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RELATIONSHIP BETWEEN SEASON OF THE YEAR, CULTURE MEDIUM AND *IN VITRO* OOCYTE COMPETENCE IN DROMEDARY CAMELS

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Summary

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The effect of season and hormones in the culture medium on the competence of camel oocytes to mature *in vitro* was conducted on 67 ovarian pairs. The ovaries were collected during breeding season (BS; n=24), at early non-breeding season (ENBS; n=20) and at late non-breeding season (LNBS; n=23). The follicles (2–8 mm) were aspirated, then oocytes were qualified into 4 grades: Q1 (very good), Q2 (good), Q3 (bad) and Q4 (very bad). The effect of follicle-stimulating hormone and equine chorionic gonadotropin (0.5 µg/mL FSH or 10 IU/mL eCG) on cumulus expansion and *in vitro* maturation of oocytes was assessed. The numbers of healthy follicles were significantly ($P<0.01$) higher during BS compared to both ENBS and LNBS. The left ovary showed a higher ($P<0.01$) activity than the right one regarding the number of healthy follicles. The number of Q1 to Q3 oocytes and recovery rate were significantly ($P<0.01$) higher during BS than NBS at early or late months. A significantly ($P<0.01$) higher number of Q1 to Q3 oocytes were obtained during the ENBS than in LNBS months. The left ovary had higher ($P<0.01$) number of Q1 and Q2 oocytes compared to the right one. Only during the BS, oocytes cultured in medium containing eCG exhibited a higher ($P<0.05$) percentage of expansion and maturation (metaphase-II) rates than the medium containing FSH. In conclusion, dromedary camels showed better ovarian activity and oocyte status during BS compared to NBS, and displayed ovarian activity during early as well as late non-breeding months. Further detailed studies are required to establish the reproductive efficiency of dromedary camels throughout the non-breeding season.

Key words: breeding, camel, culture, eCG, FSH, maturation, non-breeding, oocytes

INTRODUCTION

The one-humped camel (*Camelus dromedarius*) is the only animal that can survive for several weeks without water and still provide humans with meat and milk (Yagil, 1982). It is unique producer of food in the arid and semi-arid zones of the world. In tropical climates, the temperature effects seemed to be dominant, and the variations in relative humidity, nutrition and length of daylight seemed to be also involved (Glimore, 1981; Marai *et al.*, 2002). The seasonality of reproduction in the camelidae has long been suggested mainly on the basis of seasonal distribution of births and the status of ovarian activity. Many investigators consider the dromedary camel as a seasonally polyoestrous animal with a relatively short breeding season (Zeidan *et al.,* 2001). Outside the breeding season, the mating

activity ceases and the ovaries are inactive or show a limited number of small follicles (Shalash, 1980; Khatir *et al.,* 2007). Daylight and temperature are the two main climatic factors influencing the annual sexual cycles. In addition, the ovarian activity in she-camels was found to be mainly follicular rather than luteal (Musa & Abusineina, 1978). However, corpora lutea may be occasionally present without pregnancy. Some studies on the ovarian activity in she-camels related to the different seasons of the year were documented, especially when the early or late non-breeding season or daylight were taken in consideration. In addition, recently techniques of *in vitro* maturation and subsequent fertilization and embryo-transfer can be employed in camels to overcome some problems in the reproductive efficiency (Ismail *et al.,* 1993; Abdoon *et al.,* 2007). Thus, the present study aimed to investigate the effect of reproductive season and type of culture medium on the competence of oocytes to mature *in vitro* in dromedary camels.

MATERIALS AND METHODS

This study included 67 ovarian pairs from non-pregnant dromedary camels at 5 to 10 years of age. The ovaries were collected from Belbies and Zagazig Abattoirs, Sharkiya Province, Egypt. The experimental work was carried out in the IVF-ET laboratory, Department of Theriogenology, Faculty of Veterinary Medicine, Zagazig University, Egypt during May 2005 till December 2007.

