Improvements of ram semen quality using cactus seed oil during liquid preservation in Tris egg yolk and skim milk based extenders

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A B S T R A C T
The purpose of this investigation was to evaluate the effect of cactus seed oil (CSO) on ram semen quality parameters during liquid storage. Semen samples collected from five adult fertile Boujâad rams were pooled and diluted to a final concentration of 0.8 × 10^6 spermatozoa/ml in Tris egg yolk (TEY) and skim milk (SM) extenders supplemented with CSO at 0, 1, 2, 5, and 10% v/v. The samples were then stored at 5 °C and several sperm parameters (motility, viability, morphology, peroxidation and DNA fragmentation) were assessed after 8, 24, 48 and 72 h. Results showed that the supplementation of TEY and SM extenders with 1% and 2% CSO, respectively, increased sperm motility and viability and decreased the level of peroxidation and DNA fragmentation during storage. Therefore, we concluded that 1% and 2% CSO are the optimal concentrations that can help to improve ram semen quality during storage at 5 ºC.

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1. Introduction

Successful liquid storage of ram spermatozoa is largely dependent on extender composition. Currently, skim milk (SM) and Tris egg yolk (TEY) are two commonly used extenders for ram semen liquid storage. Nonetheless, these diluents are not able to completely prevent sperm parameter changes occurring under in vitro conditions. A decline in motility during liquid storage has been recorded over time, especially when exceeding 12 h (Paulenz et al., 2002, 2003). Previous studies reported that this decrease could be related to the action of the reactive oxygen species (ROS) generated by the cellular components of semen (the superoxide anion radical (O2•−), the hydrogen peroxide (H2O2), nitric oxide, peroxynitrite anion) and the lipid hydroperoxides formed via lipid peroxidation of the spermatozoa membranes (Del Maestro, 1980; Alvarez and Storey, 1984, 2005; Sikka, 1996). To solve such problems, many natural antioxidants are added with a view to preventing or decreasing this process and preserving liquid ram semen quality (Martí et al., 2003; Ashrafi et al., 2011; Cámara et al., 2011). Among them, vegetable oils lead to improvements of sperm quality in chicken (Al-Daraji, 2012), humans (Aboa et al., 2009) and bovine (Towhid and Parks, 2012). In a recent study, the addition of argan oil to skim milk and Tris egg yolk extenders allowed to maintain ram sperm quality at 5 ºC and 15 ºC for up to 48 h of storage (Allai et al., 2015). To the best of our knowledge, this study is the first report investigating the roles of cactus seed oil (CSO) used to supplement TEY and SM extenders on ram liquid semen.

Opuntia ficus-indica belongs to the Opuntioidae subfamily among the Cactaceae. In Morocco, the area cropped with Opuntia covers 120.000 ha approximately (Arba, 2009), with Opuntia ficus-indica being the main species (El-Mostafa et al., 2014). Recently, cactus seed oil (CSO) or prickly pear seed oil) is harvested from Opuntia ficus-indica seeds. The most important fatty acids present in this oil are linoleic acid, palmitic, oleic, stearic acid (Sawaya and Khan, 1982; Ramadan and Morsel, 2003; Enoouri et al., 2005) and sterols (El Kharrassi et al., 2014). It also contains antioxidants such as vitamins, β-carotene and phenolic compounds (Stintzing et al., 2014).
2. Materials and methods

2.1. Chemicals

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

2.2. Cactus seed oil extraction

Cactus seed oil (CSO, Opuntia ficus-indica L. Mill.) was freely provided by Cactus seed Oil Associations, at Rhamna, Morocco. Seeds were separated from fresh fruits collected in the summer season (June-August), dried at room temperature and cold-pressed using an oil extraction machine. Extraction was performed without employing any solvent system. Extracted CSO was stored in the dark at 5°C in 40 ml glass bottles until use.

