The Influence of Oocyte Diameter and Culture Media Type on *in vitro* Nuclear Maturation in Camel

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ABSTRACT

This study aimed to determine the influence of oocyte diameter and culture medium (tissue culture medium (TCM-199) and minimal essential medium (MEM)) on *in vitro* nuclear maturation in camel (*Camelus dromedarius*). The recovered oocytes (523 oocytes) were classified into three diameter categories: including ≤120, 120-150, and ≥150 µm. The results revealed that Oocytes matured in TCM-199 had a significantly ($p<0.05$) higher percentage of oocytes reaching the metaphase II (MII) (46.34%) than oocytes matured in MEM medium (38.63%). Percentage of oocytes arrested at MII was significantly ($p<0.05$) higher for oocytes with diameter of ≥150µm (53.25%), moderate for 120-150µm (43.36%) and lowest for ≤120µm (24.16%) oocytes, regardless of the type of maturation medium. The interaction between culture medium and oocyte diameter was insignificant, except for interaction between TCM-199 medium and ≥ 150 µm of oocyte diameter, reflecting a higher percentage of oocyte arrested at MII (58.88%). In conclusion, oocytes with a diameter of ≥ 150 µm matured in TCM-199 medium have the potential to improve the *in vitro* maturation of camel oocytes.

Keywords*:* camel; oocyte diameter; culture medium type; nuclear maturation.

INTRODUCTION

Camels are one of the domestic animals that have a great opportunity to adapt to global warming and climate change. So, there is great attention to this animal as a source of meat, milk, skin, racing, and carrying. Female dromedary camels have many barriers to reproductive efficiency in natural conditions (induced ovulatory, seasonality, long calving period, shorter breeding seasons) compared with other domestic animal species (Bello and Bodinga, 2020), long lactation period that reflects on inhibition of ovarian functions (Nagy and Juhász, 2019). As a result, attention has been paid to using assisted reproductive technologies to improve the fertility rate in this species (Tukur et al., 2020).

In addition, the *in vitro* maturation (IVM) of camel oocytes is the main step for improving embryo production *in vitro* (Mesbah et al., 2016). IVM is a common technique to enhance the maturation of oocytes in different farm animal species. The efficiency of IVM is affected by follicle size, oocyte retrieval method (Khatir & Anouassi, 2006), the follicular fluid's progesterone concentration (Hazeleger et al., 1995), and stage of atresia (De Wit and Kruip, 2001). Other factors controlling the quality of oocyte maturation include the shape of the cumulus oophorus cells, oocyte competency (Boni et al., 2002), morphology of the corona radiata cells (Laurincik et al., 1996) and oocyte diameter (Raghu et al., 2002, and Schoevers et al., 2007).

Diameter of oocyte can be used as an indicator of oocyte growth, due to the intensive RNA synthesis during this phase that causes an increase in size (Crozet et al., 1981 and Lazzari et al., 1994). Studies on cattle have shown a relationship between oocyte size and its capacity to continue and finish meiotic division during IVM (Otoi et al., 1997) and buffaloes (Raghu et al., 2002). In cattle ovaries, oocytes collected from peripheral follicles, without paying attention to their size, showed equal meiotic capacity, in contrast, those collected from cortical follicles reflect a size-linked capability (Arlotto et al., 1996). Previous studies have shown that smaller oocytes are more likely to have abnormal meiotic maturation, which can disrupt maturation (Lechniak et al., 2002). In pigs, the degree of oocyte nuclear maturation is related to the diameter of both the oocyte and follicle (Lucas et al., 2002). In dromedary camels, previous studies reported that the component of Follicular fluid varied according to follicular size and breeding season (Ali et al., 2008; Rahman et al., 2008; Ali et al., 2011). The developmental competence of follicular oocytes *in vitro* may be affected by the follicular sizes in goat (Majeed et al., 2012) and camel (El Shaahat et al., 2013). Also, Wani et

al., (2013) reported that a factor inherent in oocytes harvested from smaller follicles limited their further development, so follicle size is considered a crucial factor that affects oocyte developmental competence. Thus, both ingredients of the incubation media and culture conditions can influence the meiotic steps of mammalian oocytes (Kito and Bavister 1997).

