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In vitro Fertilization and Embryo Production in Dromedary Camel Using Epididymal Spermatozoa

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Abstract: The general objective of this study was to produce dromedary embryos from cumulus-oocyte complexes (COCs) that were matured, fertilized and cultured *in vitro*. The aspirated COCs with a compact cumulus cells and evenly granulated ooplasm were selected and cultured at 38.5°C, in an atmosphere of 5% CO_2 and maximum humidity (95%) for 36h. After 36 h of culture, matured COCs were inseminated with epididymal spermatozoa stored at 4°C for different days. Inseminated oocytes were cultured in different media and in different monolayers. Although the penetration, fertilization and the cleavage rates were similar for oocytes inseminated with epididymal spermatozoa stored for 0, 4 and 6 days, more blastocysts were obtained from oocytes that inseminated with epididymal spermatozoa at day 0 as compared with those inseminated with 6-days stored epididymal spermatozoa (16.33 vs 1.25%, respectively). Culture of camel oocytes in potassium simplex optimized medium (KSOMaa) augmented blastocyst development as compared with synthetic oviduct fluid medium (SOFaa) media (11.29 vs 1.19%, respectively). Furthermore, more blastocyst stage was obtained from oocytes co-cultured with oviductal versus granulosa and uterine cells (15.59 vs 7.27 and 2.63%, respectively, $P < 0.05$). In conclusion, dromedary epididymal spermatozoa can survive storage at 4°C for 4 days in tris-lactose egg yolk extender and maintain their fertilizing potentials. KSOMaa culture media with oviductal cell monolayer appeared to be the most suitable conditions for *in vitro* dromedary camel embryo production.

Key words: Dromedary · Camel · Oocytes · Epididymal spermatozoa · IVF · Co-culture

INTRODUCTION

There are several reports regarding reproductive physiology in camels [1], as well as assisted-reproduction techniques, including AI and embryo transfer [2]. However, the only *in vitro* fertilization (IVF) reported in camelids was production of llama (Llama glama) embryos, using techniques similar to those used to produce bovine embryos [3]. Successful IVF in the Arabian camel has the potential to overcome several problems, including infertility due to oviductal abnormalities and the low yield of embryos collected after superovulation [2]. In addition, IVF technology in the dromedary will improve the understanding of the fundamental mechanisms of fertilization and early embryo development and promote the development of other technologies, including cryopreservation of oocytes and embryos, embryo sexing and somatic cloning and transgenesis.

There are several factors affecting the *in vitro* camel embryo production, such as source of oocytes, source and preparation of semen, culture media and culture conditions. There are few reports on the *in vitro* oocyte maturation [4] in camelids; however information available on *in vitro* fertilization and development of IVP embryos are very limited [5]. There are few reports on the use of stored ejaculated semen for IVF in dromedaries mainly because of the difficulties in semen collection, the gelatinous nature of ejaculated semen and the lack of suitable extenders for its storage. Keeping these in view, problems use of epididymal spermatozoa could be an alternative. Moreover, several techniques have been used for *in vitro* culture (IVC). These include the use of oviduct epithelial cell co-cultures [6, 7] oviduct cell culture supernatants [8], granulosa cells [7, 9], synthetic oviduct fluid (SOF) medium without cell supplementation [10], Charles Rosenkrans CR2 medium [11] and potassium simplex optimized (KSOM) medium [12].

The objectives of the present study were to assess the effect of storing camel epididymal spermatozoa in tris-lactose egg yolk extender for different days, on their fertilizing ability and subsequent embryo development and to determine the efficiency of *in vitro* development of the fertilized oocytes in different culture media and different cell monolayers.

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MATERIALS AND METHODS

All chemicals and media obtained were from Sigma (Steinheim, Germany) unless otherwise indicated. Fetal calf serum (FCS) was from Gibco (BRL, Germany). bFSH and bLH were from Sioux (Sioux, Biochemical Inc., Sioux Center, IA, USA).

Collection and Maturation of Cumulus Oocyte Complexes: Ovaries were collected from camels of unknown reproductive history from a local slaughterhouse (El-Bassatein) immediately post-slaughter and brought to the laboratory in a thermos flask containing warm normal saline solution (NSS) fortified with 100µg/ml streptomycin at 37°C. On arrival in the laboratory, ovaries were washed 2-3 times with NSS and COCs were harvested by aspirating follicles (2-6mm diameter) using an 18G needle attached to a 10ml sterile disposable syringe containing PBS. The oocytes were evaluated under a sterio-microscope. Good quality oocyte with two layers of compact cumulus cells and homogenous granulated ooplasm were selected for *in vitro* maturation. The selected oocytes were washed once in PBS with 10% FCS and twice in maturation medium. Pooled COCs were randomly distributed in the maturation drops (10-15COCs/drop) containing 50µl of the maturation medium tittered in polystyrene culture dishes (35 x 10 mm) and cultured at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity (95%) for 36h [7]. Maturation medium was consisted of TCM-199 with Earle's salts supplemented with 5µg/ml bFSH, 10µg/ml bLH, 1µg/ml estradiol and 10% FCS.

