Effect Of Media Types And Different Additives On The Nuclear Maturation Of She-Camel (*Camelus Dromedarius*) Oocytes *In Vitro*

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ABSTRACT

The present study was conducted to investigate the effect of media type and different media additives on the competence of dromedary camel oocytes to mature *in vitro*. A total number of eighty two of healthy She-camels and aged 8-10 years were used in the present study. Two experiments were performed in this work, the first experiment was conducted to assess the ability of oocytes (n=533) to mature *in vitro* in different media and to suggest a suitable practical method for assessment of oocytes viability (cumulus expansion and extrusion of 1st polar body). Oocytes were aspirated from ovaries collected from a local slaughterhouse. Only oocytes with more than two layers of cumulus cells and homogenous ooplasm were cultured into 50µl droplets of four different culture systems: Tissue culture medium-199 (TCM-199); Ham's F-12; TCM-199+20% follicular fluid (FF); and Ham's F-12+20% FF in 35 mm Petri dish. The droplets were covered with warm (39°C) mineral oil and incubated in a CO_2 incubator (39°C, 5% CO_2 in air, 90–95% relative humidity) for 36h. The maturation rate was assessed by evaluation of degree of cumulus cells expansion and identifying first polar body extrusion into the perivitelline space, as well as by reaching the oocyte to metaphase-II (M-II) stage of cell division. The second experiment was designed to identify the possible effects of different additives [3mg/ml bovine serum albumin (BSA), 20% male serum (MS), oviductal secretion and cells (OSC) and 0.5mg/ml Vitamin E (VE)] addition to the TCM-199 medium on cumulus expansion and nuclear maturation of camel's oocytes (n=744). Results showed that in the first experiment the TCM-199 medium is superior to Ham's F-12 medium for the maturation of dromedary oocytes, and the addition of FF did not improve the maturation percentage. The second experiment proved that the addition of BSA, OSC and VE had a beneficial effect on oocyte maturation *in vitro*. In conclusion, TCM-199 and BSA or OSC as medium additives could improve the *in vitro* environment for maturation of dromedary oocytes.

INTRODUCTION

Low reproductive performance of dromedary camel has become the major impediment in multiplication and genetic improvement of this species. The dromedaries attain puberty at a late age; have a short breeding season and along gestation period of 13 months. Estrous behavior is very vague, difficult to interpret it does not often relate to follicular development in the ovaries *(1)*. There is relatively limited information available on follicular growth, oocyte physiology and oocyte maturation *in vivo* and *in vitro* studies in she-camel *(2,3,4)*. Artificial insemination, embryo transfer and development of other assisted reproductive techniques have been slow in these species. The *in vitro* embryo production system (IVP), which has been successfully applied in a number of animal species *(5)*, can be used for the genetic improvement of this species. It can also contribute to improving basic understanding of oocyte biology, fertilization and early embryonic development *(5)*. A prerequisite, however, to the development of this technology is the availability of functionally competent oocytes. Ovaries from slaughterhouse being the cheapest and most abundant source of oocytes are used for large-scale production of mature oocytes in most of the animal species. As such, extensive studies on *in vitro* oocyte maturation of many domestic species have lead to improved culture conditions so that a large percentage of oocytes successfully complete nuclear

maturation *(6)*. There are few reports on *in vitro* maturation *(3,4)* and fertilization in the dromedary camel *(7,8)* but detailed and well-defined studies on the kinetics of oocyte maturation are lacking.

There has neither been any attempt to find out a suitable method for harvesting the oocytes efficiently nor have any studies been carried out to compare different media, to find out a suitable basic medium for maturation of oocytes in this species. Therefore, this experiment was planned to compare different media for *in vitro* maturation of the dromedary oocytes and identify the possible effects of different additives [bovine serum albumin (BSA), male serum (MS), oviductal secretion and cells (OSC) and Vitamin E (VE)] to the TCM-199 medium on the *in vitro* cumulus expansion and nuclear maturation of dromedary camel's oocytes.

MATERIAL AND METHODS

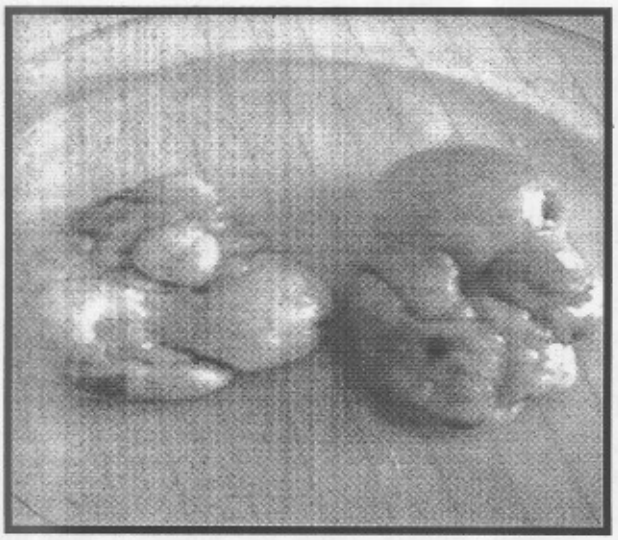
Animals and chemicals

A total number of eighty two healthy She-camels with unknown reproductive history were used in the present study. Animals were obtained from an abattoir at Belbies city, located at a distance of 20 km from Zagazig city. Two types of maturation media were used for oocytes washing and maturation (TCM-199 and Ham's F-12). Media were obtained in a liquid form (from Egyptian Organization for Biological Product and Vaccine, Agoza) and stored in the refrigerator at 5°C till usage. All chemicals were from Sigma (Steinheim, Germany).