One of the main factors influencing the rate of oocyte maturation and quality of development is culture media. (Pereira et al. 2019). Therefore, a culture medium, which is a foreign environment for the *in vitro* produced embryo, must be selected to minimize stress for the cultured embryo (Wani, 2021). Different media have been used for *in vitro* maturation of camelid oocytes with varying rates of oocyte nuclear maturation. Tissue culture medium 199 (TCM-199) is widely spread among all IVM labs. (Zhao et al. 2009).

In camel, maturation rate of oocytes was generally improved in Tissue Culture Medium-199 (TCM) compared to other media. For instance, Nowshari (2005) reported that TCM could improves the rate of mature nuclei of camel oocytes, compared with modified Connaught Medical Research Laboratories medium-1066 (CMRL) or CR1aa (CR1) medium. In this connection, Smetanina et al., (2000) observed that TCM-199 triggers the nuclear maturation of the bovine oocytes than Ham's F-10 and DMEM.

Limited investigations are available to compare between TCM and MEM media and their effect on camel oocytes IVM. According to the noticeable variability in camel's oocyte dimension collected from peripheral follicles (2- 8 mm of diameter), the present investigation aims to examine the influence of two maturation media, and oocytes diameter on the maturation of camel oocyte *in vitro*.

MATERIALS AND METHODS

This study was carried out at the International Livestock Management Training Center, Sakha, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt. All chemicals used in this study were purchased from Sigma (Madrid, Spain), unless otherwise indicated.

Oocyte Collection

Ovaries were collected from slaughtered of Sudanese she-camel with unknown reproductive history in an abattoir in Cairo, during breeding season. Immediately after slaughtering, ovaries were placed into thermos containing normal saline, 100 IU/mL penicillin and 100 μg/mL streptomycin sulphate maintained at 25-30°C until oocyte recovery. The collected ovaries were washed twice in distilled water and once in freshly prepared saline.

Oocytes were collected (523 oocytes) by slicing techniques from visible follicles (2-8 mm) on the ovarian surface. Ovaries were placed into a glass Petri dish containing 5 mL of Dulbecco's phosphate buffer solution (DPBS). Each ovary was held with forceps, and incisions were made along the entire ovarian surface using a scalpel blade. Oocytes were examined under stereomicroscope. Oocytes enclosed in a compact cumulus with evenly granulated cytoplasm (cumulus-oocyte complex, COCs) were selected and washed trice in Phosphate Buffer Saline (PBS).

In vitro Maturation

Two media were used for the maturation of camel's oocytes TCM-199 and Minimum Essential Medium (MEM), (purchased from the Egyptian Organization for Biological Product and vaccine, Agoza, Egypt). Both media were supplemented with 10% fetal dromedary camel serum (FDCS), 20 IU/mL PMSG (Gonaser, Laboratory Hipra, S.A.17170 Amer, Spain), 10 IU/mL hCG (Epifasi, Egyptian Int. Pharmaceutical Industries Co, Egypt), 1 μg/mL estradiol-17β, 20 mmol final concentration of pyruvate, 100 IU penicillin and 100 μg streptomycin /mL.

About 200 μL from the prepared maturation medium was placed into a sterile Petri dish (35mm) and covered by sterile mineral oil. Before placing oocytes in a culture dish, Petri dishes were incubated in Co_2 incubator (5% Co_2) at 38.5°C and high humidity (95%) for at least 1 h for equilibration. Both media were adjusted to pH of 7.2-7.4 and osmolarity of 280-300 mOsmol/kg and filtered by 0.22 μm-Millipore filter.

Oocytes Maturation

Oocytes were washed three times in PBS plus 2% Bovine Serum Albumin (BSA) and two times in maturation medium. Thereafter, about 10-20 oocytes per droplet (100 μL) were allocated by pasture pipette and cultured. Petri dish was incubated for about 42h in Co_2 incubator (5% Co_2) at 38.5°C and high humidity for 42h.