Recovery and Preparation of Epididymal Sperm for IVF: Testes were collected from mature males from a local slaughterhouse, immediately after slaughter and transported to laboratory within 2 h post slaughter in normal saline solution on ice. At the laboratory, testes were washed with sterile NSS and the cauda epididymides were isolated. The areas below the deferent ducts were trimmed free of tissue coverings and again washed with sterile NSS. A prick was made on the convoluted tubules with a sterile needle and the gushing fluid, rich in spermatozoa, was aspirated in syringes containing 2-3ml Tris-lactose egg yolk extender. The epididymal sperm suspension was incubated for 10 minutes at 39°C in high humidity atmosphere (95%) with 5 % Co_2 then was transferred to 15 ml centrifuge tube. For IVF, the sperm suspension was washed twice with Sperm TALP [13] by centrifugation at 250xg for 10min each. The pellet was overlaid with fertilization medium (F-TALP supplemented with 4mg/ml BSA, 50IU/ml penicillin and 10µg/ml heparin) and allowed to swim up for 30 minutes in CO_2 incubator. The matured oocytes were washed three times in fertilization medium and were randomly distributed to in polystyrene culture dishes (35 x 10 mm; 5-10 COCs/drop) containing 50µl of the fertilization medium. The oocytes were incubated in CO_2 incubator for about 1 hour, until the motile spermatozoa were added to the oocytes at the concentration of approximately 2×10^6 sperm cells/ml. Sperm and oocytes were co-incubated at 39°C in a moist atmosphere of 5% CO_2 in air, after 24 hours sperm-oocyte co-incubation, some oocytes were examined for evidence of sperm penetration and fertilization. Oocytes were categorized as fertilized (Sperm/swollen sperm head in ooplasm, a male and/or female pronuclei), unfertilized (With no signs of sperm penetration), polyspermic (With more than one sperm in ooplasm or more than two pronuclei) and degenerated (With scattered, degenerated, invisible chromatin or with vacuoles). Penetration and fertilization of the oocytes were proved by staining of *in vitro* fertilized oocyte with acto-orcein stain.

***In vitro* Culture:** Following IVF, presumptive zygotes were vortexed in PBS supplemented with 50µg/ml gentamicin to remove spermatozoa or cumulus cells still attached to the zygotes. All zygotes were washed three times in PBS before being transferred into culture drops. Culture took place at 39°C in culture media (Droplets of 50µl) under mineral oil in a humidified atmosphere of 5% Co_2 in air and maximum humidity. The first cleavage (Two to eight cells) was assessed 48h post-insemination, whereas development to the morula and/or blastocyst stage was assessed on days 4-7 of culture. Culture medium was refreshed every 48 h by replacing half of the original medium with similar volume of fresh medium [14].

Experimental Design

Time of Epididymal Spermatozoa Storage at 4°C: Spermatozoa from the cauda epididymides of 2-3 different camels were collected in Tris-lactose extender and evaluated for motility before aliquots were stored at 4°C for 0, 4 or 6 days.

Comparison between Simple and Complex Culture Media for *In Vitro* Camel Embryos Development: All the presumptive zygotes were washed three times in PBS before being transferred into culture drops. Embryo was cultured in either simple media, the mKSOMaa, SOFaa or in complex culture media (TCM-199).

Comparison between Co-Culture with Oviductal, Granulosa or Uterine Cells: Three co-culture systems were used for *in vitro*-camel embryos development: dromedary granulosa, oviductal epithelial or uterine cells.

Statistical Analysis: The proportions of oocytes fertilized, cleaved and those reaching to blastocyst stage from oocytes inseminated with epididymal spermatozoa in different culture regimens were compared with each other using 2-way ANOVA. Level of significance was set at P<0.05. Experiments were replicated three times.