Experimental design

The present study was designed to study the effects of breeding (December to May) and non-breeding seasons (BS and NBS respectively) in the early (June to August) and late months (September to November) on the ovarian activity of dromedary camels. During the BS and NBS, the number of corpora lutea, number of healthy and atretic follicles was studied. The status of follicular oocytes i.e. quality and recovery rate in different seasons was also investigated. Moreover, two trials were carried out to investigate the effect of culture condition (media type and hormonal additives) on progression of oocytes maturation *in vitro*.

Collection of the ovaries

The ovaries were collected during breeding (n=24), early non-breeding (n=20) and late non-breeding (n=23) months of camels. Two ovaries (right and left) from each camel were collected within 30–60 min after slaughter, then washed by sterile warm saline solution (0.9% NaCl) at 28–32 ºC containing 100 IU/mL penicillin-G sodium and 100 µg/mL dihydrostreptomycin sulphate (Sigma Chemical Co., St. Louis, USA). The ovaries were transported to the laboratory within 1–2 hours for processing.

Classification of the ovarian structures

All the antral follicles (∅ 2–8 mm) in both ovaries were counted. The follicles were differentiated according to the external morphology and the nature of follicular fluid (Pavlok *et al.,* 1992) into healthy follicles (less vascularized, turgid, transparent, almost spherical, easily squeezable and thin-walled) and atretic follicles (highly vascularized, opaque, nearly spherical and relatively thick-walled).

Collection of the oocytes

Oocytes were collected individually from the follicles by means of aspiration. A 20-gauge needle attached to 10 mL sterile syringe containing 0.5 mL physiological

saline solution (0.9% NaCl) at 28–32 ºC (Pavlok *et al.,* 1992) was used. Before aspiration, the needle and syringe were first primed with approximately 0.25 mL aspiration medium. In the laboratory, the ovaries were washed once with 70% ethanol and at least 3 times in saline solution containing 100 IU/mL penicillin-G sodium and 100 µg/mL dihydro-streptomycin sulfate. After aspiration, the contents of the syringe was slowly dispelled into sterile Petri dishes (30×60 mm) with minimum disruption of the cumulus oocytes complexes. Repeated aspirations of follicles were performed to collect oocytes into the syringe. The oocytes were assessed under a stereo-microscope. Recovery rate of oocytes was determined as the percentage of recover oocytes vs the total number of aspirated follicles

Qualification of the collected oocytes

The oocytes were evaluated in respect to both investment and ooplasma granulation of cumulus cells (Pavlok *et al.,* 1992) as followed:

- Q1 (Quality 1, very good): Oocytes with complete compact dense cumulus oophorus >6 layers and transparent homogeneous cytoplasm.
- Q2 (Quality 2, good): Oocytes with complete compact dense cumulus oophorus >6 layers and transparent homogeneous cytoplasm with small dark zones at periphery.
- Q3 (Quality 3, bad): Oocytes with <3–5 layers of cumulus cells not completely surrounding the oocyte and with less transparent cytoplasm containing dark zones.
- Q4 (Quality 4, very bad): Oocytes either denuded from cumulus or surrounded by expanded layers of cumulus cells appearing as scattered clamps in the matrix.

Preparation of the culture media

Culture medium (TCM-199) was obtained in a liquid form from the Egyptian Organization for Biological Products and Vaccines, Dokki, Giza, Egypt and then stored in refrigerator at 5 ^{0}C until used. Media were supplemented with 10 mg/100 mL L-glutamine, 100 IU/mL penicillin G sodium and 50 µg/mL dihydro-streptomycine sulfate. The pH was measured by a pH meter and adjusted to 7.4 using 1 N NaOH (Sigma Chemical, St Louis, USA). The medium was sterilized by using 0.2 µm Millipore biological filtres and equilibrated in CO_2 incubator containing 5% CO_2, with maximum humidity 95% at 38.5°C for at least 2 hours prior to use.

