engineering and naturel sciences, Departement of Fiber and polymer Enginnering ,Bursa technical university, Bursa

Noureddine Louanjli
Labomac IVF Centres and Clinical Laboratory Medicines, Casablanca

Boubker Nasser
Laboratoire de Biochimie et Neurosciences, FST de Settat, Université Hassan 1er, Settat

mehmet Ozturk
Departement of Chemistry, Faculty of Sciences Mugla Sitki Kocman University: Mugla

BOUCHRA EL AMIRI (bouchraelamiri@hotmail.com)
National Institute of Agricultural Research https://orcid.org/0000-0003-3443-5988

Research

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Abstract

Background

Recently, researchers have focused on the use of natural antioxidants to improve semen quality as a key element for successful artificial insemination. In this context the first aim of this study was to determine the antioxidant activity and composition (minerals, vitamins, and sugars) of *Opuntia ficus-indica* cladode ethanolic extract (ETHEX). A further purpose of the study was to investigate the effect of ETHEX supplementation on the quality of liquid ram semen extended with skim milk (SM) at 5°C. The antioxidant activity of ETHEX was studied using $DPPH^{\bullet}$ assay. The mineral composition and the sugar and vitamin contents of ETHEX were determined using an inductively coupled plasma optical emission spectrometry (ICP-OES), and HPLC-DAD-RID analytical instruments. As a second part, semen was collected from five Boujaâd rams with an artificial vagina. The ejaculates with more than 70 % motility were pooled, extended with skim milk (SM) extender without (control) or supplemented with 1–8 % of ETHEX (37°C; 0.8×10^9 sperm/mL). Sperm quality parameters were assessed at 8, 24, 48 and 72 h.

Results

The results showed that ETHEX had a higher antioxidant activity compared to those of ascorbic acid and butylated hydroxytoluene (BHT). Furthermore, ETHEX contains a considerable amount of minerals, vitamins, and sugars. The inclusion of 1 or 2 % ETHEX in SM increased the sperm motility, viability, membrane integrity and decreased the abnormality, spontaneous and catalyzed lipids peroxidation ($P < 0.05$) up to 72 h. In addition, semen diluted with 1 and 2 % ETHEX decreased the level of DNA fragmentation compared to the control group ($P < 0.05$).

Conclusions

In conclusion the ETHEX could be recommended to improve the quality of liquid ram spermatozoa. However, its effects on sperm physiology and artificial insemination should be further studied.

Background

Artificial insemination with cryopreserved ram is not common for sheep industry [1] because cryopreservation leads to a decrease in fertility. During cryopreservation process, all sperm parameters extremely decrease compared to the liquid storage. The acceptable results have only been obtained with laparoscopic. For that reason, liquid storage seems to be the alternative to frozen-thawed semen. Therefore, some studies have motivated the use of liquid semen preservation for different species including boar [2, 3], cattle [4], antelope [5], horse [6] and sheep [7, 8]. One of the main factors associated with the low sperm quality during liquid storage and cryopreservation is the production of reactive oxygen species (ROS), which occurs as an abnormal consequence of sperm metabolism [9]. To prevent such

stress, the semen needs to be diluted in a suitable extenders and conditions. However, the addition of extenders generates a significant reduction in the antioxidant capacity of spermatozoa and seminal plasma. In fact, the spermatozoa and seminal plasma system comprising taurine, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) to prevent oxidative damage. The antioxidant system of spermatozoa is compromised during semen processing [10]. For those reason, the need of extender supplementation is become necessary. Many additives with antioxidative properties have been reported to reduce the impact of ROS-induced and cold shock damages on sperm cells [11] and thus improve ram [12–14], bull [15, 16], cat [17], rabbit [18] and boar [3, 19] sperm quality. Antioxidant molecules could reduce the impact of oxidative stress, and thus improve semen quality after liquid storage.

In this context, the effects of several plant extracts have been demonstrated in many animal species [20]. However, it well known that the antioxidant property of plant extract is correlated with their free radical scavenging properties [21]. The cactus *Opuntia ficus indica* is cultivated in both hemispheres. It is abundant in North and South America (Mexico, United States), Mediterranean countries including Morocco and South Africa [22–24]. Ethanol extract from *Opuntia ficus-indica* cladode is a natural antioxidant source with a free radical scavenging potential due to its constituents such as tocopherol, polyphenols, flavonoids, quercetin, phenolic acids (caffeic and gallic acid), minerals and sulfur amino acids, (taurine, methionine, arginine and cysteine) [25–27]. A report has shown the beneficial effect of ETHEX on lipoperoxidation of low-density lipoprotein reduction and DNA oxidation [28]. Besides, several studies have reported its efficiency in the treatment of several diseases. It exhibited anti-tumoral [29] antiviral [30], anti-inflammatory [31, 32] and antioxidant effects [33, 34].