First experiment

1. Ovarian collection

Ovaries were removed from slaughtered animals within 15 minute after slaughter (Plate 1, 2), placed into warm (37°C) physiological saline (9 g NaCl in 1 L Mili-Q H_2O) and were transported to the laboratory. The temperature of the saline containing the ovaries ranged between 30 to 32 °C on arrival to the laboratory. Ovaries were washed twice with physiological saline and oocytes were collected within 30-45 minute after ovarian collection.



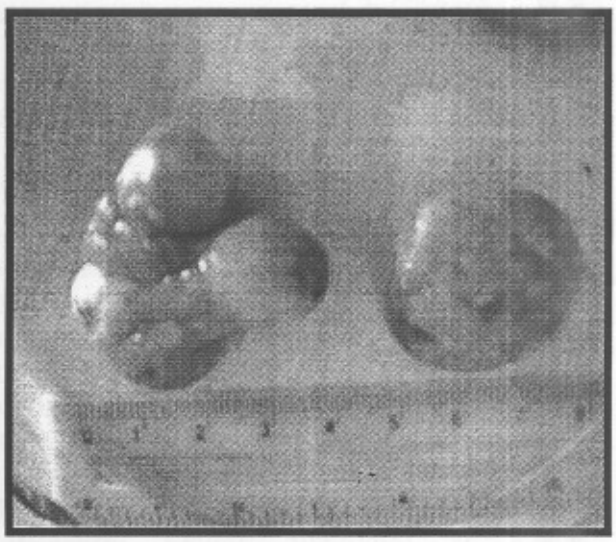


Plate 1 and 2: The dromedary camel ovaries collected from slaughtered animals, the developing follicles and corpora lutea project from the main contour of the ovary and give it a lobular shape.

2. Oocytes collection and *in vitro* maturation

Follicular contents were aspirated from follicles (3-10 mm) with an 18-gauge needle attached to a disposable syringe. During aspiration, a rotatory movement within the follicle was used to dislodge the cumulus oocyte complex (COC), at times, adhered to the follicular wall. A total of 533 oocytes were selected under stereomicroscope for use in the first experiment.The COC's were washed three times in Hepes buffered washing medium (TCM-199 or Ham's F-12 medium +0.25 mM sodium pyruvate + penicillin 100 IU/ mL and streptomycin 50 µg/ ml).

Oocytes with homogenous and evenly granulated cytoplasm and 3-4 or more layers of cumulus cells (Plate 3) were placed into drops (50 µl) of maturation medium under mineral oil, and cultured in 35 mm Petri dishes at 39°C under an atmosphere of 5% CO_2 in air, 95% humidity for 36 h. The oocytes were matured in Hepes buffered TCM-199 or Ham's F-12 media with the addition of 0.8 mg/mL sodium bicarbonate, 100 IU/ mL penicillin and 50 µg/mL streptomycin (control) or with addition of 20% follicular fluid (FF).

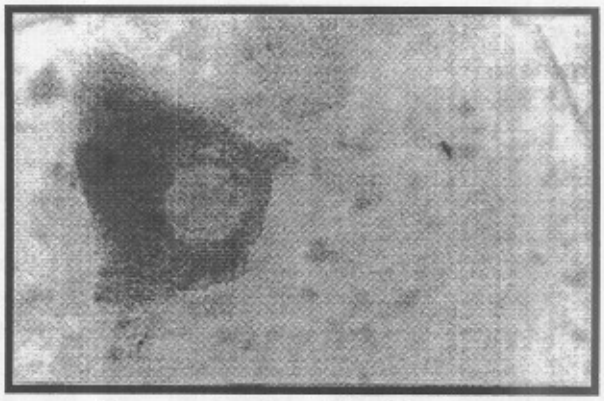


Plate 3. Compact cumulus cells of the dromedary she-camel oocyte showing many tight layers of cumulus cell.

3. Assessment of oocytes maturation

The judgment of oocytes maturation was based on cumulus expansion, extrusion of the 1st polar body and nuclear maturation (reaching the metaphase-II stage of cell division).