RESULTS

Experiment 1: Data presented in Tables 1 and 2 show that, the penetration, fertilization and first cleavage (Two to eight cells) rates were statistically similar for oocytes inseminated with epididymal spermatozoa stored at 4°C for 0, 4 and 6 days. However, insemination with epididymal spermatozoa at day 0 enhanced significantly (P<0.05) the morula and blastocyst development (21.32 and 16.33%, respectively) as compared with those inseminated with epididymal spermatozoa stored for 6 days (4.48 and 1.25%, respectively). The current results also, revealed that storing camel epididymal spermatozoa in tris-lactose egg yolk extender up to 4 days could preserve its fertilizing ability (43.21%) and subsequent embryo development to the blastocyst stage (9.71%). First cleavage was seen as early as 16h after IVF, morula had developed by day 4 and blastocysts after day 5 of culture (Fig. 1).

Experiment 2: The fertilized oocytes were cultured in either semi defined simple medium (KSOMaa), defined simple medium (SOFaa) or complex culture medium (TCM-199). There was non significant difference among the three groups for the cleavage rate, but the rate of blastocyst development was the highest for oocytes cultured in KSOMaa as compared with SOFaa (11.29 vs 1.19%, respectively, P<0.05).

Table 1: Fertilization rate of the dromedary camel oocytes inseminated with epididymal spermatozoa stored for different days at 4°C

Insemination time	No.of oocytes	Penetration rate	Fertilization rate	Polyspermy
0 day	56	58.71 ± 7.17^{a}	47.78 ± 7.78^{a}	10.92 ± 3.37^{a}
4 days	62	57.61 ± 7.78^{a}	43.21 ± 9.23^{a}	14.39 ± 2.39^{a}
6 days	49	51.17 ± 2.09^{a}	36.67 ± 3.77^{a}	14.52 ± 2.09^{a}

Table 2: Development of the dromedary camel oocytes inseminated with epididymal spermatozoa stored for different days at 4°C

Insemination time	No. of oocytes	Cleavage rate	Morula	Blastocyst
0 day	63	37.68 ± 3.19^{a}	21.32 ± 3.39^{a}	16.33 ± 3.05^{a}
4 days	67	35.62 ± 7.32^{a}	11.83 ± 6.77^{ab}	9.71 ± 4.33^{ab}
6 days	71	25.11 ± 1.79^{a}	4.48 ± 1.79^{b}	1.25 ± 0.69^{b}

Values in the same column with different superscript letters differ significantly at (P < 0.05)

Table 3: Developmental rate of *in vitro* fertilized dromedary oocytes cultured in different media

Treatment	No. of oocytes	Cleavage rate	Morula	Blastocyst
KSOMaa	64	36.75 ± 5.45^{a}	17.43 ± 4.49^{a}	11.29 ± 2.50^{a}
TCM-199	76	34.14 ± 4.00^{a}	9.12 ± 3.17^{ab}	5.15 ± 4.30^{ab}
SOFaa	69	36.46 ± 1.24^{a}	5.28 ± 2.68^{b}	1.19 ± 1.18^{b}

Values in the same column with different superscript letters differ significantly at (P < 0.05).

Table 4: Developmental rate of *in vitro* fertilized dromedary oocytes co-cultured with oviductal, granulosa or uterine cells monolayer

Treatment	No. of oocytes	Cleavage rate	Morula	Blastocyst
Oviductal cells	83	48.05 ± 3.25^{a}	22.75 ± 2.50^{a}	15.59 ± 2.88^{a}
Granulosa cells	79	35.04 ± 10.32^{a}	11.75 ± 4.98^{ab}	7.27 ± 2.35^{b}
Uterine cells	76	32.95 ± 2.77^{a}	8.30 ± 1.16^{b}	2.63 ± 1.52^{b}

Values in the same column with different superscript letters differ significantly at (P < 0.05).

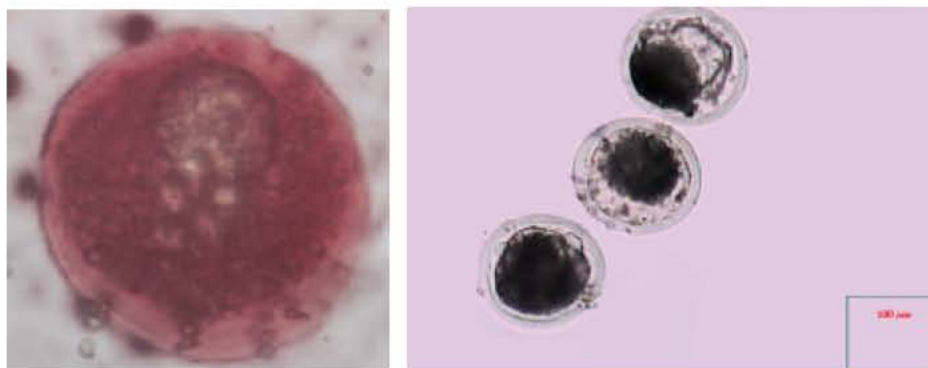

Fig. 1: (Fertilized oocyte showing male and female pronuclei formation A) (B) compact morula and blastocyst stages

Experiment 3: Data presented in Table 4 reveal that, there was no significant difference among the three groups for the cleavage rate, but the rate of blastocyst development was the highest for oocytes co-cultured with oviductal epithelial as compared with granulosa and uterine cells (15.59 vs 7.27 and 2.63%, respectively, $P < 0.05$).