2.3. Animals and semen collection

Fifty ejaculates were collected from five adult Boujâd rams using an artificial vagina. Ejaculates with volume (0.9–2 ml), concentration (>2 × 10^6 sperm/ml), motility (>70%) and morphology (<10% total sperm abnormalities) were pooled to minimize individual variations and then the samples were evaluated. The experimental campaign lasted for 10 weeks from July to September 2014 corresponding to the breeding season of Boujâd rams.

2.4. Extender preparation

A skim milk (11 g of skim milk in 100 ml distilled water, heated for 10 min to 95°C) and a Tris egg yolk (2.666 g Tris, 0.44 g glucose, 1.398 g citric acid, egg yolk 12% v/v) at pH 6.8 extenders were prepared daily. Penicillin and streptomycin (0.5 mg/ml) were added to prevent bacterial growth. Ejaculates were diluted at 37°C to reach a final concentration of 0.8 × 10^6 sperm/ml with base extenders (SM or TEY) containing CSO (1, 2, 5 and 10%) or no additive (control). Extended ram semen was stored at 5°C and then assessed after 8, 24, 48 and 72 h of storage.

2.5. Experimental design

2.5.1. Experiment 1. Effects of storage period, extenders, CSO concentrations and interactions (antioxidant/extend)er

The aim of this experiment was to determine the effect of storage period (8, 24, 48 and 72 h), extender (SM vs. TEY), concentrations of CSO (0, 1, 2, 5 and 10% v/v) and interactions (extender x CSO concentrations) on total motility, progressive motility, viability, morphology and lipid peroxidation of ram semen during liquid storage.

2.5.2. Experiment 2. The effect of optimal concentrations of CSO on DNA fragmentation

The most beneficial concentrations of CSO yielding the best protective effects on sperm progressive motility in experiment 1 was selected to assess the DNA fragmentation at 0, 8, 24, 48 and 72 h of storage at 5°C.

2.6. Semen evaluation

2.6.1. Sperm motility

For each sample, the sperm concentration was adjusted with NaCl 0.9% to 20 × 10^6 sperm/ml. Sperm motility was assessed at 37°C using a 10× negative phase contrast objective on a UB203 microscope (UOP/Proiser, Paterna, Valencia, Spain). The percentages of total motility (TM) and progressive motility (PM) in each sample, were determined using a computer-assisted sperm analysis system (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain) as described by Yániz et al. (2008). A spermatozoon was defined as non-motile if average path velocity (VAP) was lower than 10 μm/s and it was considered progressively motile when VAP was >75 μm/s and straightness index (STR) was >80%. A minimum of 200 sperm cells in at least three to four different fields was assessed.

2.6.2. Sperm viability

Sperm sample viability (VIAB) was assessed using eosin-nigrosin staining (eosin Y 1.67 g, nigrosin 10 g and sodium citrate 2.9 g, dissolved in 100 ml of distilled water) according to Evans and Maxwell (1987). Sperm motility was performed by mixing 3 μl (20 × 10^6 sperm/ml) of the semen sample with 6 μl of the stain on a warm slide. Viability was assessed by counting 200 spermatozoa under a bright-field microscope at ×400 magnifications. Non-viable spermatozoa had red or dark-pink heads while viable sperm have white or faintly-pink heads (Fiser and Marcus, 1989).

The results were expressed in terms of mean percentage of viable spermatozoa.

2.6.3. Sperm morphology abnormalities

Sperm morphology (ABN) was evaluated using the Diff-Quik kit (Diagnostic Systems S.L. Barcelona (Spain)). An aliquot of 4 μl of diluted semen (20 × 10^6 sperm/ml) was smeared on a glass slide and dried at room temperature. The slide was then immersed in the fixative solution for 1 min and then in the first and second stain solutions for seven to ten times. Between the fixing step and each of the staining steps, the excess solutions were removed from slides by placing slides vertically on absorbent paper. At least 200 spermatozoa were assessed under a light microscope at 1000× magnification using UB203 microscope (UOP/Proiser, Paterna, Valencia, Spain).