To the best of our knowledge, this is the first report focusing on the effect of *Opuntia ficus-indica* cladode ethanolic extract on Boujaâd ram sperm quality parameters. The objective of this study was to determine the antioxidant properties, mineral, vitamin and sugar composition of ETHEX and to assess its inclusion to SM on sperm total and progressive motility, viability, abnormality, membrane integrity, lipid peroxidation levels, and DNA fragmentation during liquid storage at 5°C up to 72 h.

Materials And Methods

Chemicals

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Merck (Merck, Schuchardt, OHG, Germany). Ultrapure water obtained from a Millipore Milli-Q system (Bedford, MA, USA).

Cactus extraction and DPPH inhibition method

Nopal cactus cladodes were collected from the experimental station of Regional Center-INRA Settat, Morocco. They were washed with distilled water, dried at 55 °C in an oven and mechanically milled. The obtained powder was stored in a closed container at room temperature until use. In order to prepare the ethanol extract, a fine dried powder (5 g) was extracted by stirring at room temperature with 100 mL of

ethanol /water mixture (70: 30 v/v) for 4 days at 4 °C according to method of Radi et al. [35]. After the solution was filtered, then the solvent was evaporated using a vacuum rotary evaporator (Buchi R-210) at 38 °C. The remained part was lyophilized and stored at 4 °C until used.

Radical scavenging activity (RSA) of ETHEX was measured using the free radical 1,1- diphenyl-2-picrylhydrazyl (DPPH) as described by [36]. A known amount of the dry ETHEX (0.1 mL) was centrifuged in methanol (2.9 mL) at 5000 rpm for 15 minutes followed by filtration through filter paper (Whatman No.1). Methanolic solution of DPPH (0.5 mL, 100 µM) was added to the tubes including the supernatant of each concentration. After vigorous shaking of the tubes, they were incubated at room temperature for 45 minutes in the darkness. Ascorbic acid and BHT were used as reference compounds. Lower absorbance of the reaction mixture indicated the higher free radical scavenging activity.

The percentage of free radical scavenging activities of the samples were calculated using the following formula [37]:

$\% \text{ RSA} = [(A_D - A_E)/ A_D]*100$

Where A_D and A_E is the absorbance of the DPPH blank sample and the absorbance of the test solution, respectively. A_E was the difference between the absorbance value of the test solution and its blank.

Total sugar content

The sample (2 g) was extracted with ultrapure water (20 mL) in an ultrasonic bath (Elmasonic, Germany) for 10 minutes at room temperature. Then it was filtered through 0.45 µm PTFE filter.

Total sugar content of sample was determined using a high-performance liquid chromatography (HPLC) system (Agilent Technologies, USA) equipped with a refractive index detector (RID). The sugars were separated by NH_2 column (Inertsil, 5 µm, 4.6 mm × 250 mm, GL Sciences) maintained at 30°C. The injection volume was 10 µL. The mobile phase was acetonitrile (80%) with the flow rate of 1.0 mL min^{-1}. Standard solutions were injected to get the retention time and standard curve for each sugar. The results were evaluated with the ChemStation Software and expressed as mg g^{-1} dry weight.

Vitamin content

The sample (1 g) was extracted with ultrapure water (10 mL) in an ultrasonic bath (Elmasonic, Germany) for 10 minutes at room temperature. After, 1 mL of 2 M NaOH and 12.5 mL of 1 M phosphate buffer (pH=5.5) were added and fulfilled to 25 mL with ultrapure water. Then, the sample was filtered through 0.45 µm PTFE filter before injection.

The vitamin content of sample was determined using a high-performance liquid chromatography (HPLC) system (Agilent Technologies, USA) equipped with a diode array detector (DAD). The vitamins were separated on ODS column (5 µm, 4.6 mm × 250 mm, Inertsil, GL Sciences) maintained at 30 °C. The injection volume was 10 µL. The mobile phases were the aqueous solution of trifluoroacetic acid (0.025%,

v/v), solution A; and acetonitrile, solution B. The elution program was the gradients of Solvent B as following :0.0% solvent B (0-5th min), 25 % solvent B (6-11th minutes), 45% solvent B (11-19th minutes), 40 % solvent B (19-20th minutes), 0% solvent B (20-22nd minutes). Standard solutions of vitamins (Vitamins C, B2, B3, B5, and B9) were injected to get the retention time and standard curve for each vitamin. The results were evaluated with the ChemStation Software and expressed as mg /100 g dry weight.

Mineral content

The digestion of sample was performed on microwave digestion system (CEM MARS5 (USA). Approximately 0.5 g of dry sample was transferred to a PTFE digestion tube containing 6 mL of nitric acid (65 %) and 2 mL of hydrogen peroxide. The operating conditions of microwave oven were as follow: temperature (150-200 °C), ramp (20 min), time (2 min) and the power (100 %) for each step. The digested sample was cooled to room temperature, filtered and the filtrate was diluted by adding 100 mL of ultra-pure water. The mineral concentration was determined by the inductively coupled plasma optical emission spectrometry (ICP-OES), (Agilent 5100, USA). The nitrogen content was estimated by the Kjeldahl method according to the Altuntas et al. [38].

