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Original article

In vitro production of Sudanese camel (*Camelus dromedarius*) embryos from epididymal spermatozoa and follicular oocytes of slaughtered animals

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Abstract

Application of assisted reproductive technology in camelidea, such as artificial insemination (AI) and embryo transfer, has been slow in comparison to that for other livestock species. In Egypt, there are few attempts to establish *in vitro* maturation (IVM) and fertilization (IVF) techniques in dromedary camel. The present study was carried out to produce Sudanese camel embryos using *in vitro* matured oocytes and epididymal spermatozoa. Dromedary camel ovaries were collected from abattoirs and then, the oocytes were aspirated from all the visible follicles on the ovarian surface (~2-8 mm in a diameter). Meanwhile, Fetal Dromedary Camel Serum (FDCS) was obtained from camel fetuses after slaughtering. Thereafter, only Cumulus Oocyte Complexes (COCs) were matured *in vitro* in the Tissue Culture Medium (TCM-199) complemented with 10% FDCS. Spermatozoa required for *in vitro* fertilization were collected from testes (epididymal cauda) of the slaughtered camel bulls. The results clearly showed that the maturation rate of oocytes at metaphase II was about 59.5% while the fertilization rate was around 70.4%. Intriguingly, the embryo rates determined were 13.1%, in 2-cell; 0.0%, in 4-cell; 34.7%, in 8-16% cell; 39.1%, in morula and 13.1% in a blastocyst stage. This study represented a successful *in vitro* production of Sudanese dromedary camel embryos from epididymal sperm cells and *in vitro* matured oocytes recovered from slaughtered camels.

Key words: blastocyst, cleavage, dromedary camel, epididymal sperm, *in vitro* maturation, oocytes

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Introduction

Dromedary camel is one of the most important animals that live in hot, dry or both climates, since it well adapted to the arid conditions. It supports people who live under drastic conditions by diet (milk and meat), in addition to affording precious welfare benefits (fiber, transportation and sports) in these areas (Jianlin 2011).

The *in vitro* production (IVP) emerged as an effective reproductive approach for cattle species (Gordon 2003). For example, various studies reported the significant effect of the IVP on cattle such as Holstein and Nelore (Batista et al. 2016, Jin et al. 2016). Inconsistent of cattle, dromedary camels are seasonally breed animals (Allam et al. 2013), have a long pregnancy period (13 months), give a single calve every two years (Jianlin 2011). Moreover, Abdalla et al. (2015) reported that camels have a wide range of milk production (3.5-20 kg/d). Therefore, it was recommended to make a selection for this species. At a time, people in developing countries slay the camels without caring for their genetic values. Nowadays, the assisted reproductive technologies in camelidae such as artificial insemination (AI) and embryo transfer (ET) are relatively slower in comparison with that of their counterparts of equine, cattle, sheep and goats species (John 2011). This is ascribed to the significant importance of cattle in human livestock feeding relative to camels because they are not geographically distributed in the most advanced countries (Jianlin 2011). There are currently few data available on follicular growth and maturation in camels (Ratto et al. 1999, Kandil et al. 2014). For instance, the *in vitro* maturation (IVM) and fertilization (IVF) rate of dromedary camel oocytes were investigated under various conditions (Abdoon 2001, Ali and Abdel-Razek 2001, El-Harairy et al. 2007, Zeidan et al. 2015). These studies have revealed that further *in vitro* production research is needed on dromedary camels. Inspired by this, the present study was carried out to *in vitro* produce camel embryos upon usage of spermatozoa from epididymal cauda and follicular oocytes which are both recovered from slaughtered Sudanese camel bull and she-camel. Interestingly, the experiments were conducted in non-breeding season, which is highly required for developing countries due to the great importance of camel in their livestock feeding.

Materials and Methods

This study was jointly planned by the Animal Production Department, Faculty of Agriculture, Mansoura University and Animal Production Research Institute, Dokki, Giza, in cooperation with the Animal Production Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt. The study was carried out at the Laboratory of Physiology and Biotechnology belonging to the Animal Production Department, Faculty of Agriculture, Mansoura University. All chemicals and media used in this study were purchased from Sigma (Madrid, Spain) without otherwise specified.

