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Effect of Season and Culture Media on the Competence of Dromedary Camel Oocyte to Mature *In vitro*

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Abstract: The present study was conducted on sixty seven ovaries of camels to investigate the effect of season and type of culture medium on the competence of oocytes to mature *in vitro*. 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 Ø) were aspirated, then oocytes were qualified into 4 grades; Q1 (very good), Q2 (good), Q3 (bad) and Q4 (very bad). The effect of hormonal additives (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 than NBS (early or late months). The left ovary appeared higher (P<0.01) activity than right one regarding the mean number of healthy follicles. The number of Q1 to Q3 oocytes (but not Q4) and their recovery rates were significantly (P<0.01) higher during BS than NBS at early or late months. During the LNBS months, a significant (P<0.01) higher number of Q1 to Q3 oocytes obtained than ENBS months. The left ovary appeared higher (P<0.01) number of Q1 and Q2 oocytes compared to the right one, but not with Q3 to Q4. Only during the BS, the oocytes cultured in medium containing eCG appeared a higher percentage of expansion and maturation (Metaphase-II) rates than culture in medium containing FSH. *In conclusion,* dromedary camels showed better ovarian activity and oocytes status in BS than NBS and displayed ovarian activity during early as well as late non-breeding months. Therefore, further detailed studies are required to establish the reproductive efficiency of dromedary camels throughout the non-breeding season under Egyptian condition.

Key words: Camels · Breeding · Non-breeding · Season · Oocytes · Maturation

INTRODUCTION

One-humped camel (*Camelus dromedarius*) is the only animal that can exist for several weeks without water and remaining to provide human with meat and milk [1]. 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 [2, 3]. In addition, seasonality of reproduction has long been suggested for the Camelidae which based mainly on the seasonal distribution of birth and the status of ovarian activity. Many investigators consider the dromedary camel as a seasonally polyestrous animal with a relatively short breeding season [4]. Outside the breeding season, mating activity ceases and the ovaries are inactive or show a limited number of small follicles [5]. However, daylight and temperature are the two main climatic factors influencing the annual sexual cycles. In addition, the ovarian activity in she-camel was found to be mainly follicular rather than luteal [6]. However, corpora lutea may be occasionally present without pregnancy. Detailed studies on ovarian activity in she-camels related to the different seasons of the year are not fully understood, especially if early or late non-breeding season months or daylight length were taken in consideration. In addition, recently techniques of *in vitro* maturation and subsequent fertilization and embryo-transfer can be employed in camel to overcome some problems in the reproductive efficiency [7]. These techniques can impregnate as many of females as possible at the start of breeding season become pregnant. 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.

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MATERIALS AND METHODS

This study included sixty seven 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 designed to study the effects of breeding (December to May) and non-breeding seasons either early (June to August) or late months (September to November) on the ovarian activity of dromedary camel. During the BS and NBS, the number of healthy and atretic follicles was studied. The status of follicular oocytes in different seasons was also investigated, i.e. quality and recovery rate. Moreover, two trials were carried out to investigate the effect of culture condition (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 minutes after slaughter, then washed by sterile warm saline solution (0.9% NaCl) at 28-32°C containing 100 IU/ml penicillin-G sodium and 100µg/ml dihydro-streptomycin sulphate (Sigma Chemical Co.-P.O. pox 14508 St Louis, Mo- 63178 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 Ø) in either left or right ovary were counted. The follicles were differentiated according to the external morphology and the nature of follicular fluid [8] 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 (2-8 mm Ø) on the left and right ovaries using aspiration process. A 20-gauge needle attached to 10 ml sterile syringe and contained 0.5 ml physiological saline solution (0.9% NaCl) at 28-32°C [8] was used. Before commencing 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% ethyl alcohol and at least 3 times in saline solution containing 100 IU/ml penicillin-G sodium and 100 µg/ml dihydro-streptomycin sulphate. After aspiration, the contents of the syringe were slowly dispelled into sterile petri dishes (30x60 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 stereo-microscope. Recovery rate of oocytes was determined as the percentage of oocytes in proportions to the total follicles by the following formula:

Recovery rate = No. of recovered oocytes / No. of aspirated follicles X 100

Qualification of the collected ooyctes: The oocytes were evaluated in respect to both investment and ooplasma granulation of cumulus cells as follows:

Q1 (Quality 1, very good): The oocytes with complete compact dense cumulus oophorus >6 layers and transparent homogeneous cytoplasm.

Q2 (Quality 2, good): The oocytes with complete compact dense cumulus oophorus >6 layers and transparent homogeneous cytoplasm with small dark zones at periphery.

