Effect of Semen Extender on Cryopreservation and Fertilization Rates of Dromedary Camel Epididymal Spermatozoa

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ABSTRACT

The last decade showed great interest in the application of assisted reproductive technologies in camel reproduction particularly in racing and milking animals which represent part of the Arab heritage. The present work was designed to assess the cryopreservation and fertilization rates of dromedary camel epididymal spermatozoa using different extenders. In experiment 1: 16 testicles from mature dromedary camel were collected at Cairo slaughterhouse, transported to the lab at 4 ºC. Spermatozoa were extracted from epididymides using the retrograde flushing technique, washing sperm cells in a retrograde direction from the ductus deferens through the cauda epididymidis with a syringe loaded with warmed (37ºC) extender. Retrieved epididymal spermatozoa were diluted in Ovixcell®, in Tris-fructose-egg yolk and sodium citrate-egg yolk semen extenders. Sperm motility was evaluated after 15 minute of incubation in a water bath at 37ºC under phase-contrast microscopy using a pre-warmed (37ºC) Makler Chamber. In experiment 2: epididymal spermatozoa extended in Ovixcell® and tris-fructose-egg yolk was processed for freezing and stored in liquid nitrogen at –196 ºC. Sperm Post-thawing motility was evaluated after 15 minute of incubation in a water bath at 37ºC under phase-contrast microscopy using a pre-warmed (37ºC) Makler Chamber. Epididymal spermatozoa were used for in vitro fertilization of in vitro matured camel oocytes. Cleavage rate and development to the blastocyst stage were determined. Initial epididymal sperm motility was 52.8 ± 0.7%, 41.2 ± 33.7% and 7.5± 2.5% for Ovixcell®, Tris-fructose-egg yolk semen extender and sodium citrate-egg yolk, respectively. Post thawing motility was similar in Ovixcell® and tris-fructose-egg yolk extenders (47.5± 5.0% and 45.0 ± 7.5%, respectively. Also, cleavage rate was similar for in vitro mature dromedary oocytes fertilized by frozen thawed epididymal spermatozoa extended in Ovixcell® or Tris-fructose-egg yolk semen extenders (79.3 and 83.8%, respectively. Morulae and blastocyst rates were 58.1% and 52.2 for spermatozoa cryopreserved inOvixcell®, Tris-fructose-egg yolk semen extender, respectively. In conclusion, Ovixcell®, Tris-fructose-egg yolk semen extenders are suitable to use in cryopreservation and in vitro fertilization of camel epididymal spermatozoa.

Key word: Fertilization Rates, Camel Epididymal, Spermatozoa.

INTRODUCTION

Cryopreservation of epididymal spermatozoa is a useful tool to preserve genetic material of valuable animals after emergency castration or unexpected death and it can contributes
to the expansion of reproductive techniques, such as artificial insemination (AI) and in vitro embryo production (IVP). However, the progress in semen preservation and related techniques in the camelidae family has been slow in comparison to other livestock species (Bravo et al., 2000a). This could partly be due to the lack of proper semen extender to maintain the viability of spermatozoa for short and long term.

The epididymal spermatozoa from several animal species such as cattle (Foote and Igboeli, 1968), horses (Morris et al., 2002), goats (Blash et al., 2000), rabbits (Paufller and Foote, 1968) and dogs (Marks et al., 1994) and dromedary camel (Wani, 2008) have been preserved or used successfully for artificial insemination or for in vitro fertilization. In addition, it is reported that extender type used for dilution of semen is an important and effective factor for successful storage and survival rates of frozen and non-frozen spermatozoa (Paulenz et al., 2002).

Despite attempts using lactose-, sucrose-, citrate- and fructose-based buffers in addition to the commercially available extenders manufactured for other livestock species such as Green buffer® (IMV, L’Aigle, France), Biladyl®, Androhep® and Triladyl® (Minitube, Tiefenbach, Germany), the post-thaw motility of frozen-thawed camelid spermatozoa averages 20% (von Baer and Hellemann, 1999; Deen et al., 2003; Vaughan et al., 2003; Santiani et al., 2005) and is rarely >40% (Bravo et al., 2000b; Niasari-Naslaji et al., 2007; El-Bahrawy et al., 2010). Tris-fructose extender was suggested to be better than lactose (Vyas et al., 1998) and Bicephos (Deen et al., 2004) for the preservation of camel semen at refrigerated temperature. Also, Niasari-Naslaji et al., (2006) reported that tris-fructose and Green buffer were better than lactose and sucrose buffer for storage of Bacterian camel semen at 5 °C. Tris buffer seems to have value in prolonging the life span of sperm at ambient temperature, 5 and at -196 °C (Bearden et al., 2000). Recently, Khalifa and Lymberopoulos (2013) recorded that soybean lecithin based extenders (Ovixcell®) is superior to milk-egg yolk extender in preserving chromatin stability and motility of ram spermatozoa.