2.6.4. Lipid peroxidation

Spontaneous lipid peroxidation was determined according to Maia (2006). A diluted semen sample was supplemented with 1 ml of the TBA reagent (15% trichloroacetic acid, 0.25N HCl, 0.375% thiobarbituric acid in distilled water) and 1% (v/v) 50 mM BHT (Butylated hydroxytoluene) solution in ethanol. These samples were then incubated at 100°C for 15 min in a boiling water bath, cooled in an ice bath and centrifuged at 1000 × g for 10 min. The supernatant was maintained on ice during spectrophotometrical measurements. To induce lipid peroxidation, a 0.24 mM Fe (II) sulfate heptahydrate (FeSO₄·7H₂O) solution was added to each sample and the semen samples were incubated in a water bath at 37°C for 15 min. Each sample was mixed with 1 ml TBA solution and 1% (v/v) 50 mM BHT solution in ethanol and processed as described above. The concentration of thiobarbituric acid reactive substances (TBARS) was determined by comparing the sample’s absorbance at 532 nm with a created standard curve for malondialdehyde (MDA) estimation. The results were expressed in nmol TBARS/10^6 sperm.

2.6.5. DNA fragmentation

For the TUNEL technique, we used the In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s protocol with slight modifications performed in ram by Nur et al. (2010). Semen...
Table 1

<table>
<thead>
<tr>
<th>Extenders</th>
<th>GROUP</th>
<th>TM (%)</th>
<th>PM (%)</th>
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<tr>
<td></td>
<td></td>
<td>8h</td>
<td>24h</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>CONTROL</td>
<td>84.8 ± 0.6</td>
<td>77.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>CSO 1</td>
<td>87.9 ± 0.6</td>
<td>83.0 ± 0.8</td>
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<tr>
<td></td>
<td>CSO 2</td>
<td>88.2 ± 0.6</td>
<td>82.3 ± 1.1</td>
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<tr>
<td></td>
<td>CSO 5</td>
<td>81.6 ± 0.6</td>
<td>78.4 ± 1.0</td>
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<td></td>
<td>CSO 10</td>
<td>85.8 ± 0.6</td>
<td>76.7 ± 1.2</td>
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<tr>
<td>T E Y CONTROL</td>
<td>85.5 ± 0.6</td>
<td>80.9 ± 0.6</td>
<td>76.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>CSO 1</td>
<td>90.5 ± 0.5</td>
<td>85.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>CSO 2</td>
<td>90.9 ± 0.6</td>
<td>85.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>CSO 5</td>
<td>84.6 ± 0.5</td>
<td>81.6 ± 1.1</td>
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<tr>
<td></td>
<td>CSO 10</td>
<td>85.8 ± 0.6</td>
<td>81.1 ± 1.1</td>
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<table>
<thead>
<tr>
<th>Extenders</th>
<th>VIAB (%)</th>
<th>ABN (%)</th>
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<tbody>
<tr>
<td></td>
<td>8h</td>
<td>24h</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>SM</td>
<td>CONTROL</td>
<td>89.0 ± 0.5</td>
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<tr>
<td></td>
<td>CSO 1</td>
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<td></td>
<td>CSO 2</td>
<td>91.5 ± 0.7</td>
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<td></td>
<td>CSO 5</td>
<td>90.5 ± 1.4</td>
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<tr>
<td></td>
<td>CSO 10</td>
<td>88.0 ± 1.1</td>
</tr>
<tr>
<td>T E Y CONTROL</td>
<td>88.8 ± 0.8</td>
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<tr>
<td></td>
<td>CSO 10</td>
<td>89.7 ± 1.2</td>
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</table>

TM: total motility; PM: progressive motility; SM: Skim milk; T E Y: Tris egg yolk; CSO: Cactus seed oil. Values are expressed as mean ± SEM. a, b, c, d, e Different letters show significant differences between extenders within each duration of storage (p < 0.05). A, B, C, D Different superscripts within rows indicate a significant effect of duration within each concentration of CSO (p < 0.05). * Asterisks show a significant effect of CSO concentration using the control as a reference within each duration of storage (p < 0.05).