Effect of hormonal (follicle-stimulating hormone or equine chorionic gonadotropin) additives

The effect of hormonal additives on the potential of oocytes to mature *in vitro*, during the BS and NBS, was studied. The selected oocytes (for each hormone, n=20 during BS and n=40 during NBS) were washed three times in the maturation medium using fine polished Pasteur pipettes before being injected finally into four wells culture dishes, each containing 400 µL of TCM-199 supplemented with 0.5 µg/mL FSH (Folltropin, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) or 10 IU/mL eCG (Novormon, Bioniche Animal Health Canada Inc.), to culture for 36 hours in a CO_2 incubator (5% CO_2), 95% relative humidity and 38.5 °C (Gomez *et al.,* 2002). The percentage of germinal vesicles, germinal vesicle breakdown, matured and degenerated cumulus oocyte complexes (COCs) were recorded.

Assessment of the cumulus expansion and nuclear maturation

The cumulus expansion was determined after oocytes incubation for 36 hours under a stereomicroscope. The assessment of the cumulus expansion were done by the criteria of Pavlok *et al.* (1992). Expanded cumulus cell mass was extended away from the zona pellucida, while non-expanded cumulus cell mass was tightly adherent to the zona pellucida.

For the evaluation of nuclear maturation, oocytes were transferred to a small plastic tube containing 3% sodium citrate solution followed by repeated agitation for the denudation of oocytes (Carolan *et al.,* 1996). The contents of the tube was then transferred to a new 35 mm Petri dish and the demanded oocytes were mounted on a glass slide with a cover slip supported by droplets of paraffin vaseline mixture, then fixed with ethanol/acetic acid (3:1) at 4 ^{o}C for 24 hours. The oocytes were stained with aceto-orcein (1% orcein in 40% acetic acid) for 25 min, rewashed with a fresh fixative and examined under light microscope. The stage of nuclear maturation was described as follows:

- Immature: Germinal vesicle stage with intact nucleus or germinal vesicle breakdown stage with loose nuclear membrane;
- Mature: Metaphase-I (paired or bivalent chromosomes were observed within the nucleus of the oocyte) or metaphase-II (two groups of unequally spread chromosomes were observed and the polar body set was clustered together).
- Degenerated: The cytoplasm was highly shrinked and destructed.

Statistical analysis

Analysis of data was performed (Snedecor & Cochran, 1982). Significant differences between the means were evaluated utilizing Duncan's Multiple Rang Test (DMRT) (Duncan, 1955).

Table 1. Numbers (mean ±SEM) of normal and atretic follicles of the left and right ovaries during the breeding season, the early non-breeding season and the late non-breeding season in dromedary camels

Season	Ovarian structures	
	Healthy follicles	Atretic follicles
Breeding season		
Left ovary	34.26±1.59	6.19±0.49
Right ovary	21.56±1.38	7.33±0.47
Mean	27.91±1.40^{A}	6.76±0.42^{C}
Early non-breeding season		
Left ovary	16.67±1.75	7.94±0.54
Right ovary	12.06±1.53	8.82±0.52
Mean	14.37±1.64^{C}	8.38±0.53^{A}
Late non-breeding season		
Left ovary	31.06±1.43	7.11±0.44
Right ovary	18.13±1.25	8.61±0.42
Mean	24.60±1.38^{B}	7.86±0.43^{B}

Means bearing different letters within same column and row are significant (P<0.01).

RESULTS

Regarding the number of healthy follicles, the results in Table 1 showed a significantly (P<0.01) higher number during the BS vs both ENBS and LBNS. Similarly, the number of the normal follicles was significantly (P<0.01) higher in the late than in early non-breeding months. The highest number of healthy follicles was recorded in BS and the lowest number – during ENBS. However, the number of the atretic follicles was significantly (P<0.01) higher during ENBS vs both LNBS and BS. With regard to side of ovary, the number of the normal follicles in the left ovary was significantly (P<0.01) higher than that in the right one, and atretic follicles in the right ovary were significantly (P<0.01) more numerous compared to the left ovary (Table 1). The total number of follicles per ovary collected was significantly (P<0.01) higher during the BS than in ENBS. However, the effects of breeding or non-breeding seasons at the late months on the total number of follicles were similar. With the regard to side of ovary, the total number of follicles was significantly (P<0.01) higher in the left ovary than in the right one (Table 1).