Fixation, Staining and Examination of Oocytes

After maturation period, oocytes were washed using PBS containing 1 mg/mL hyaluronidase to remove the cumulus cells. Oocytes diameter was measured using eye piece micrometer. The oocytes were classified into three size groups including small <120, medium 120–150, and large >150 μm in diameter. Each experiment consisted of at least five replicates. Then, oocytes were washed twice in PBS supplemented with 3% BSA and loaded on clean slide. Slides were placed into fixation solution (3 ethanol: 1 glacial acetic acid) overnight. Thereafter, oocytes were stained with 1 % orcein in 45% acetic acid and examined for maturation under phase-contrast microscopy as the following: Matured oocytes: Chromosome at metaphase II (MII) and polar body exhausted in perivitelline space, oocytes with germinal vesicle (GV): Chromosomal in disk in cytoplasmic with intact membrane of nuclei, oocytes with germinal vesicle breakdown (GVBD): Chromosomal in disk in cytoplasmic but intact membrane of nuclei is breakdown, oocytes at metaphase 1 (M1) and metaphase (MII) and degenerated oocytes: Oocytes were vacuolated or cytoplasmic shrunken or chromatin condensed (Shamiah, 2004).

Statistical analysis

Statistical analysis for the obtained data was analyzed using General Linear Model of SAS (2001). The differences among the treatments mean were performed using Duncan New Multiple Range Test (Duncan, 1955). The percentage values were adjusted to arcsine transformed before performing the analysis of variance. We used the following statistical model:

$Y_{ijk} = \mu + \alpha_i + + (\alpha\beta) + \varepsilon_{ijk}$.

Where:

Y_{ijk} = Observed values

μ = Overall mean

αi = Medium type

β_j = Oocytes diameter

$\alpha\beta_{ij}$ = Interaction Medium type × Oocytes diameter

ε_{ijk} = Random error

RESULTS AND DISSECTION

Data of the effect of medium type on *in vitro* maturation of camel oocytes are depicted in Table 1. The maturation rate in terms of the percentage of oocytes arrested at MII stage was significantly ($p<0.05$) higher in TCM-199 (46.34%) than in the MEM medium (38.63%). A similar trend was observed for the percentage of degenerated oocytes, but the differences were not significant ($p<0.05$). On the contrary, percentages of oocytes at germinal vesicle (GV), germinal vesicle breakdown (GVB) stage and mediated stages (MS) were slightly higher in the MEM treatment ($p<0.05$).

These results are in line with the results reported by Nowshari (2005) who found that maturation rates of camel oocytes were significantly ($P<0.05$) highest with TCM (61%) than CMRL (50 %) or CR1 (47%) medium. Likewise, Mahmoud et al. (2003) noted that the maturation rate of dromedary she-camel was highest with TCM-199 supplemented with fetal calf serum compared to that with pregnant camel serum (45.34 vs. 40.10%). According to the present results, culture conditions of mammalian oocytes can influence the meiotic phases (Kito and Bavister, 1997). Therefore, the reported differences in TCM-199 and MEM effects on oocyte maturation in the present study may be due to the components of each medium.

Data of the effect of oocyte diameter on *in vitro* maturation of camel oocytes are depicted in Table 2. Regardless of the type of the maturation medium, oocyte diameter significantly ($p<0.05$) affected oocytes IVM in camel. Oocytes with ≥150μm in diameter had the highest percentages of maturation rate in terms of oocyte arrested at MII (maturation rate) (53.25%, $P<0.05$) followed by a diameter of 120-150 μm (43.36%), and diameter of ≤120 μm (24.16%). However, oocytes at other maturation stages showed an opposite trend as affected by oocyte diameter.

Our results indicated a strong relationship between oocyte diameter and the resumption of meiotic division of camel oocytes. The highest maturation rate was recorded for oocytes with diameter ≥150 μm, while the lowest rate was for those with ≤120 in diameter. Several studies concluded that oocyte diameter is directly proportional to follicle diameter and as both increase the developmental capability of the oocytes improves in goat (Anguita et al., 2007), and cows (Gandolfi et al., 2005). In the present study, we confirm this fact. This finding agrees with that of Armstrong (2001) who found that meiotic capability is highly affected by oocyte diameter, which in turn is associated with ovarian follicle size. In this respect, the