DISCUSSION

Successfully IVF requires appropriate preparation of sperm and oocytes as well as the culture conditions that are favorable to the metabolic activity of sperm and oocytes [15]. The source of spermatozoa is an important topic in IVF, because of the difficulties in semen collection, the gelatinous nature of ejaculated semen and the lack of suitable extenders for its storage, epididymal spermatozoa were used in the present study [16]. The results of this study show that epididymal spermatozoa of dromedary camel maintain their ability to fertilize mature oocytes *in vitro*. This result supports the previous results on llamas [3]; camels [17, 5]; cattle [18] and pigs [19]. This may be attributed to, epididymal spermatozoa easily capacitated than ejaculated sperm [16]. The results of this study show that epididymal spermatozoa of dromedary camel maintain their ability to fertilize mature oocytes *in vitro* after storage at 4°C in tris-lactose for at least 4 days. However, Wani [20] indicated that camel epididymal spermatozoa maintained their fertilizing ability throughout the storage period of 8 days. The fertilization rates of epididymal spermatozoa may vary due to number of reasons such as the site of semen collection, age of the animals and their use in natural or artificial breeding, methods of semen preparation and also developmental competence of the oocytes. This might reflect a difference of extenders type used and/or storage conditions. A high proportion of dromedary epididymal spermatozoa are immature as evidenced by a proportion of about 62% with cytoplasmic droplets at the time of collection; of these, about 34-67% is lost on storage [17].

Blastocyst development is only one-step along the roads to the production of live offspring [21].The current results demonstrated that, simple culture media, mKSOMaa, could support the development of the fertilized camelids oocytes *in vitro* up to the blastocyst stage in a rate comparable with the complex culture media, TCM-199. These results are in agreement with Khatir *et al.* [22] who demonstrated that, a major breakthrough of the deprived *in vitro* camelids development was through the utilization of mKSOMaa as IVC medium, which markedly improved development during culture. The potassium simplex optimized medium (KSOM) improve development of mouse per-implantation embryos *in vitro* [23]. Supplementation of KSOM with amino acids and serum provides an environment similar to that occurring in the oviduct in vivo and promotes development of the preimplantation embryo in mouse [24, 25] and bovine [26, 27]. The limited blastocyst obtained in the present study may be related to the heterogeneity of follicles size (3-10mm) used in this experiment. It is possible that, oocytes contained in the smallest follicles (3-5mm in diameter in camelids) may have a reduced ability to develop after *in vitro* fertilization.

Several previous studies assumed that, culturing embryos with oviductal cells in cattle [28] or llamas [3] or with granulosa cells in cattle [29] or goats [30] improved both the yield and the quality of blastocysts. These findings may emphasize the current results which revealed that, the two co-culture used systems are equally suitable to sustain development of fertilized oocytes to the morula stage. However, the oviductal cell co-culture system was better than granulosa cell co-culture, in terms of development to blastocyst stage. This may be attributed to, the oviduct represents the microenvironment whereas fertilization and early embryonic development occur [31].

Therefore, oviductal cell co-culture may provide better conditions than granulosa cells for *in vitro* embryo development. Moreover, this beneficial effect may be due to the production of some factors, which can be fixed to the embryos, as reported in sheep by Gandolfi *et al.* [32] and may play a role in their development. The existence of a specific glycoprotein secreted by porcine oviduct was found to increase the cleavage rate of zygotes and had a beneficial effect on *in vitro* development of fertilized oocytes to blastocyst stage [33]. In this regard, the low developmental rate obtained with the granulosa or the uterine cells co-culture system may have been due to the high concentration of cells in the mono-layer; perhaps the larger number of cells results in greater competition for nutrients or their metabolic waste may impair embryo development.

In conclusion, the current results demonstrated that dromedary epididymal spermatozoa can survive in storage for at least 4 days in tris-lactose egg yolk extender at 4°C. These spermatozoa maintain fertilizing ability and may be suitable for use in IVF. KSOMaa media with oviductal cell co-culture and appeared to be the most suitable conditions for *in vitro* dromedary camel embryo production.

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