The objectives of this study were to evaluate: 1) the effects of different semen extenders on motility and viability of dromedary camel epididymal spermatozoa stored after extraction 0 h, 24 h and 48 h at 4°C; 2) the effects of cryopreservation of dromedary epididymal spermatozoa at –196 °C on their ability to fertilize in vitro matured camel oocytes.

MATERIALS AND METHODS

Experiment 1: Effect of type of semen extender on motility, viability and cryopreservation of dromedary camel epididymal spermatozoa

Collection of dromedary camel epididymal spermatozoa:

Testicles from 16 mature dromedaries were collected post mortem at a local abattoir (El-Basatein) in Cairo, Egypt during the breeding season (November). Both testicles from each animal were removed on average 1.30 h after slaughter and transported to the laboratory in a Styrofoam box at refrigeration temperature with a portable refrigerator. Approximately 180 min after collection, testes and epididymides were removed from the scrotal sac and afterwards caudae epididymides were isolated from testes and from surrounding connective tissue. Epididymal sperm were collected using the retrograde flushing technique as performed by Turri et al., (2012). Briefly the lumen of the ductus deferens was cannulated, before flushing, with a blunted 22-gauge needle to avoid losses of material. Cauda epididymidis and ductus deferens were isolated from the rest of the
epididymidis by making a cut with a scalpel near the junction of the corpus and the proximal cauda. Sperm cells were then flushed in a retrograde direction from the ductus deferens through the cauda epididymidis with a syringe loaded with approximately 2 ml of warmed (37°C) extender (Figure 1).

**Figure 1: Retrograde retrieval method for recovery of spermatozoa from the tail camel cauda epididymidis**

For each testicles, it was randomly decided which epididymis to process with the following semen extender; (1) The Tris-fructose extender (3.63 g Tris, 0.50 g fructose, 1.99 g citric acid, 20 ml egg yolk, 7% glycerol and double distilled water to 100 ml, Salamon, and Maxwell 2000); 2) Ovixcell® extender without animal protein (IMV Technologies, L'Aigle, France); and 3) Sodium citrate diluent (2.37 g sodium citrate, 0.50 g glucose, 20 ml egg

**Assessment of progressive motility and viability:**
Immediately after extraction the epididymal sperm evaluation was performed. Progressive sperm motility was evaluated after 15 minute of incubation in a water bath at 37°C under phase-contrast microscopy using a pre-warmed (37°C) Makler Chamber. Also, sperm viability was assessed by Eosin Y-Nigrosin staining according to Emilson et al. (1978).

**Freezing and thawing of epididymal spermatozoa:**
After collection, epididymal semen samples were diluted to achieve a final concentration of 60 x10⁶ sperm/ml. Samples were then refrigerated to 5°C on average within 2 h. After reaching the 5°C, samples were loaded into 0.5 ml straws. Then straws were frozen on nitrogen vapour by placing on a rack at 4 cm above the nitrogen level for 15 min. Subsequently straws were plunged into liquid nitrogen and stored in liquid nitrogen tanks. After cryo-storage, semen straws were thawed at 37°C for 2 min to determine the post-thaw epididymal semen quality.

**Experiment 2: Assessment of in vitro fertilization rate of cryopreserved dromedary camel epididymal spermatozoa**

**Ovaries and Oocyte collection:**
Dromedary camel ovaries were collected at a local slaughterhouse (El-Basatein) in Cairo transported to the laboratory within 2-3 hr in physiological saline solution (0.9% NaCl) at 28-35°C. In the laboratory, ovaries were washed once with 70% ethanol and at least 3 times in saline solution supplemented with 100 IU/mL penicillin and 100 µg/mL
streptomycin. Camel cumulus oocytes complexes (COCs) were collected by aspiration of 2-8 mm diameters follicles using 20-gauge needle attached to 10 ml sterile syringe. After collection, COCs were classified according to their morphology into 4 grades according to the number of cumulus cell layers and homogenous of the cytoplasm. COCs with at least 1 layer of cumulus cells and homogenous cytoplasm were used in this experiment. COCs were washed at least 3 times in TCM-199 HEPES buffered supplemented with 10% fetal calf serum (FCS) + 50 µg/mL gentamycin and once in maturation medium. COCs were matured in TCM-199 supplemented with 25 mM sodium bicarbonate + 10% foetal calf serum (FCS) + 10 µg/mL follicle stimulating hormone (FSH) + 20 ng/ml epidermal growth factor (EGF) + 50 µg/mL gentamycin. COCs were cultured for 40 hr at 38.5°C under atmosphere of 5% CO₂ in air and high humidity.