The number of Q1 and Q2 oocytes was significantly (P<0.01) higher during the BS than in both ENBS and LBNS. Similarly, they were significantly (P<0.01) higher in the late than in the early months of NBS. The highest numbers of Q1 and Q2 oocytes were recorded during the BS and the lowest – ENBS. The Q3 oocytes were significantly (P<0.01) more numerous during BS than in NBS. The effects of hot-humid and hot-dry months on Q4 were similar. However, the incidence of Q4 oocytes was not influenced by season.

Table 2. Numbers (mean ± SEM) of follicular oocytes recovered from the right and left ovaries during the breeding season, the early non-breeding season and the late non-breeding season in dromedary camels

Season	Morphology of follicular oocytes				Oocytes RR
	Q1	Q2	Q3	Q4	
Breeding season					
Left ovary	23.78±1.64	6.57±0.75	2.33±0.41	1.17±0.36	83.68
Right ovary	10.94±1.22	4.00±0.80	2.94±0.55	1.83±0.33	68.22
Mean	17.36±1.44^{A}	5.29±1.77^{A}	2.64±0.50^{A}	1.50±0.34^{A}	77.27^{A}
Early non-breeding season					
Left ovary	9.94±1.81	2.39±0.83	1.11±0.45	0.83±0.32	57.98
Right ovary	6.33±1.34	1.50±0.88	1.78±0.61	1.74±0.29	54.36
Mean	8.14±1.63^{C}	1.95±0.85^{C}	1.44±0.54^{B}	1.29±0.31^{A}	56.35^{C}
Late non-breeding season					
Left ovary	17.94±1.48	4.44±0.67	1.22±0.37	1.17±0.39	64.89
Right ovary	10.22±1.10	2.56±0.72	1.89±0.50	1.89±0.36	61.93
Mean	14.08±1.26^{B}	3.50±0.69^{B}	1.56±0.44^{B}	1.53±0.37^{A}	63.68^{B}

Means bearing different letters within the same column and row, differ significantly (P<0.01); Q= quality; RR = recovery rate

Table 3. Effect of FSH or eCG on the maturation of dromedary camel oocytes after culture *in vitro* in TCM-199 during the breeding and the non-breeding seasons of the year

Morphology of oocytes	Hormonal effect				χ^2
	FSH		eCG		
	Breeding season	Non-breeding season	Breeding season	Non-breeding season	
	n=20	n=40	n=20	n=40	
Expanded oocytes	16 (80.0%)	30 (75.0%)	20 (100%)	30 (75.0%)	n.s.
Germinal vesicles	1 (5.0%)	6 (15.0%)	0 (0.0%)	2 (5.0%)	n.s.
Germinal vesicle breakdown	2 (10.0%)	4 (10.0%)	1 (5.0%)	6 (15.0%)	n.s.
Matured (metaphase-II)	15 (75.0%)	20 (50.0%)	18 (90.0%)	27 (67.5%)	P<0.05
Degenerated oocytes	2 (10.0%)	10 (25.0%)	1 (5.0%)	5 (12.5%)	n.s.

n.s. = non significant.

The recovery rate of oocytes was significantly (P<0.01) increased in BS as compared to the NBS either in the early or late months (Table 2). The highest recovery rate was recorded during the BS and the lowest in ENBS. Regarding the side of ovary, the mean numbers of Q1 and Q2 oocytes and recovery rate were significantly (P<0.01) higher in the left ovary, while the Q3 and Q4 were significantly (P<0.01) more numerous in the right ovary (Table 2).