Oocyte collection

Ovaries were collected from 53 slaughtered Sudanese she-camels with unknown reproductive history in the Elbasatein Abattoir, Cairo, during non-breeding season. Immediately after slaughtering, they were placed in saline solution (0.9% NaCl) complemented with antibiotics (100 IU penicillin and 100 µg streptomycin/ml) at about 25°C and transported to the laboratory. Thereafter, the redundant tissues were cut from the ovarian stalk and the ovaries were washed three times using Phosphate Buffer Solution (PBS) at 28°C, added to 2 mg/ml of Bovine Serum Albumin (BSA), 100 IU penicillin and 100 µg streptomycin/ml to eliminate adhering tissues or blood clot. Then, the ovaries were washed one-time using ethanol 70% to discard any contamination and the oocytes were collected by aspiration using a 20-gauge hypodermic needle linked to a sterile disposable 5 ml syringe containing 2 ml PBS as harvesting medium (Pavlok et al. 1992). After aspiration, the syringe contents were slowly dispelled into sterile Petri dish (60 mm) for searching oocytes under a stereo-microscope. The oocytes collected were washed three times in PBS, evaluated under an inverted microscope and classified into compact oocytes with ≥5 layers of complete cumulus cells and evenly granulated dark ooplasm (COCs), partial denuded, denuded, shrunken and fragment oocytes (Madison et al. 1992). Only, COCs were used for *in vitro* maturation.

In vitro oocyte maturation (IVM)

The basal maturation medium (BMM) used in this study was TCM199 as a liquid medium (Egyptian Organization of Biological Products and Vaccine, Giza), supplemented with 10% (v/v) Fetal Dromedary Camel Serum (FDCS), 10 µg/ml FSH, 10 IU/ml hCG (Pregnyl, Nile, Co. for Pharm. Cairo, ARE), estradiol (E-17 β), 20 mMol Na Pyruvate, 100 IU/ml Na Penicillin G and 100 µg/ml Streptomycin. About 200 µl from prepared BMM was placed into a sterile Petri

dish (60 mm), covered by sterile mineral oil, then Petri dishes were incubated in CO_2 incubator (5% CO_2) at 38.5°C and high humidity (90-95%) for at least 1 h for equilibration before placing oocytes in culture dish. BMM was adjusted to pH value from 7.2 to 7.4 and osmolarity from 280 to 300 mOsmol/kg, and filtered by 0.22 µm millipore filter (Milieux GV, millepore, Cooperation Bedford, MOA).

To prepare FDCS, blood samples were collected from three camel fetuses immediately after slaughtering pregnant dromedary she-camels through the umbilical cord puncture into 15 ml centrifuge tube and placed into an ice box, then transported to the laboratory within 3-4 h. Blood samples were centrifuged two times at 2800 *xg* for 15 min to aspirate clear serum by a pasture pipette which was placed in other 15 ml sterile centrifuge tubes transferred in a water bath at 56°C for 30 min and left to cool for obtaining heat-inactivated serum. Thereafter, the serum was placed into 1.5 ml Eppendorf tubes and frozen until usage.

Only COCs which presented a compact, non-atretic cumulus oophorus-corona radiata and an oocyte with homogeneous cytoplasm were selected for *in vitro* maturation. All selected COCs were washed thoroughly three times in the collection medium and two times in BMM to remove substances from the follicular fluid which may hinder maturation. Then, about 25-30 oocytes per droplet were placed by a pasture pipette in petri dishes containing the maturation medium. Petri dishes containing oocytes and BMM were incubated in a carbon dioxide incubator (5% CO_2) at 38.5°C and high humidity for 40 h. At the end of the maturation time, COCs were fixed and stained. COCs were placed in PBS containing hyaluronidase (1 mg/ml). The oocytes were then pipetted onto a slide. A cover slip, spotted with a paraffin wax-vaseline (10:1) mixture at each corner, was placed directly over the center of the drop containing the oocytes. Oocytes were fixed by placing the slides in acetic acid: ethanol (1:3) for overnight, and stained with a drop of aceto-orcein (1% orcein in 60% acetic acid w/v) for 4-5 min, and then washed by aceto-glycerol (3:1). Estimation of nuclear maturation was carried out under a phase contrast microscope using x200 and x500 magnification, and expressed as the percentage of oocytes arrested at the metaphase II.

Collection of epididymal spermatozoa

Testes of 3 mature camel bulls were collected from the same abattoir of ovaries collection. The testes were collected immediately after slaughtering, placed in plastic bags in an icebox (5°C) and transported to the laboratory within about 3-4 h. Each testicle was isolated from its *tunica vaginalis* and other attached extraneous tissues, washed 3 times using tap water and finally washed using ethyl alcohol 70%. Different incisions in the tail of the epididymis were performed with a scalpel and then, by pressing that region manually the epididymal spermatozoa were released and collected by aspiration with sterile disposable syringe (5 ml) containing 2 ml PBS as an extender (Deen et al. 2003). The epididymal spermatozoa collected were examined under a low power microscope using a hot stage adjusted at 37°C. Initial motility was estimated and only samples with ≥60% initial motility were used.