Animals, semen collection and processing

Five Boujaâd mature rams (weight: 80-85 kg, aged between 3 and 4 years) were used as semen donors. They were maintained at the station of animal Reproduction Biology at the Research Regional Center, Settat INRA-Morocco. A total number of 50 ejaculates were collected from the rams using an artificial vagina, during the breeding season (July to September) and then the semen was pooled to minimize individual variation. After semen collection, the semen (10 µL) was placed in a glass slide without coverslip and the wave motion of semen evaluated (0–5 scale) after judging five different microscopic fields. The sperm concentration was determined by a spectrophotometer. Ejaculates, which met the following criteria, volume of 0.5–2 mL; good wave motion (≥3 on a 0–5 scale), ≥2.5 × 10^9 spermatozoa/mL and ≥70 % motile sperm were evaluated and used for the next step. All ejaculates were pooled in order to eliminate individual differences and diluted with a skim milk (SM) as the base extender. It was prepared by diluting skim milk (11 g) in distilled water (100 mL) and then heated at 95 °C for 10 minutes. Penicillin and streptomycin (0.05 mg/mL) were added to the extender. The semen was diluted with the base extenders at 37 °C, containing 0 (control), 1, 2, 4 and 8 % of ETHEX to reach a final concentration of approximately 0.8 × 10^9 sperm/mL (single step dilution).

The semen samples were then cooled from 37 to 5 °C. Sperm total motility, progressive motility, viability, abnormality, membrane integrity and lipid peroxidation were determined at 8, 24, 48 and 72 h. The combination extender × antioxidant concentration giving the best protective effects on sperm progressive motility was selected to assess DNA fragmentation at 8, 24, 48 and 72 h using tunnel technique.

Evaluation of semen characteristics

Sperm total and progressive motility were assessed by a computer-assisted sperm analysis system (ISAS, version 1.0.17, Proiser, Valencia, Spain). The semen was diluted with PBS-BSA to reach 20 × 10^6 sperm/mL at 37 °C. The sperm motility was assessed using a 10× negative phase contrast objective on a UB203 microscope (UOP/Proiser, Paterna, Valencia, Spain).

For viability assessment, eosin-Y (1.67 g), nigrosin (10 g), sodium citrate (2.9 g), dissolved in distilled water (100 mL) and then nigrosin–eosin stain (10 µL) and sperm dilution (5 µL) were mixed together on glass slide [39]. The mixture was smeared and examined with bright-field microscopy (400x). A total number of 200 spermatozoa were counted. Sperm showing partial or complete purple heads were considered non-viable or dead and only sperm showing strict exclusion of the stain were considered alive [40].

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm plasma membrane according to Jeyendran et al. [41]. Solution for HOST assay consisted of fructose (9 g), sodium citrate (4.9 g) per liter of distilled water. For sperm tail assessment, semen (30 µL) was mixed with hypoosmotic solution (300 µL, 100 mOsM) and incubated for 50-60 minutes at 37 °C. After incubation, 10 µL of the mixture was spread with a coverslip on a warm slide. A total number of 200 spermatozoa were counted at least using five different microscopic fields at 400x. The percentage of spermatozoa with swollen and curved tails was recorded [42].

The morphology of sperm was evaluated using the Diff-Quik kit (Diagnostic Systems S.L. Barcelona, Spain). Briefly, 3 µL of diluted semen was smeared on a glass slide and allowed to air-dry. The slide was then dipped into a fixative solution for 1 min and into first and second solutions seven to ten times. Between the fixing step and each of the staining steps, the excess solutions were dried from the slides by placing them vertically on absorbent paper. At least 200 sperms were evaluated under light microscopy at 1000× magnification using UB203 microscope (UOP/Proiser, Paterna, Valencia, Spain).

Measurement of lipid peroxidation

Malondialdehyde (MDA) concentrations, as an indice of lipid peroxidation (LPO) in the semen samples, were measured using the thiobarbituric acid reaction [43]. The thiobarbituric acid reactive substances (TBARS) were measured in the semen spontaneous LPO or after incubation with 0.24 mM of $FeSO_4$ at 37 °C in a water bath for 60 minutes (iron catalyzed LPO). The TBARS concentration was determined by comparing the sample's absorbance at 532 nm with a standard curve prepared using MDA. The results were expressed in nmol TBARS/10^8 sperm.

Assessment of DNA fragmentation

For the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique, the *in Situ* Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) has been used according to Nur et al. [44]. Briefly, different aliquots of semen samples were diluted with phosphate-

buffered saline (PBS) and centrifuged at 400 x *g* for 10 minutes. One drop of re-suspended spermatozoa was smeared on a glass slide and fixed with 10 % formaldehyde for 30 minutes at room temperature.