Q3 (Quality 3, bad): The 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): The oocytes were 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 Egyptian Organization for Biological Products and Vaccines, Dokki, Giza, Egypt and then stored in refrigerator at 5°C until used. Media were supplemented with 10mg/100ml L-glutamine, 100 iu/ml Penicillin G-Sodium and 50 µg/ml Dihydro-streptomycine sulfates. The pH was measured by a pH meter and adjusted to pH 7.4 using NaOH (1.0N) (Sigma, Chemical P.O Box 14508 ST Louis, MO 63178, USA). The medium was sterilized by using 0.2 µm

Millipore biological filters and equilibrated in an atmosphere of 5% CO_2 in air in CO_2 incubator, with maximum humidity 95% at 38.5°C for at least 2 hours prior to use.

Effect of hormonal (FSH or eCG) additives: The effect of hormonal additive 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 pipette 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 CO_2 incubator at 5% CO_2, 95% relative humidity and 38.5°C [9]. The percentage of germinal vesicle, germinal vesicle breakdown, matured and degenerated oocytes were recorded.

Assessment of the cumulus expansion and nuclear maturation: The cumulus expansion was determined after oocytes incubation for 36 hours under steriomicroscope. The criteria to assess the cumulus expansion were done. Expanded cumulus cell mass expanded away from the zona pellucida, while non-expanded cumulus cell mass was tightly adherent to the zona pellucida.

For the judging of nuclear maturation, oocytes were transferred to small plastic tube containing 3% sodium citrate solution followed by repeated agitation for the denudation of oocytes [10]. The contents of the tube were 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°C for 24 hours. The oocytes were stained with aceto-orcein (1% orcein in 40% acetic acid), for 25 minutes, 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 loosed nuclear membrane.

Mature: Metaphase-I [paired or bivalent chromosomes were observed within 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 is highly shrinked and destructed.

Statistical analysis: Analysis of the data was performed [11]. Significant differences between the means were evaluated utilizing Duncan's Multiple Rang Test (DMRT) [12].

RESULTS

Regarding the number of healthy follicles, the results obtained in Table 1 and Fig. 1 showed a significantly ($P<0.01$) higher number during the BS than NBS either the early or late months. Similarly, number of the normal follicles was significantly ($P<0.01$) higher in the late than early months. The highest number of the healthy follicles was recorded in BS and the lowest number was recorded during ENBS. However, the number of the atretic follicles was significantly ($P<0.01$) higher during the early than the late months of NBS, as well as, during the BS. The highest number of the atretic follicles was recorded during ENBS while, the lowest number was recorded during the BS. With regard to side of ovary, number of the normal follicles in the left ovary was significantly ($P<0.01$) higher than the right one, while number of the atretic follicles in the right ovary was significantly ($P<0.01$) higher than the left ovary (Table 1). The total number of follicles per ovary collected was significantly ($P<0.01$) higher during the BS than NBS at the early months. However, the effects of

Table 1: Mean values (M+SE) of normal and atretic follicles of the left and right ovary during BS, ENBS and LNBS in dromedary camel

		Ovarian structures	
Season of year		Healthy follicles	Atretic follices
BS:	-LO	34.26±1.59	6.19±0.49
	-RO	21.56±1.38	7.33±0.47
Mean		27.91±1.40^{A}	6.76±0.42^{C}
ENBS:	-LO	16.67±1.75	7.94±0.54
	-RO	12.06±1.53	8.82±0.52
Mean		14.37±1.64^{C}	8.38±0.53^{A}
LNBS:	-LO	31.06±1.43	7.11±0.44
	-RO	18.13±1.25	8.61±0.42
Mean		24.60±1.38^{B}	7.86±0.43^{B}
Total Mean:	-LO	27.33±5.41^{A}	7.08±0.51^{B}
	-RO	17.25±2.78^{B}	8.25±0.47^{A}

Means bearing different letters within same column and row are significant ($P<0.01$). LO=Left Ovary; RO=Right Ovary; BS=Breeding Season; ENBS=Early Non-breeding Season; LNBS=Late Non-breeding Season

Table 2: Mean values (M+SE) of follicular oocytes recovered from the right and left ovary during BS, ENBS and LNBS in dromedary camels

		Morphology of follicular oocytes				
Season of year		Q1	Q2	Q3	Q4	Oocyte RR
BS:	-LO	23.78±1.64	6.57±0.75	2.33±0.41	1.17±0.36	83.68
	-RO	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}
ENBS:	-LO	9.94±1.81	2.39±0.83	1.11±0.45	0.83±0.32	57.98
	-RO	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}
LNBS:	-LO	17.94±1.48	4.44±0.67	1.22±0.37	1.17±0.39	64.89
	-RO	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}
Total	-LO	17.22±4.01^{A}	4.47±1.21^{A}	1.55±0.39^{B}	1.06±0.11^{B}	70.62^{A}
Mean:	-RO	9.16±1.43^{B}	2.09±0.72^{B}	2.20±0.37^{A}	1.82±0.04^{A}	59.88^{B}