antrum size of the ovarian follicle at which the oocyte obtains meiotic competence is species-specific (Wickramasinghe, and Albertini, 1993). According to Abdoon, et al., (2007), oocyte diameter of dromedary she-camel is approximately 166.2±2.6 μm. In other species such as buffalo, resumption of meiosis evidenced by GVBD is increased as oocyte diameter increased and about 70% of ≥100 μm oocytes reached MII (Danilda H. Duran, 2008). In cattle, oocytes obtain the capability to complete GVBD stage and meiosis just when the diameter of the follicular antrum reaches 2-3 mm (Lonergan, et al., 1994). Also, Otoi et al. (1997) mentioned that meiotic capacity is affected by oocyte diameter because bovine oocytes need to be 110 μm in diameter to reach the MII phase of nuclear maturation. Moreover, Fair et al. (1996) found that the *in vitro* developmental capacity of cattle oocytes to achieve MII stage was directly correlated with their diameter. For the oocyte to complete meiotic division, it is required to reach a minimum diameter of 110 μm. Moreover, Khatir and Anouassi, (2006) found that the oocytes recovered from small follicles (3-5 mm in diameter in camelids) may have a reduced ability to develop after IVM, *in vitro* fertilization (IVF), *in vitro* culture (IVC). In this study the maximum ($P<0.05$) value of degenerated camel oocytes was noted with the IVM of ≤120 μm oocytes. These results agree with that of Danilda, (2008) who reported that buffalo oocytes with a diameter of less than 100 μm had a greater degeneration (28.9%). Also, Alsaadoon et al (2021) indicated a higher percentage of *in vitro* maturation of sheep oocytes was recorded with large follicles 38.0±1.71 % than medium and small follicles (29.57±2.06 %,18.5± 0.27 % respectively). After IVM, oocytes in this category neither reached the MII phase nor cleave after IVF due to high degeneration incidence in small (<100 μm) oocyte before IVM that could be the main reason for the decreased efficiency rate of IVM. Also in cattle, the high rate of oocyte degeneration was noticed in <100 μm oocytes (Crozet et al., 1986; and Fair et al., 1996) that shows a stable storage of large molecules necessary for the continuation of both meiosis and early growth of the zygote (Sirard et al., 1992; De Smedt et al., 1994). This might be the reason for the inability of oocytes with a diameter of less than 100 μm to achieve the MII phase after IVM.

Follicular size has been shown to affect estrogen contents in many species. In cattle, estrogen concentrations increased as the size of the follicle also increased (Henderson et al., 1982). El-Shahat et al., (2013) showed a positive correlation between estradiol 17-β concentrations in the follicular fluid and follicle size of dromedary camels. Also, in goats, Al-Rubaei and Radhi (2023) showed that the concentrations of estradiol 17β, calcium, and sodium increased ($p<0.01$), while FSH and potassium decreased with an increase in the follicular size. Estradiol 17-β effect is important in initiating LH receptor expression and responsiveness (Segaloff et al., 1990), antrum formation (Wang and Greenwald, 1993), and prevention of atresia (Billing et al., 1993).

In comparison with camel, oocytes reach complete meiotic competence at a diameter of 115 μm and the ability to fully developmental at a diameter of 120 μm in Nili Ravi buffaloes (Yousaf and Chohan, 2003), in cattle (Otoi et al., 1997), and 125 μm (Anguita et al., 2007) or 135 μm (Martino et al., 1994) in goats. In Indian buffaloes, oocytes at a diameter of 145 μm collected from follicles at ≥8 mm have full meiotic competence (Raghu et al. 2002). The previous findings on other species and those reported in our study on camel indicated that these variations might be caused by the breed type and the methodology of oocyte diameter measurement.

The influence of interaction between culture medium and oocyte diameter (Table 3) in all phases of the division was not significant, except interaction between medium type TCM-199 and diameter of oocytes ≥ 150 μm was higher reflecting higher percentage of oocyte arrested at MII for ≥150 oocytes matured in TCM-199 (58.88%) and lower percentage of oocytes at GVB stage (7.48%).

CONCLUSION

Oocyte diameter and consequently follicular diameter as well as the type of maturation medium must be put into consideration to obtain the highest successful maturation rates during *in vitro* camel oocyte maturation. According to the experimental conditions in this study, the best results for *in vitro* maturation of camel oocytes were obtained for oocytes with diameter ≥150 μm in TCM-199. As a result, it's important to work out and optimize culture systems that consider all the main items and components to improve the maturation of she-camel oocytes *in vitro*.

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Table 1: Effect of medium type on *in vitro* maturation of camel oocytes

Medium	N	GV		GVB		MS		MII		DG	
		n	%	n	%	n	%	n	%	n	%
TCM-199	246	24	9.76	28	11.38	47	19.11	114	46.34[a]	33	13.41
MEM	277	34	12.27	39	14.08	65	23.47	107	38.63[b]	32	11.55

a and b: Means denoted within the same column with different superscripts are significantly different at p<0.05.