The slides were washed three times with PBS (5 minutes each), treated in a humidified chamber with proteinase K for10 minutes, washed with PBS, treated with 3 % H_2O_2 in distilled water for 10 minutes at room temperature and washed again with PBS. The slide was permeabilized with 0.1 % Triton X-100 for 5 minutes on ice. The slides were incubated in dark at 37 °C for one hour with the TUNEL reaction mixture. After labeling, samples were washed with PBS and analyzed immediately using fluorescence microscopy (Zeiss eurostar, Germany 100×). The percentage of TUNEL positive sperm was determined by the evaluation of at least 100 sperm.

Statistical methods

Data were analyzed statistically using JMP15.0 (SAS Institute Inc., Cory, NC, USA) program. All dependent variables were submitted to the Shapiro–Wilk test normality and homogeneity analysis. Dependent variables with normal distribution were evaluated using variance analysis with the comparison of means using Tukey's test. Results were expressed as the mean ± SEM (standard error meaning). Differences with values of $P< 0.05$ were considered to be statistically significant.

Results

Radical-scavenging activity

The scavenging ability of antioxidant substances was evaluated by the DPPH assay. The results on the scavenging activities of ETHEX, ascorbic acid and BHT are presented in Table 1. The results indicate that the antioxidant activity of the ETHEX was higher than ascorbic acid and BHT in all tested concentrations.

Sugar, vitamin and mineral contents

As shown in Table 2, the major sugars presented in ETHEX were saccharose (2.85 mg g^{-1}) and glucose (2.67 mg g^{-1}). The fructose content was found to be 0.56 mg g^{-1}. As seen in Table 2, vitamins C, B2, B9 and B3 were detected in ETHEX. Among them, the major vitamin was B2 (1384 ± 5.04 mg/100 g), followed by vitamin C (963.1 ± 3.04 mg/100 g), and vitamin B3 (91.79 ± 0.19 mg/100 g). The vitamin B5 was undetected in the cactus.

The mineral content of ETHEX (Table2) revealed the presence of boron, calcium, copper, iron, manganese, magnesium, nitrogen, phosphorus, potassium, sodium and zinc. The copper (0.004 g/100 g); manganese (0.005 g/100 g); zinc (0.003 g/100 g); and boron (0.003 g/100g) concentrations were found to be lower. The most abundant mineral was potassium followed by nitrogen, calcium, and magnesium. The data showed that ETHEX contains iron (0.020 g/100 g).

Microscopic sperm parameters

The effects of ETHEX on sperm motility, viability, membrane integrity and abnormality of ram semen during different storage times at 5 °C are presented in Fig. 1 - 3.

The effect of ETHEX on sperm total motility and progressive motility was significantly higher in SM supplemented with 1 and 2 % compared to the control groups (Fig.1). However, no difference was found between SM with 4 % ETHEX and the control group (Fig.1).

Regarding the viability, a significant difference was recorded between the control and the SM with 1 and 2 % ETHEX ($P< 0.05$) after 48 h of storage (Fig.2). In contrast, no difference was found between the control and other samples (SM with 4 % and 8 % ETHEX) (Fig.2). Whereas the enrichment of the basic extender with 1% ETHEX resulted in higher percentage of membrane integrity compared to the control groups (Fig.2).

The results for abnormality up to 72 h of incubation with different concentrations of ETHEX in SM are presented in Fig.3. These results showed that addition of 1 % of ETHEX to SM decreases the percentage of abnormality compared to the control ($P< 0.05$).

Malondialdehyde concentration

The spontaneous and catalyzed lipid peroxidation levels in ram semen containing different concentration of ETHEX for different storage periods at 5 °C are given in Table 3. The level of spontaneous and catalyzed LPO in control groups were significantly higher when compared to the experimental groups supplemented with 1 and 2 % of ETHEX during the storage period at 5 °C in SM. The skim milk with 1 % of ETHEX maintained a better effect when compared to the other concentrations. While no effect ($P< 0.05$) was observed when the concentration exceeded 2 % compared to the control group.

DNA Fragmentation levels

The effect of ETHEX on DNA fragmentation of liquid stored ram semen at different time periods is shown in Table 4. It was determined that using the selected concentrations was statistically significant in sperm DNA damage of ram semen during liquid storage. The DNA fragmentation significantly reduced, in comparison to the controls, with addition of 1 % ETHEX after 8 h of storage and 2 % ETHEX to SM after 24 and 72 h of storage.

Discussion

Tris egg yolk and skim milk are the commonly used extenders for sperm preservation [45, 46] However, skim milk-based extenders give satisfactory results in storage of ram semen, compared to Tris based extenders [47]. It is worthy that even with SM, handling fresh semen depends on time and temperature of storage [48] It has already been demonstrated that ram semen quality decrease extremely in cold liquid storage after 3–5 days [7].