3. Results

3.1. Effects of storage period, extenders, CSO concentrations and interactions (antioxidant/extender)

Skim milk (SM) and Tris egg yolk (TEY) based extenders were supplemented with different concentrations of CSO (0, 1, 2, 5 and 10% v/v) and sperm quality was assessed at 8, 24, 48, and 72 h of storage at 5 °C (Tables 1 and 2). Overall, all studied parameters decreased with the advancement of storage time. However, the sperm motility (TM and PM) and viability did not differ among 24 and 48 h of preservation, particularly when SM or TEY are supplemented with CSO at 1% and 2%. The most significant declines were recorded starting from 48 h of storage. Compared with control groups, greater sperm TM and PM were recorded during all storage periods when SM and TEY extenders were supplemented with CSO at 1% and 2% (Table 1). However, the interaction of extenders and CSO concentrations showed that TEY supplemented with 1% and 2% CSO and SM supplemented with 1% of CSO maintained better sperm TM compared to all treatments (Table 1). Regarding PM, higher percentage were observed with SM supplemented with 1% and 2% CSO compared to all used concentrations (Table 1).

Sperm viability was improved by 1% and 2% of CSO compared to the controls regardless of the base extenders from 24 h of preservation onwards (Table 2). The interaction of extender and CSO on sperm viability showed that higher percentages were observed in TEY extender with 1% and 2% CSO compared to all tested treatments.
Progressively over storage time (specially from 48 h to 72 h) sperm motility and viability, as well as abnormality decreased significantly when adding 1% and 2% of CSO in SM and 1% in TEY at 5 ºC, respectively (Table 2). Combining extender and CSO revealed that CSO at 1% in SM and TEY decreased the abnormality rate compared to all other concentrations used in this study. All beneficial effects of CSO were lost when its concentration reached 10%.

3.2. Effects of cactus seed oil (CSO) concentrations on sperm spontaneous and catalyzed lipid peroxidation

Fig. 1 shows the spontaneous and catalyzed lipid peroxidation levels in ram semen extended with TEY and SM using different concentrations of CSO at 5 ºC at different storage periods. These levels were reduced when the extenders (SM or TEY) were supplemented with 1% and 2% CSO during storage at 5 ºC compared to the controls. In contrast, no differences were observed when CSO concentration reached 5% and 10% compared to the control groups.

3.3. Effects of the optimal cactus seed oil (CSO) concentration on DNA fragmentation

Fig. 2 shows the effects of CSO concentration on DNA fragmentation of stored liquid ram semen. The DNA fragmentation dropped significantly by 2% and 1% of CSO in SM and in TEY respectively, after 24 h compared to the controls. This effect became more evident after 48 h of storage.