In vitro fertilization (IVF)

The collected epididymal spermatozoa in PBS were washed three times using TALP medium (Tyrod's Albumin Lactate Pyruvate) (Parrish et al. 1986) by centrifugation at 2000 *xg* for 10 min each time. The washed sperm samples were diluted with fertilization medium (TALP, Tyrod's Albumin Lactate Pyruvate) supplemented with 100 IU/ml Penicillin, 4 mg/ml BSA, 100 µg/ml Streptomycin and 10 µg/ml heparin (capacitated substance) up to the final concentration of about 50×10^6 sperm/ml (determined by haemocytometer) and allowed to swim up for 1 h in an incubator (5% CO_2 and 38.5°C) at an angle of 45°. The *in vitro* matured oocytes were washed twice in the fertilization medium and randomly distributed to 4-well culture plates (40-50 COCs/well) containing 490 µL of the fertilization medium. Whereas, the motile spermatozoa (10µL) were added to the oocytes in the fertilization medium at the condensation of approximately 1×10^6/mL. Both sperms and oocytes were co-incubated at 38.5°C in a moist atmosphere of 5% CO_2 in air for 24 h.

In vitro culture

After fertilization period (24 h), the presumptive zygotes were removed from the fertilization medium, washed three times in an embryo culture medium consisting of TCM199 supplemented with 100 IU/ml penicillin, 100 µg/ml Streptomycin and 10% FDCS. Thereafter, the fertilized oocytes were cultured in 500 µl of the culture medium in 4-well dishes covered by sterile mineral oil and incubated in a carbon dioxide incubator at 38.5°C in a moist atmosphere of 5% CO_2 in air for 7 days. The culture medium was renewed by a fresh medium every 48h. The first cleavage was recorded on day 2, whereas development to blastocyst

Table 1. Percentages of camel follicular oocytes at different phases *in vitro* post maturation.

Oocyte phase	N	%
Germinal vesicles (GV)	23	15.0
Germinal vesicles breakdown (GVB)	8	5.2
Metaphase I (MI)	8	5.2
Metaphase II (MII)	91	59.5
Degenerated oocytes	23	15.1
Total oocytes	153	100

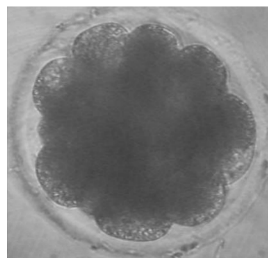

Plate 1. Embryo at morula stage.

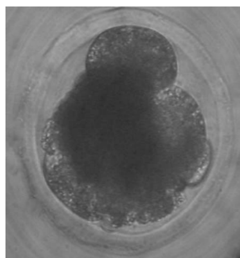

Plate 2. Embryo at 8-16 cell stage.

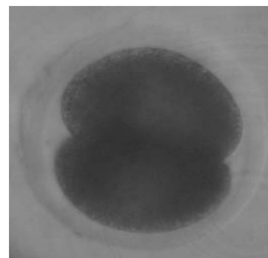

Plate 3. Embryo at 2-cell stage.

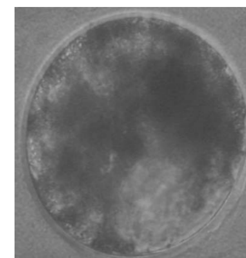

Plate 4. Embryo at early blastocyst stage

Table 2. Fertilization rate of *in vitro* matured oocytes and embryonic stages of *in vitro* development of camel embryos.

Item	N
In vitro fertilization:	
Total oocytes*	165
Mature oocytes**	98
Unfertilized oocytes	67
Fertilized oocytes	69
Fertilization rate, % [(1)]	41.8
Fertilization rate, % [(2)]	70.4
Embryonic stage***:	
2 cell	9 (13.1%)
4 cell	–
8-16 cell	24 (34.7%)
Morula	27 (39.1%)
Early blastocyst	9 (13.1%)

* Immature oocytes, ** Calculation based on maturation rate (59.5%), *** Based on the number of fertilized oocytes, [(1)]: Based on the total number of oocytes, [(2)]: Based on the calculated number of *in vitro* mature oocytes.

stage was recorded on days 4-7 of culture using inverted microscope. The fertilized oocytes were examined after fertilization under an inverted microscope to determine the cleavage rate.

Results

The results presented in Table 1 showed that the *in vitro* maturation rate of camel oocytes in term of oocytes parentage arrested at the metaphase II (M II) was 59.5% in TCM-199 supplemented with 10% FDCS. Whereas, when the maturation rate included the percentage of oocytes at the metaphase I (5.2%), it was increased to 64.7%.