To overcome the loss of semen quality during storage, several extracts form plant have been listed as natural antioxidants that could target such effects [49–53]. Even, to the best of our knowledge, there is no report in the literature on the supplementation of ram semen with ETHEX. Thus, the present study was carried out to test the ability of ETHEX at four concentrations (1, 2, 4 and 8 %) to prevent the harmful effect of handling liquid ram semen in SM at 5°C up to 72 h .The main finding that emerges from this study was that the beneficial influence of ETHEX resulted in higher overall quality of ram semen. In fact, the inclusion of 1 and 2 % ETHEX to the ram semen extended in SM at 5°C increase the sperm motility, viability, and membrane integrity and decrease the sperm abnormality. In contrast, concentrations above this threshold (2 %) showed no significant results on sperm quality.

The beneficial effects recorded while using 1 % and 2 % ETHEX are probably due to synergistic effects of multiple constituents of natural extracts and not to the single purified active compounds [54]. The results of a study showed that the most herbs and herb-derived products offer a large variety of one or more active compounds [55]. The ETHEX, characterization showed a considerable radical scavenging activity exceeding that of BHT and ascorbic acid (Table 1). Besides, the beneficial effects of ETHEX on the lipid peroxidation and DNA fragmentation were also recorded.

Numerous researches have already pointed out that ETHEX contains an important antioxidant compounds ranging from flavonoids to phenols [24, 56, 57]. Some of them have a potential to act as scavengers, superoxidase, and hydroxyl and peroxy radicals released from oxidative phosphorylation. All together can avoid the free radical's formation and protects the mitochondria, DNA and plasma membrane of sperm [44, 58–60]. Prevent oxidative damage could be achieved by a variety of different mechanism, such as free radical scavenging, transition metal chelation and interactions with lipid membranes, proteins and nucleic acids [27, 28]. In addition to previously mentioned compounds, ETHEX contains sugars (glucose, fructose, and sucrose) and vitamins (C, B2, B3, and B9). The inclusion of such compounds in extenders has been shown to improve ram semen quality [61, 62]. More precisely, sugars and vitamins have beneficial antioxidative and protective effects against the cold shock and freeze–thaw damage of sperm [61, 63, 64]. Sugars act as plasma membrane protector and increase its integrity percentage in several species [65–67]. Furthermore, the sugars provide the main energy source, which spermatozoa require for the development of their metabolic processes [19]. Regarding the vitamins, an improvement of ram semen quality observed in the current study could be attributed to the presence of Vitamin C. In fact, previous studies stated the improvement of the sperm viability, motility and decrease the percentage of abnormality [68–70]. Coming back to minerals, the characterization of the ETHEX revealed that it the contained calcium, magnesium, sodium, and zinc (Table 2). Specially, zinc have been reported to protect spermatozoa against oxidative stress and subsequent improving male fertility [71]. Supplementations of zinc (Zn) enhanced the serum Zn level and improved overall hormone production, the sperm quality during handling stages such as fresh semen, equilibration and post thawing and fertility [72, 73]. Furthermore, Zn showed a positive outcome regarding antioxidant activity [74] as well as the stabilization of the final assembly of DNA nucleoproteins and sperm plasma membrane [65]. It plays an important role in controlling motility by controlling the use of energy through adenosine triphosphate systems and by regulating the energy stores of phospholipids [75]. Additionally, to zinc, calcium triggers

the acrosomal reaction in mammalian spermatozoa is also involved in sperm motility [76], and inhibits the enzyme phosphodiesterase, which prevents cAMP degradation and enhances sperm motility [77].

Conclusion

In conclusion, the findings of the present study imply that supplementation of SM with ETHEX at 1 and 2% level has a beneficial effect on liquid ram semen quality parameters during liquid storage up 72h, which may be due to the antioxidant effects of one or more active compounds presented in ETHEX. However, additional studies are required to reveal the active components existing in ETHEX and test the impacts of this extract inclusion in the freezing media and success of AI in sheep.

Abbreviations

AI: Artificial insemination; GSH: Reduced glutathione, GSH-Px: glutathione peroxidase; catalase: CAT; SOD: superoxide dismutase; RSA: Radical scavenging activity; DPPH: Free radical 1,1- diphenyl-2-picrylhydrazyl; BHT: Butylated hydroxytoluene; ICP-OES: Inductively coupled plasma optical emission spectrometry (ICP-OES); HPLC : high performance liquid chromatography; CASA: Computer assisted sperm analysis; HOST: The hypoosmotic swelling test; MDA: Malondialdehyde concentrations; LPO: Lipid peroxidation; TBARS: The thiobarbituric acid reactive substances.

ROS: Radical oxygen species; TUNEL: Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick-end labeling.

Declarations

Ethics approval and consent to participate

Animal studies were conducted in accordance with the protocols of Animal Use and Care of the University of Hassan 1st, Settat, Morocco.

Consent for publication

Not applicable.