**In vitro fertilization of matured oocytes:**
Cryopreserved dromedary camel epididymal spermatozoa in Tris-fructose and Ovixcell® were used in the present study. In both groups, after thawing at 37 for 40 sec. motile spermatozoa were separated by laying out the thawed semen on a Percoll discontinuous gradient (2 ml of 45% over 2 ml of 90%) then centrifugation for 30 min at 1500 r.p.m. Sperm pellet was re-suspended in Sperm-Tyrodd’s Albumin Lactate Pyruvate medium (Sp-TALP) supplemented with 6 mg/ml bovine serum albumin (BSA) + 50 µg/ml gentamycin. Sperm suspension was centrifuged for 10 min at 2000 r.p.m. The supernatant was removed and the sperm pellet was suspended in 1 ml Fert-TALP medium supplemented with 6 mg/ml BSA F-V (fatty acid free, FAF). Spermatozoa were counted using haemocytometer and diluted with Fert-TALP medium supplemented with 6 mg/ml BSA (F-V, FAF) + 50 µg/ml gentamycin + 10 µg/ml heparin to give a final concentration of 2 x 10⁶ spermatozoa/ml.

Matured oocytes were washed 3 times in Fert-TALP medium then divided into two groups. In the first group, oocytes were transferred (20-25 oocytes) into 4-well culture dish containing 300 µl Fert-TALP medium covered with mineral oil. Sperm suspension from Tri-fructose (Group 1) was used for in vitro fertilization. In the second group, oocytes were fertilized using spermatozoa cryopreserved in Ovixcell® semen extender. In both groups oocytes and spermatozoa were co-incubated for 18 to 20 h at 38.5°C under 5% CO₂ in air with high humidity.

In both groups, after in vitro fertilization the presumptive zygotes were washed 3 times then in SOFaa medium supplemented with 5% FCS + 50 µg/ml gentamycin + 10 ng/ml insulin, incubation was performed for 9 days at 38.5°C under 5% CO₂ in air and high humidity. SOFaa medium was changed every 2 days. Cleavage rate and embryo development to blastocyst stage were checked on Days 2, 4 and 6.

**Statistical Analysis:**
All results were expressed as mean ± standard deviation (mean ± SD). The conformity of variables with the normal distribution was examined using Shapiro-Wilk W-test. Duncan test was employed to assess statistical differences between extenders (p<0.05).

**RESULTS**
The effects of Ovixcell®, tris-fructose and citrate-egg yolk semen extenders on motility and viability of dromedary camel epididymal spermatozoa are presented in Table 1. Results indicated that motility evaluated immediately after epididymal extraction (0 h) was significantly (P<0.05) higher in Ovixcell® than in tris-fructose and citrate-egg yolk extenders. Initial motility was higher (P<0.05) in Tris-fructose than citrate-egg yolk.
After 24 and 48 h of storage at 4 °C, spermatozoa diluted in Ovixcell® showed significantly (P<0.05) higher motility than tris-fructose. Sperm motility completely stopped in citrate-egg yolk. Sperm viability at 0, 24 and 48 h was similar in Ovixcell® and Tris-fructose semen extenders. However, initial sperm viability was lower (P<0.05) in citrate-egg yolk compared with Ovixcell® or Tris-Fructose semen extender. Sperm motility and viability were zero after 24 h cooling at 4 °C and spermatozoa extended in citrate-egg yolk. Post-thawing motility and viability were similar between Ovixcell® and Tris-fructose semen extender.

Table 1: Effect of type of semen extender on progressive motility and viability of camel epididymal spermatozoa stored at different temperatures

<table>
<thead>
<tr>
<th>Time Extender</th>
<th>Motility</th>
<th>Viability</th>
<th>Motility</th>
<th>Viability</th>
<th>Motility</th>
<th>Viability</th>
<th>Motility</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h at 37 °C</td>
<td>52.8±0.7a</td>
<td>83.0±3.4a</td>
<td>48.1±2.9a</td>
<td>78.2±1.6a</td>
<td>44.3±3.1a</td>
<td>68.6±3.7a</td>
<td>47.5±5.0a</td>
<td>73.1±2.4a</td>
</tr>
<tr>
<td>24 h at 4 °C</td>
<td>41.2±3.7b</td>
<td>79.0±2.3b</td>
<td>37.4±1.2b</td>
<td>75.3±2.9b</td>
<td>32.7±1.4b</td>
<td>61.5±2.4b</td>
<td>38.0±3.1a</td>
<td>71.7±4.6a</td>
</tr>
<tr>
<td>48 h at 4 °C</td>
<td>7.5±2.5c</td>
<td>12.1±4.6c</td>
<td>0.0c</td>
<td>0.0c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Post-Thawing</td>
<td>47.5±5.0a</td>
<td>73.1±2.4a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a,b within the same column differ significantly at P<0.05
b,c within the same column differ significantly at P<0.05