The effect of hormonal additives in culture media on the ability of oocytes to mature *in vitro* was illustrated in Table 3. During the BS, eCG resulted in a significantly (P<0.05) higher rate of oocytes expansion and maturation to metaphase-II stage in comparison to the FSH. A significant difference was obtained with the maturation of oocytes when eCG was added during the BS compared to the NBS.

DISCUSSION

In this study, the number of healthy follicles was significantly higher in BS than NBS (early or late months). The LNBS appeared to be more active when compared to ENBS. These findings are in agreement with other studies in dromedary she-camels (Amer, 2004). The highest number of the normal follicles in dromedary camels was recorded in the BS while the lowest number – in ENBS. However, the number of the atretic follicles was significantly higher during the ENBS than in both LNBS months and BS. A similar trend was previously reported (Abdoon, 2001; Amer, 2004; Khatir *et al.,* 2007). It was shown that ovary side had a highly significant effect on the number of normal follicles and insignificant effect on the number of atretic follicles. However, there were no differences in the total number of follicles collected from the right or left ovaries (Basiouni, 1997; Abdoon, 2001; Abdoon *et al.,* 2007). The left ovary weight was insignificantly higher than that of the right one during spring, summer and autumn seasons, while in winter, an opposite trend was observed (Amer, 2004). In general, the results obtained in the present study, showed that pregnancy corpus luteum did not inhibit follicular growth, whether these follicles produce

estrogen which may be required for either maintenance or persistence of the corpus luteum or for implantation (Nawito *et al.,* 1967). The absence of corpora lutea in all non-pregnant camels and their presence only during pregnancy shows that ovulation in the camel may not be spontaneous but could be induced by copulatory stimulus or by cervical stimulation.

Normally, the mature follicles of she-camels became atretic in the absence of mating and follicular rupture in this species. The atresia results from the substitution of ovum and granulosa cells by proliferating fibroblasts and theca cells which progressively became theca-luteinic cells (Abdoon *et al.,* 2007). Also, there was an atrophy and degenerative changes in the granulosa cells of the cystic follicles as well as thickening in the theca externa with enlargement of the blood vessels.

The mean number of oocytes with Q1 to Q3 and their recovery rate were significantly higher in BS than NBS at the early or late months. With this regard, the differences of bad quality oocytes decreased between BS and NBS, contrary to oocytes with good quality. At the same time, the ENBS months exhibited significantly more Q1 to Q3 (but not Q4) occytes than LNBS months. The left ovary had higher Q1 and Q2 oocyte numbers compared to the right one, but not Q3 to Q4. It has been recorded that the highest numbers of oocytes complexes and partial denuded oocytes were obtained during autumn and winter and the lowest numbers during summer and spring season (Amer, 2004). Also, the highest expanded and denuded numbers were recorded during spring and winter and the lowest during summer and autumn. The ovary side had significant effect on the cumulus oocytes compact and expanded cumulus oocytes, while the effect of side of ovary on the number of oocytes was insignificant.

In general, it is interesting to notice that, the superiority of the total number of follicles and oocytes recovery in both left and right ovaries of dromedary camels was recorded during the BS as compared to the NBS (either the hot-humid or hot-dry months). This could be probably due to the fact that the gonadotropic hormonal balance was in favour of the follicular growth stimulation oocyte status in the breeding season but not in favour of ovulation process.

The kinetics of *in vitro* oocyte maturation has not been critically studied in camelids, but has been examined in some detail in cattle (Lonergan *et al.,* 1997). Although maturation of the oocyte is not required for sperm penetration or for sperm nuclear decondensation under *in vitro* conditions, exposure of the sperm to immature oocytes was associated with decreased embryo development. In addition, the period of *in vitro* culture required for an oocyte to undergo nuclear maturation is reflective of its subsequent developmental competence.