Availability of data and materials

The data analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

We declare that each author contributed to the article. Pr. Larbi Allai: Extraction of *Opuntia ficus-indica* ethanolic, evaluation, analysis of semen and write the manuscript. Pr. Mehmet Ozturk and Pr. Pinar Terzioğlu: Analyze of antioxidant activity, the mineral, sugar and vitamin and the correction of the manuscript. Dr. Noureddine Louanjli: Analyse of DNA Fragmentation. Dr. Bouchra El Amiri, Dr. Xavier Druart and Pr. Boubker Nasser: Designed the experiment, supervised the experiments and revised the manuscript.

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Tables

Table 1. Radical scavenging activity (Inhibition %) of ETHEX, Ascorbic acid and BHT, by DPPH method.

Concentration (µg/ml)	ETHEX	Ascorbic acid	BHT
0.5	40.10	34.03	24.69
1	46.50	38.78	32.10
4	60.00	59.99	54.35
8	82.10	79.26	70.27
16	97.66	90.80	77.10
32	99.85	93.35	91.23

Table 2. Sugar, vitamins and mineral composition of ETHEX[a].

Sugars	concentration (mg/g)	Vitamins	Concentration (mg/100g)	Minerals	Content (g/100 g)
Sucrose	2.85±0.02	C	963.1±3.04	Nitrogen	2.49±0.04
Glucose	2.67±0.01	B2	1384 ±5.04	Phosphorus	0.35±0.004
Fructose	0.56±0.001	B9	12.18±0.11	Potassium	4.55±0.06
		B3	91.79±0.19	Calcium	1.93±0.03
		B5	-[b]	Magnesium	1.25±0.02
				Iron	0.020±0.001
				Copper	0.004±0.001
				Manganese	0.005±0.001
				Zinc	0.003±0.001
				Boron	0.003±0.001
				Sodium	0.724±0.001

[a] The results were the average of three parallel measurements ± SEM (P<0.05).

[b] not detected.

Table 3. The effect of ETHEX on lipid peroxidation after 8, 24, 48 and 72 hours of ram liquid semen stored at 5°C in SM.

	Groups	Storage time (hours)			
		8	24	48	72
SLPO	CONTROL	0.40 ± 0.02^{bc}	0.89 ± 0.04^{a}	1.75 ± 0.03^{a}	2.34 ± 0.03^{ab}
	ETHEX 1%	0.28 ± 0.02^{c}	0.38 ± 0.02^{b}	0.86 ± 0.04^{d}	1.35 ± 0.09^{d}
	ETHEX 2%	0.47 ± 0.05^{b}	0.80 ± 0.05^{a}	1.33 ± 0.09^{c}	1.63 ± 0.03^{c}
	ETHEX 4%	0.55 ± 0.07^{ab}	0.84 ± 0.05^{a}	1.54 ± 0.06^{bc}	2.13 ± 0.05^{b}
	ETHEX 8%	0.72 ± 0.03^{a}	0.88 ± 0.05^{a}	1.67 ± 0.04^{ab}	2.44 ± 0.07^{a}
ILPO	CONTROL	1.56 ± 0.12^{a}	1.66 ± 0.06^{a}	2.04 ± 0.03^{a}	3.40 ± 0.10^{a}
	ETHEX 1%	0.64 ± 0.03^{c}	0.75 ± 0.06^{c}	1.52 ± 0.08^{b}	1.78 ± 0.09^{c}
	ETHEX 2%	0.68 ± 0.05^{c}	1.33 ± 0.07^{b}	1.79 ± 0.06^{ab}	2.47 ± 0.08^{b}
	ETHEX 4%	1.08 ± 0.07^{b}	1.35 ± 0.06^{b}	2.03 ± 0.07^{a}	3.20 ± 0.06^{a}
	ETHEX 8%	1.05 ± 0.08^{b}	1.63 ± 0.04^{a}	2.06 ± 0.07^{a}	3.41 ± 0.08^{a}

SLPO: Spontaneous LPO; ILPO: Induced LPO. Values are expressed as mean ± SEM. Different superscripts $^{(a,b)}$ within the same column indicate a significant effect of the extender within each duration of storage.

Table 4 The effect of ETHEX on DNA fragmentation index after 8, 24, 48 and 72 hours of ram liquid semen stored at 5°C in SM.

Group	Storage time(h)			
	8	24	48	72
CONTROL	4.70 ± 0.48^{a}	9.75 ± 1.11^{a}	14.5 ± 0.29^{a}	15.75 ± 0.63^{a}
ETHEX 1%	1.75 ± 0.41^{b}	3.50 ± 0.29^{b}	6.25 ± 0.25^{b}	8.50 ± 0.50^{b}
ETHEX 2%	3.50 ± 0.50^{ab}	5.40 ± 0.63^{b}	8.80 ± 0.45^{b}	10.75 ± 0.80^{b}

The DNA fragmentation index (%) was analyzed for semen stored during 72 hours at 5°C in skim milk supplemented with selected ETHEX concentrations. Values are expressed as mean ± SEM. Values are expressed as mean ± SEM. Different superscripts $^{(a,b)}$ within the same column indicate a significant effect of the extender within each duration of storage.