The effects of type of semen extender on cleavage rate and development of in vitro fertilized camel oocytes are presented in Table 2, Fig. 2. Cleavage rate and embryo development were similar for in vitro matured dromedary oocytes in vitro fertilized using frozen-thawed spermatozoa cryopreserved in Ovixcell® or Tris-fructose semen extender.

Table 2: Effect of cryopreservation of camel epididymal spermatozoa in different types of semen extender on cleavage rate and development of in vitro fertilized camel oocytes

<table>
<thead>
<tr>
<th>Item</th>
<th>Fertilized oocyte</th>
<th>Cleavage rate (%)</th>
<th>Embryo development (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extender</td>
<td></td>
<td>2-4-cell</td>
<td>8-16-cell</td>
</tr>
<tr>
<td>Ovixcell®</td>
<td>112</td>
<td>89 (79.4)</td>
<td>21 (23.6)</td>
</tr>
<tr>
<td>Tris-fructose</td>
<td>128</td>
<td>107 (83.6)</td>
<td>27 (25.2)</td>
</tr>
</tbody>
</table>

Figure 2: In vitro produced dromedary camel embryos after fertilization using cryopreserved camel epididymal spermatozoa
DISCUSSION

In the present work, progressive motility at 0 h and for that stored for 24 h and 48 h at 4 °C was higher (P<0.05) for camel epididymal spermatozoa stored in Ovixcell® than Tris-Fructos semen extender. While, storage of camel epididymal spermatozoa in citrate-egg yolk semen extender significantly (P<0.05) decrease its progressive motility compared with Ovixcell® or tris-fructose semen extenders. Moreover, viability was similar for dromedary epididymal spermatozoa stored in Ovixcell® and Tris-fructose semen extenders at 0 h, 24 h and 48 h at 4 °C. Meanwhile, dromedary epididymal spermatozoa completely lost their motility and viability after storage at 4 °C for 24 h in citrate-egg yolk semen extender. Similarly, Khalifa and Lymberopoulos (2013), found that Ovixcell® is superior to milk-egg yolk extender in preserving ram sperm chromatin stability and motility. While, Wani (2008) concluded that dromedary epididymal spermatozoa survive in storage for at least 8 days in tris–lactose- and tris–tes egg yolk diluents at 4 °C. Progressive forward motility of frozen-thawed sperm was greater using SHOTOR (Tris-fructose) diluent compared to IMV buffers (Niasari-Naslaji et al., 2007). This difference may be attributed to difference among individual variation between bulls, type of extenders used and/or storage conditions or method of sperm collection.

Furthermore, in the present study cleavage rate and embryo development to the blastocyst stage were similar for in vitro fertilization of dromedary oocytes using frozen-thawed camel epididymal spermatozoa stored in Ovixcell® or Tris-Fructose semen extenders. In contrast, there was no significant difference (P>0.05) in the proportion of oocytes fertilized with spermatozoa stored in either of the two extenders for up to 8 days. The proportion of oocytes that cleaved (43-60%) and those that developed to blastocysts (14-21%) did not show any difference (P>0.05) either, when spermatozoa from different days of storage were used (Wani, 2008).

The proportion of normally fertilized oocytes in this study (79 to 83%) is higher to those reported in the same species (68%) with fresh ejaculated semen (Khatir and Anouassi, 2006), and that reported in llamas (29.2%) using epididymal spermatozoa (Del Campo et al., 1994). The fertilization rates of epididymal spermatozoa may vary due to method of semen collection, age of the animals or type of the source of semen, semen extender, methods of semen preparation and also the developmental competence of the oocytes or media used to culture zygotes.

Conclusion, Ovixcell® semen extender improve progressive motility of dromedary camel epididymal spermatozoa stored at 4 °C for 48 h, and sodium-citrate-egg yolk semen extender is not suitable for cold storage of camel epididymal spermatozoa. Cleavage and developmental rates are similar for in vitro matured camel oocyte fertilized using epididymal spermatozoa cryopreserved in Ovixcell® and Tris-fructose-egg yolk semen extenders.

REFERENCES