4. Discussion

The present study investigated the effects of different doses of CSO added to SM and TEY extenders on sperm motility, viability, morphology, lipid peroxidation and DNA fragmentation of ram semen stored at 5 ºC for 72 h. Our findings highlighted a significant deterioration of semen quality in relation to the time of storage, regardless of the treatments received. These results are in agreement with those previously reported by Sarlós et al. (2002) and Kasimanickam et al. (2007) indicating the functional deterioration of semen quality during long periods of liquid storage. The main factors of such deterioration are oxidative stress and endogenous free radicals production (Hammerstedt, 1993; Baumber et al., 2000; Anel et al., 2006). Hence, the addition of antioxidants to the extenders can be an option to prevent or decrease the peroxidation process. In sheep, a wide range of antioxidants has been used to achieve this goal (Sarlós et al., 2002; Kheradmand et al., 2006; Maia et al., 2010; Sicherle et al., 2011; Silva et al., 2013). In the present study, focus was on CSO extracted from Opuntia ficus-indica as naturally occurring extracts of plants with many components acting as antioxidants. In fact, Opuntia ficus-indica seed oil is characterized by a higher proportion of linoleic acid compared to certain conventional edible vegetable oils such as olive oil (3.5–21%), soy oil (49.2%), corn oil (47.7%) and sesame oil (44.5%) (Astiasarán and Candela, 2000; Mailler, 2006). However, it is worthwhile noting that the seeds derived from different varieties (green, yellow, orange and red) of Opuntia ficus-indica grown in Algeria showed differences regarding phenolic compounds, fatty acids and antioxidant activities (Chougui et al., 2013). The recorded differences can be attributed to the genetic makeup of the cultivar, the geographical origin of the fruit, the degree of maturity and storage conditions but also to the extraction protocols and analytic assays. The implication is that at any time the CSO is used to improve semen quality all cited parameters should be specified and referenced.

Before launching discussion on the current paper, we noticed lack of information related to the effects of SM and TEY supple-

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mented with CSO on ram semen or even to semen of other species. Moreover, the exact mechanism by which CSO enhanced the ram sperm quality remains unclear and is of great interest per se. For the first time, we showed that when CSO was added to SM and TEY extenders an improvement in sperm quality was achieved. More precisely, the overall results showed that the optimal concentrations leading to maintain the spermatozoa of Boujảd sheep in acceptable quality were 1% and 2% CSO. However, at higher CSO concentration levels (10%), these beneficial effects are lost. Nevertheless, the use of an appropriate antioxidant level is a prerequisite (Roca et al., 2004). Possibly, a part of the beneficial effects is due to the antioxidant, fatty acids and tocopherols present in the CSO. Supplementing extenders with fatty acids (oleic or linoleic acids) and tocopherols improved progressive motility, viability and reduced catalyzed lipid peroxidation of boar (Cerolini et al., 2000; Breining et al., 2005; Hossain et al., 2007; Jeong et al., 2009), bovine (Towhidipark, 2012; Kiernan et al., 2013; Kaka et al., 2015) and ram semen (Kheradmand et al., 2006; Maia et al., 2010; Sicler et al., 2011; Silva et al., 2013).

In addition to previously mentioned compounds, CSO contains a high level of β-carotene (Ramadan and Mörse1, 2003). This molecule has the capacity to protect the plasma membrane against lipid peroxidation and to form an important component of the antioxidant defense (Di Mascio et al., 1989; Gupta and Kumar, 2002). In fact, it inhibits the propagation of radicals initiated by lipid peroxidation (Tsuhichi et al., 1993; Krinsky, 1998; Schafer et al., 2002). Among the carotenoids present in CSO, the lycopene have been reported to improve the viability of turkey spermatozoa during liquid storage (Rosato et al., 2012) and to protect DNA integrity against cryodamage of bull spermatozoa (Tuncer et al., 2014).

More than 20 phenolic compounds are found in CSO, which confer a high antioxidant activity to this oil (Chougui et al., 2013). Among them, the ferulic acid is known to have a high antioxidant and antiradical potential with the ability to inhibit lipid peroxidation (Sánchez-Moreno et al., 1999; Srinivasan et al., 2007; Yashin et al., 2013). In human, the ferulic acid has been shown to improve sperm motility, viability and to reduce lipid peroxidation (Zhang and Zhang, 1997).

In conclusion, the results of this study imply that supplementation of SM and TEY with an appropriate level of cactus seed oil has a beneficial effect on ram sperm parameters preserved at 5 °C up to 72 h. Therefore, this oil can be considered a powerful natural antioxidant when incorporated to extenders for maintaining quality of chilled ram semen. Further work is needed to get more precise information on the CSO component with real protective capacity from different cactus varieties and origins and to identify CSO concentrations conducive to obtaining high fertility rates.

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