Figures

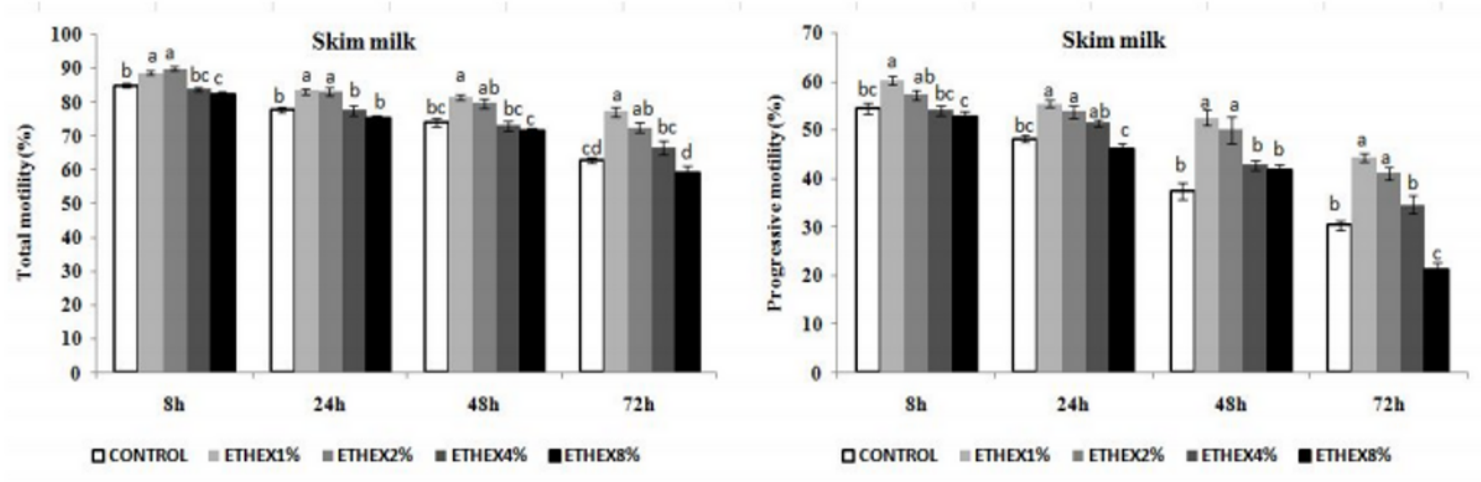

Figure 1

Effect of ETHEX on sperm motility during liquid storage in SM extender. Mean ± SEM values of sperm motility of ram semen stored for 8-72 h at 5°C in SM extender supplemented with ETHEX 1-8 % w/v). Different superscripts indicate a significant difference between ETHEX concentrations within each duration of storage (P<0.05).

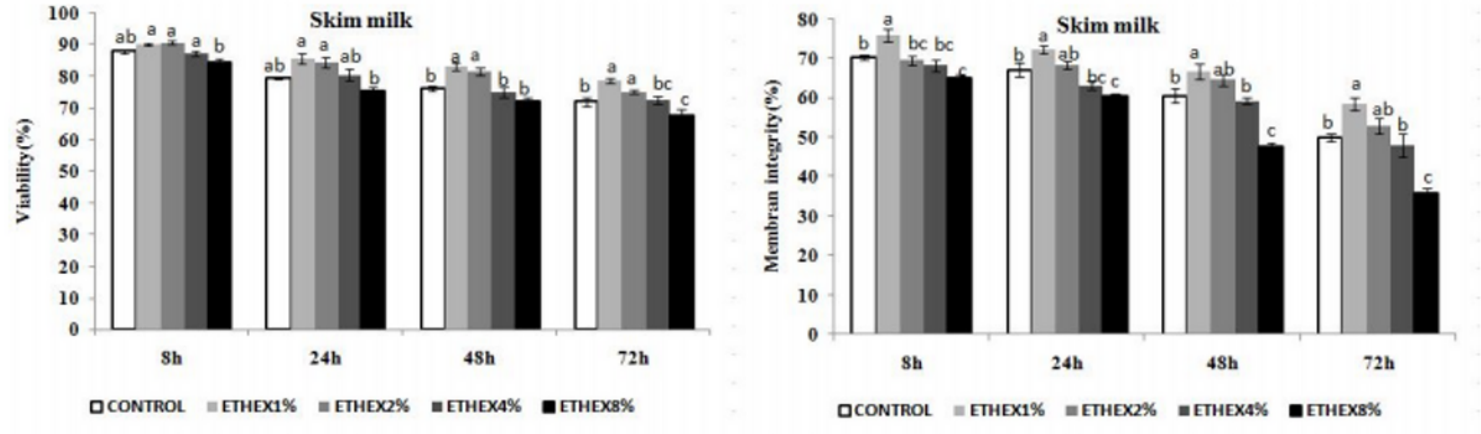

Figure 2

Effect of ETHEX on viability and membrane integrity during liquid storage in SM extender. Mean ± SEM values of viability and membrane integrity of ram semen stored for 8-72 h at 5°C in SM extender supplemented with ETHEX 1-8 % w/v). Different superscripts indicate a significant difference between ETHEX concentrations within each duration of storage (P<0.05).