Table 2: Effect of oocyte diameter on *in vitro* maturation of camel oocytes

Diameter (μm)	N	GV		GVB		MS		MII		DG	
		n	%	n	%	n	%	n	%	n	%
≤120	149	25	16.78[a]	23	15.44[a]	38	25.50[a]	36	24.16[c]	27	18.12[a]
120-150	231	15	10.49[b]	19	13.29[ab]	29	20.28[b]	62	43.36[b]	18	12.59[b]
≥150	143	18	7.79[b]	25	10.82[b]	45	19.48[b]	123	53.25[a]	20	8.66[b]

a and b: Means denoted within the same column with different superscripts are significantly different at P<0.05.
N: Total number of oocytes, GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, MS: Mediated stage, MII: Metaphase II, DG: Degenerated oocytes.

Table 3: Interaction between medium types on *in vitro* maturation of camel oocytes with different diameter

Medium	Diameter (μm)	N	GV		GVB		MS		MII		DG	
			n	%	n	%	n	%	n	%	n	%
TCM-199	≤120	66	10	15.15	11	16.67	16	24.24	17	25.76	12	18.18
	120-150	73	7	9.59	9	12.33	13	17.81	34	46.58	10	13.70
	≥150	107	7	6.54	8	7.48	18	16.82	63	58.88	11	10.28
MEM	≤120	83	15	18.07	12	14.46	22	26.51	19	22.89	15	18.07
	120-150	70	8	11.43	10	14.29	16	22.86	28	40.00	8	11.43
	≥150	124	11	8.87	17	13.71	27	21.77	60	48.39	9	7.26

N: Total number of oocytes, GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, MS: Mediated stage, M II: Metaphase II, DG: Degenerated oocytes.

تأثير القطر ونوع البيئة على الإنضاج النووي لبويضات النوق معمليا

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الملخص العربى

تهدف هذه الدراسة لمعرفه تأثير كلا من البيئة وقطرِ البويضات على الانضاج النووي لبويضات الإبل معمليا. جُمِعتْ البويضات المحاطة بالخلايا الركامية المندمجة بطريقة التشريحِ مِنْ الحويصلات المبيضية المرئية على سطحِ المبيض ذات القطرِ من 2- 8 ملليمترِ. أُستخدم في هذه الدراسة نوعين من البيئات (بيئة زراعة الأنسجة TCM-199 وبيئة الـ MEM). قسمت البويضات المتحصل عليها من حيث القطر إلى ثلاثة رتب: ≤120 , 120-150 و ≥150 ميكروميتر وتم زراعتها في حضان على درجة حرارة 38°م و5% ثاني أكسيد الكربون ودرجة رطوبة عالية لمدة 42 ساعة. تم تثبيت البويضات بعد الإنضاج وصبغها وفحصها تحت الميكروسكوب ثلاثي الأبعاد. أظهرت النتائج أن نسبة البويضات التي وصلت إلى مرحلة الإنضاج (الطور الاستوائي الثاني MII) في بيئة زراعة الأنسجة TCM-199 أعلي معنوياً (P< 0.05) من تلك التي في بيئة الـ MEM (46.34 مقابل 38.63 %). كانت نسبة البويضات التي وصلت إلى مرحلة الطور الاستوائي الثاني (MII) في بيئة زرع الأنسجة أعلي معنوياً (P<0.05) (53.25%) لذات القطر ≥ 150 ميكرو متر ومتوسطة لتلك التي قطرها 120-150 ميكرو متر (43.46%) وأقل للتي قطرها أقل من 120 ميكرو متر (24.16%). لم يكن هناك تأثير معنوي للتفاعل بين نوع البيئة وقطر البويضة على معدل الإنضاج؛ وأن أعلي معدل إنضاج للبويضات التي وصلت الي مرحلة الطور الاستوائي الثاني MII كانت للبويضات التي قطرها ≥150 ميكرومتر والمنضجة ببيئة زرع الأنسجة TCM-199 (58.88%). الخلاصة أن أفضل النتائج للإنضاج المعملي لبويضات الإبل تم الحصول عليها من تلك التي قطرها ≥150 ميكرومتر في بيئة زرع الأنسجة TCM-199.

الكلمات الاسترشادية: الابل, قطر البويضة, نوعية بيئة الاستزراع, الانضاج النووى.