Means bearing different letters within the same column and row, differ significantly (P<0.01), Q=Quality; LO=Left Ovary; RO=Right Ovary; BS=Breeding Season; ENBS=Early Non-breeding Season; LNBS=Late Non-breeding Season; 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 BS and NBS of the year

	Hormonal effect				
	FSH		eCG		
Morphology of oocytes	Breeding	Non-breeding season	Breeding	Non-breeding season	X^2
No. of oocytes	20 (00.0)	40 (00.0)	20 (0.0)	40 (0.0)	
Expanded	16 (80.0)	30 (75.0)	20 (100.0)	30 (75.0)	ns (P<6.25)
GV	1 (5.0)	6 (15.0)	0 (0.0)	2 (5.0)	ns (P<5.40)
GVBD	2 (10.0)	4 (10.0)	1 (5.0)	6 (15.0)	ns (P<1.46)
Matured (M-II)	15 (75.0)	20 (50.0)	18 (90.0)	27 (67.5)	* (P<10.53)
Degenerated	2 (10.0)	10 (25.0)	1 (5.0)	5 (12.5)	ns (P<5.29)

ns=non significant, *=significant, GV=Germinal Vesicle, GVBD=Germinal Vesicle Breakdown, M-II=Metaphase-II

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 the right one (Table 1).

The Q1 and Q2 oocytes were significantly (P<0.01) higher during the BS than the NBS either the early or late month (Table 2 & Fig. 1). Similarly, they were significantly (P<0.01) higher in the late than the early months of NBS. The highest numbers of Q1 and Q2 oocytes were recorded during the BS and the lowest numbers were recorded during ENBS. The Q3 oocytes were significantly (P<0.01) higher during breeding than non-breeding season, as well as, the effects of early and late months on Q4 were similar. However, the rate of Q4 oocytes in relation to the season was insignificant. The recovery rate of oocytes was significantly (P<0.01) increased in BS as compared to the NBS either the early or late months. The highest recovery rate was recorded the BS and the lowest was recorded in the months of ENBS. Regarding side of ovary, the means of Q1 and Q2 oocytes and recovery rate were significantly (P<0.01) higher in the left than right ovary, while the Q3 and Q4 were significantly (P < 0.01) higher in the right than the left ovary (Table 2).

It is interested to notice that, superiority of the total number of follicles, and oocytes recovery in both left and right ovaries was recorded during the BS as compared to NBS either the early or late months. These probably may be due to that the gonadotropic hormonal balance was in favor of the follicular growth stimulation oocyte status in active season but is not in favor of ovulation process.

The effect of hormonal additives in culture media on the ability of oocytes to mature *in vitro* was illustrated in Table 3 & Fig. 2. During the BS, eCG resulted in a

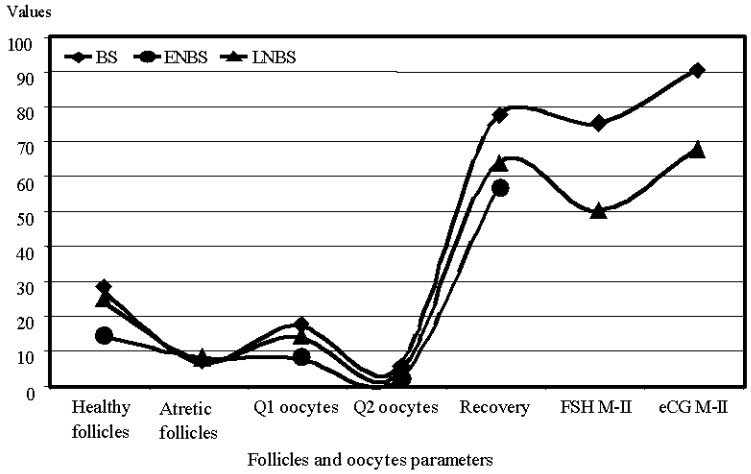

Fig. 1: A diagram showing the mean value of healthy and atretic follicles, Q1 and Q2 oocytes, recovery rate of oocytes and the percentage of Metaphase-II oocytes matured in vitro in culture media supplemented with either FSH or eCG hormones