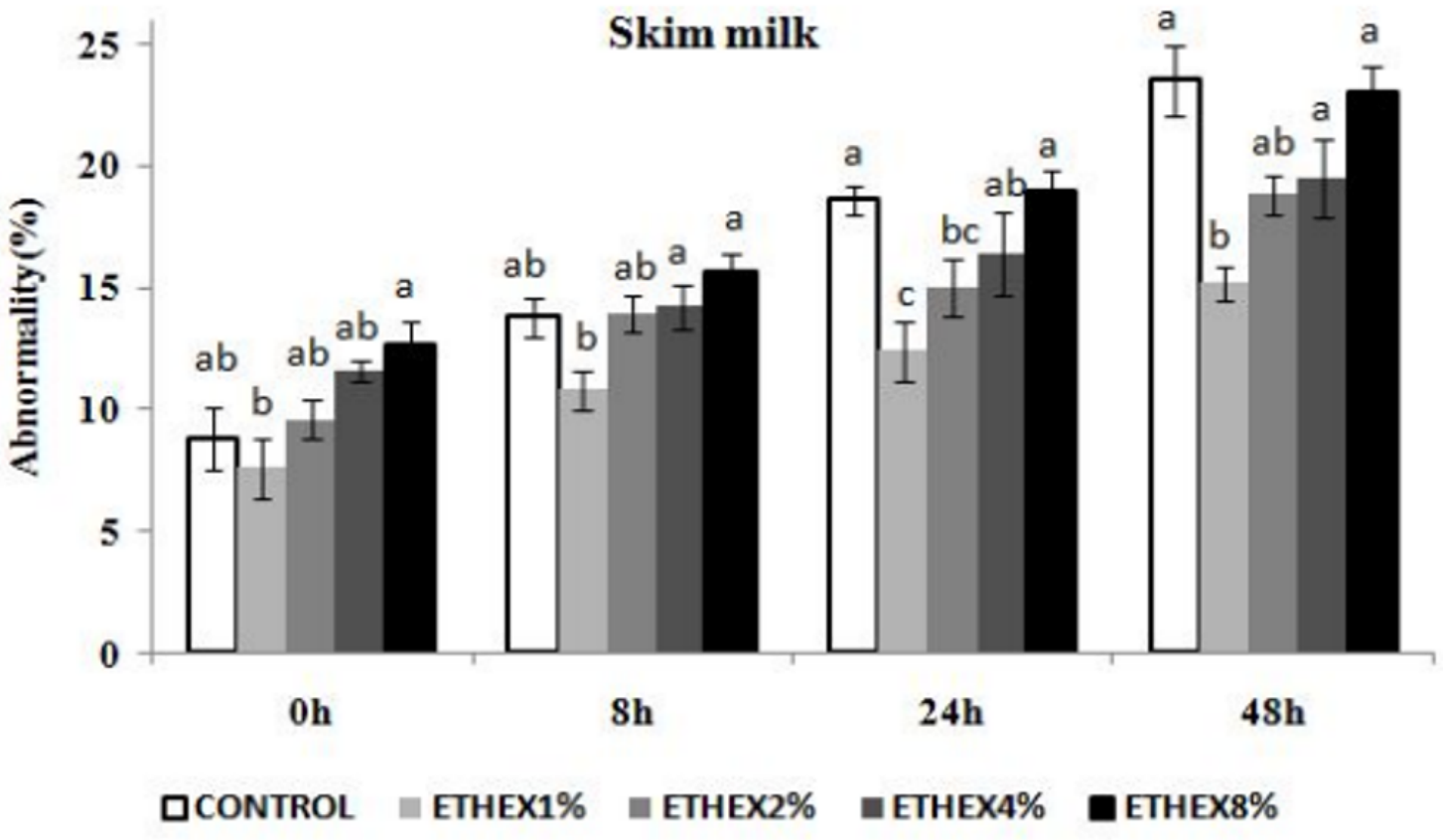

Figure 3

Effect of ETHEX on abnormality during liquid storage in SM extender. Mean ± SEM values of abnormality of ram semen stored for 8-72 h at 5°C in SM extender supplemented with ETHEX 1-8 % w/v). Different superscripts indicate a significant difference between ETHEX concentrations within each duration of storage ($P<0.05$).