In vitro fertilization

In the present study, *in vitro* fertilization rate was 41.8% based on the total number of immature oocytes, increased to 70.4% based on a calculated number of *in vitro* matured oocytes (Table 2). Concerning the embryonic stages of fertilized oocytes, the percentage of embryos at the morula stage (Plate 1) showed the highest frequency distribution of cleaved oocytes (39.1%), followed by those at 8-16 cell stage (Plate 2), while embryos at 2-cell (Plate 3) and blastocyst (Plate 4) stages represented the lowest frequency distribution, amounting to13.1% for each. Yet, no embryos at 4-cell stage were detected (Table 2).

Discussion

Comparable with the present results, a lower maturation rate (oocytes at M II) for dromedary camel oocytes was recorded (39.3%, Khalil 2005; 38.4%, Zeidan et al. 2015). In contrast, El-Nahla et al. (2014) showed a maturation rate of 50.2% using a high-quality oocytes matured in maturation medium supplemented with FSH or eCG hormones during the non-breeding season. However, Torner et al. (2003) showed a maturation rate (58.5%) of camel oocytes recovered from the ovaries bearing corpora lutea (CLs) higher than that recovered from non-bearing ovaries (51.2%). The ovaries used in this study were taken from camels with anonymous reproductive history (pregnant or non-pregnant) and based upon the previous studies, the wide variation in maturation rate may be related to the time of maturation. This may be explained by the finding of Torner et al. (2003) who reported that the oocytes of pregnant camels require 36 h of maturation to reach levels of >50% M)) in comparison with the oocytes of non-pregnant camels, where 32 h are sufficient. Regardless serum supplementation in a maturation medium, *in vitro* maturation of oocytes depends on several conditions including reproductive status, ages, side of the ovary (Amer and Moosa 2009) and method of oocytes collection (Sansinena et al. 2003, Farag et al. 2012). The last authors found that the ovaries without the corpus luteum are promising subjects for high recovery value of camel oocytes (COCs) which have a great ability to be matured *in vitro*. In this context, Nagy (2014) declared that TCM-199 medium enhanced the maturation rate of the dromedary camel oocytes more effectively than MEM medium. The higher maturation rate found in this study in comparison to that determined in the most previous studies may revealed the rich constituents of TCM-199 medium which contains numerous nutrients, as inorganic salts, antioxidant, glucose and amino-acids that may enhance follicular activation (Javed et al. 2010). Amino-acids afford energy source molecules and precursor protein synthesis (Fujihara et al. 2012), and antioxidant which is very important for protecting cells against reactive oxygen species (Andrade et al. 2014). In addition, Fetal Dromedary Camel Serum (FDCS) supplementation to the media shows better maturation rate which might be due to the compositions of FDCS serum which is composed of many immunoglobulins of the camel species (El-Hatmi et al. 2006). In comparison with the results dealing with the *in vitro* maturation rate of camels oocytes, Coleman et al. (2007) showed that the *in vitro* maturation rate of bovine oocytes matured in TCM-199 supplemented with fetal calve serum amounted to 62%. Whereas, the maturation rate was 53.8% for buffalo oocytes (Shamiah 2004).

Moreover, in accordance with the present results, Wani et al. (2008) found that the proportion of dromedary camel oocytes fertilized with epididymal spermatozoa was 43% and those that developed to blastocysts was 14%. Whereas, using freshly ejaculated semen, the rate of cleavage in dromedary camel ranged from 51 to 64% and the rate of development to blastocyst ranged from 16.5% to 23% (Khatir et al. 2005). Moreover, Khatir et al. (2008) found higher cleavage rate (69%) with the highest percentage of embryos at blastocyst stage (37%) when camel oocytes were matured in TCM199 with camel follicular fluid (2.5%) obtained from the large follicles. In another species of camel (*Lama glama*), IVP attained 17% at blastocysts stage (Conde et al. 2008). Trasorras et al. (2012) found a lower rate of blastocysts reaching 9%.Recently, blastocyst rate ranged between 34-36% using TALP culture medium in the same living species (Trasorras et al. 2014). As a result of this, cleavage rate of camel oocytes varies according to the type of culture medium (Khatir et al. 2005), the size of follicle (Khatir et al. 2007) and co-culture with granulosa or oviductal cells (Khatir et al. 2004).

Conclusion

Based on the results obtained, the present study represents a successful attempt to *in vitro* production of Sudanese dromedary camel embryo (39.1% at morula and 13.1% at blastocyst stages) from capacitated epididymal sperm cells and follicular oocytes matured in TCM-199 supplemented with 10% fetal dromedary camel serum. Both sperm cells and oocytes were recovered from slaughtered animals of Sudanese bred. Further investigations are needed for increasing rate of IVP of camels by improving maturation rate with different types of supplements to sera, hormones, temperature and time of maturation period.

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