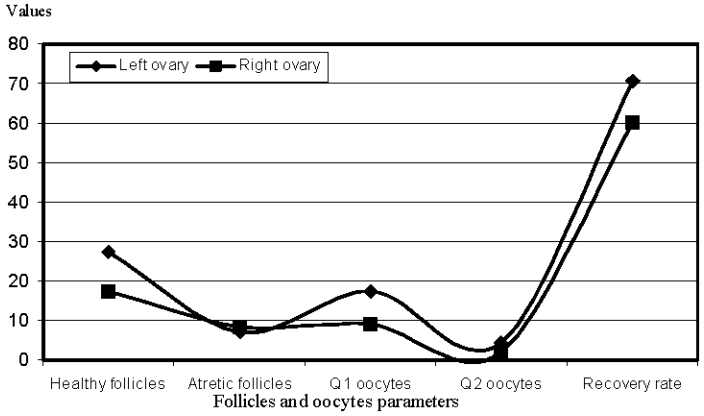

Fig. 2: A diagram showing the mean value of healthy and atretic follicles, Q1 and Q2 oocytes and recovery rate of oocytes from the left and right ovaries

significant 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 numbers of healthy follicles were significantly higher in BS than NBS (early or late months). The LNBS appeared to be more active when compared to the ENBS. These findings are in agreement with several authors in dromedary she-camels [13]. The highest number of the normal follicles of the dromedary camel was recorded in the BS and the lowest number was recorded in the ENBS. However, the number of the atretic follicles was significantly higher during the ENBS than the LNBS months and BS. Similar trend was previously reported by [13, 14]. The last authors showed that ovary side had highly significant effect on the number of normal follicles and insignificant effect on number of atretic follicles. However, there were no differences in the total number of follicles collected from the right or left ovaries [14, 15]. However, the ovary weight was insignificantly higher in the left ovary than in the right one during spring, summer and autumn seasons, while in winter season an opposite trend was observed [13]. In general, the results obtained in the present study, showed that pregnancy corpus luteum does 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 [16]. 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.

Generally, the mature follicles of she-camels normally 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 thecal luteinic cells [17]. Also, there was 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. Under this aspect, the differences of bad quality oocytes decreased between BS and NBS, controversy to oocytes with good quality. At the same time, the ENBS months appeared significantly higher means of than Q1 to Q3 (but not Q4) than LNS months. The left ovary appeared higher Q1 and Q2 oocytes compared to the right one, but not with Q3 to Q4. It has been recorded that the highest numbers of oocytes complexes and partial denuded oocytes obtained during autumn and winter and the lowest numbers during summer and spring season [13]. While, the highest expanded and denuded were recorded during spring and winter and the lowest during summer and autumn seasons. 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 interested to notice that, superiority of the total number of follicles and oocytes recovery in both left and right ovaries of the dromedary she-camels was recorded during the BS as compared to the NBS either the hot-humid or hot-dry months. These probably may be due to that the gonadotropic hormonal balance was in favor of the follicular growth stimulation oocyte status in the breeding season but is not in favor 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 [18, 19]. 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 a 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 [20] and may be attributed to the time interval between ovarian collection and oocytes aspiration (2-3 hr), or to the method of oocytes collection (follicular aspiration). It appears that *in vivo and in vitro* maturation time is 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 MII after 36 h of *in vitro* culture [21, 22], which is consistent with the reported time interval between mating and ovulation in camels (36 h). The importance of determining optimal *in vitro* oocyte maturation time is illustrated by the results of studies in cattle that show that oocyte aging may be the cause of reduced fertility if *in vivo* insemination is delayed [14]. 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 [20] 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 [20] and it is unknown, but *in vitro* maturation conditions have been shown to affect the level of maternal mRNA polyadenylation and alter the storage of mRNA necessary for the early embryo development [23]. In addition, oocytes morphology (cumulus cells and ooplasm) associated with competence to reach the second metaphase and blastocyst stage 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 [9]. Gonadotropin treatment effectively increased the number of follicles accessible for oocyte collection, as reported in cattle [24,25]. 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 >5mm) 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 [26]. Based on previous work in llamas [27], follicular wave emergence was expected 2.3±0.3 d after follicular ablation; hence, treatment was initiated 2 d 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 super stimulated alpacas [9]. Due to large individual variation, differences observed in the mean number of oocytes that reached respective maturational stages did not reach significance. However a higher proportion of expanded and matured oocytes were collected from llamas after eCG treatment. Regardless, 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 [18,25,28].

In conclusion, dromedary camels showed better ovarian follicular activity and oocyte status during breeding season (short daylight) than non-breeding season (long daylight). Dromedary camels display ovarian activity during non-breeding season either early or late months. Therefore, daylight length seemed to play major role in regulating the seasonal reproductive activity in dromedary camels. Further detailed studies are required to establish reproductive efficiency of dromedary camels throughout non-breeding season in both early and late months. Moreover, the addition of eCG plays an important role in maturation of dromedary camel oocytes *in vitro*.

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