In addition to the greater proportion of matured oocytes *in vitro*, the proportion of degenerated oocytes observed after *in vitro* maturation appeared to be lower in the present study compared to earlier reports (Del Campo *et al.,* 1994; Manzoor, 2005), and may be attributed to the time interval between ovarian collection and oocytes aspiration (2–3 h), or to the method of oocytes collection (follicular aspiration). It appears that *in vivo* and *in vitro* maturation times are similar under the conditions of the present study. However, additional study is required to determine if maturation occurs earlier than 28 h of *in vitro* culture. It is also noteworthy that most camel oocytes (85%)

reached metaphase II after 36 h of *in vitro* culture (Hyttel *et al.,* 1997; Abdoon, 2001), which is consistent with the reported time interval between mating and ovulation in camels (36 h). The importance of determining the optimal *in vitro* oocyte maturation time is illustrated by the results of studies in cattle showing that oocyte aging may be the cause of reduced fertility if *in vivo* insemination is delayed (Hunter, 1989). In addition, delayed insemination *in vitro* has been associated with oocytes that are capable of being fertilized but unable to develop into embryos as a result of deranged cortical granules and microtubules.

The dark appearance of the cytoplasm of oocytes was consistent with that previously described (Del Campo *et al.,* 1994) and may be attributed to the prevalence of lipid droplets. Furthermore, *in vitro* maturation was associated with apparent aberrations in cumulus expansion (i.e. dark clumping and spherical clusters of cumulus cells). The significance of these apparent aberrations has been documented in a previous study as well (Del Campo *et al.,* 1994) and is unknown, but *in vitro* maturation conditions have been shown to affect the level of maternal mRNA polyadenylation, and to alter the storage of mRNA necessary for the early embryo development (Pocar *et al.,* 2001). In addition, oocytes morphology (cumulus cells and ooplasm) associated with competence to reach the second metaphase and blastocyst stages has been correlated with the expression of several specific transcripts.

Only during the BS, the culture of oocytes with eCG resulted in a higher percentage of expansion and maturation (metaphase-II) stage in comparison to FSH. Superstimulatory treatments (FSH or eCG) were equally efficacious in inducing multiple follicle growth, consistent with a similar comparison made in alpacas (Gomez *et al.,* 2002) and camels (Abdoon *et al.,* 2007). Gonadotropin treatment effectively increased the number of follicles accessible for oocyte collection, as reported in cattle (Looney *et al.,* 1994; Goodhand *et al.,* 1999). The superstimulatory response was relatively consistent among animals in both groups. Of 40 llamas, only five (12%) failed to respond to gonadotropin treatment (i.e. no follicles >5 mm), and of those that responded, all had >3 follicles ≥6 mm. The consistency in response was attributed to the emergent stage of follicular wave development at the time treatment was initiated (Adams, 1994). Based on previous work in llamas (Adams, 1994), follicular wave emergence was expected 2.3±0.3 days after follicular ablation; hence, treatment was initiated 2 days after ablation in the present study.

The number of expanded oocytes observed after FSH or eCG treatment in this study was higher than that reported in superstimulated alpacas (Gomez *et al.,* 2002). Due to large individual variations, differences observed in the mean number of oocytes that reached respective maturational stages were not significant. However a higher proportion of expanded and matured oocytes were collected from llamas after eCG treatment. Over 80% of the expanded oocytes in both treatment groups were in metaphase-II. In general, the low number of degenerated oocytes may be attributed to the timing of collection; i.e. following gonadotropin treatment of a newly emerged follicular wave (Arlotto *et al.,* 1996; Goodhand *et al.,* 1999).

In conclusion, dromedary camels showed better ovarian follicular activity and oocyte status during the breeding season (short daylight) than in the non-breeding season (long daylight). Dromedary camels displayed ovarian activity during both hot-

humid or hot-dry months of the non-breeding season. Therefore, daylight length seemed to play a major role in regulating the seasonal reproductive activity in dromedary camels. Further detailed studies are required to establish the reproductive efficiency of dromedary camels throughout the non-breeding season in both hot-humid and hot-dry months.

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