3.1. Cumulus expansion and extrusion of the 1st polar body

The cumulus expansion was determined after oocytes incubation under Stereomicroscope. The criteria of assessing the cumulus expansion were done *(9)* as follow, expanded cumulus cell mass was expanded away form the zona pellucida, while the non-expanded cumulus cell mass was tightly adherent to the zona pellucida (Plate 4A,B). The extrusion of the 1st polar body was assessed by rolling of the oocytes within the medium under stereomicroscope by fine Pasteur pipette.

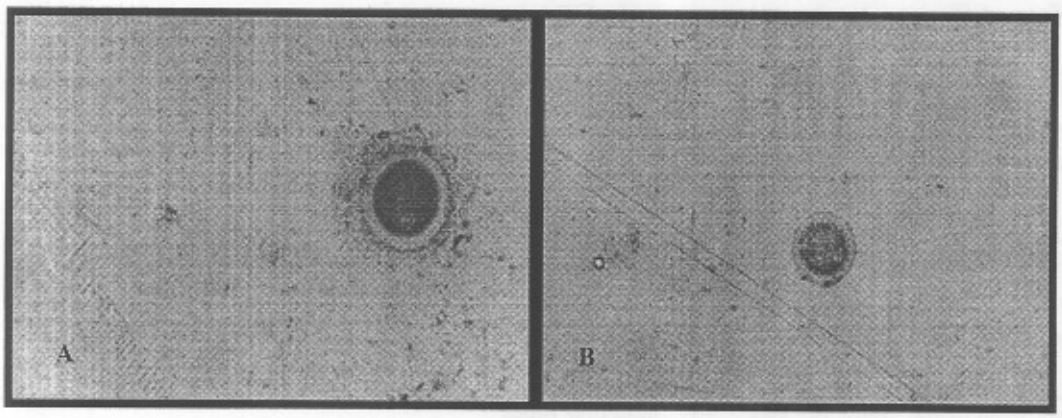


Plate 4. Cumulus expansion of the dromedary she-camel oocyte showing cumulus cells expanded away form the zona pellucida (A) or cumulus cells entirely lost (B).

3.2. Nuclear maturation

For the judging of nuclear maturation cumulus oocytes complexes (COCs) were transferred to small plastic tube containing 3% sodium citrate solution followed by repeated agitation for the denudation of the 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. Thereafter oocytes were fixed and cleared with ethanol acetic acid 3:1 at 4°C for 24 h, and stained with aceto-orcein (1% orcein in 40% acetic acid), for 25 minutes. After that oocytes were rewashed with a fresh fixative and examined under light microscope *(11)*. The stage of nuclear maturation was described as follows:

a. Immature: Germinal vesicle stage with intact nucleus or germinal vesicle breakdown (GVBD)
b. Intermediate: Paired or bivalent chromosomes were observed within nucleus of the oocyte (Metaphase-I).
c. Mature: Two groups of unequally spread chromosomes were observed and the polar body set was clustered together (Metaphase-II).

Second experiment

The oocytes (n=744) were matured in Hepes buffered TCM-199 (the best medium from the second experiment) with the addition of sodium bicarbonate, penicillin 100 IU /ml and streptomycin 50 µg /ml (control) or control with addition test substances. There were five different treatments: control, BSA (3mg/ml), MS (20%), OSC and VE (0.5 mg/ml).

Statistical analysis

The proportion of oocytes at M-II stage and those classified as others, in different maturation media, and the proportion of oocytes at GVBD, M-I and M-II from different additives to maturation media were compared with each other using Chi square test. The level of significance was set at P<0.001. Chi square test were analyzed *(12)* by the following equation: $X^2=\Sigma(f-F)^2/F$ where, f is the observed frequency in any cell and F the frequency expected in the null hypothesis of independence holds, where $F=[(row\ total)(Column\ total)]/n$ and the $df=(R-1)(C-1)$, where R=row numbers and C=Column numbers.

RESULTS

Experiment I: The effect of medium type on oocyte maturation

The results of *in vitro* maturation of oocytes in four different culture systems are summarized in Table 1. There was a significant difference (P<0.001) in the number of oocytes, which were viable for *in vitro* maturation of 36 h (76-85%) using four different culture system. A higher proportion of oocytes (P<0.001), however, attained nuclear maturity (MII-stage) after the maturation period were found when cultured in TCM-199 compared with Ham's, TCM+20% FF and Ham's+20% FF (65 versus 46, 37 and 32%, respectively)

Table 1. *In vitro* development of the she-camel oocytes cultured in different maturation media for 36 h.

Medium used	Total COCs	Viable oocytes		GVBD[a]		MI[a]		MII[a]		Degenerted	
	Number	Number	%	Number	%	Number	%	Number	%	Number	%
TCM	145	119	82	12	10	30	25	77	65	26	18
Ham's	117	100	85	20	20	34	34	46	46	17	15
TCM+ 20% FF	138	115	83	23	20	50	43	42	37	23	17
Ham's+ 20% FF	133	101	76	30	30	38	38	33	32	32	24

[a] Proportion of the viable oocytes. FF=Follicular Fluid
Chi-square value = 32.0, significantly higher (P < 0.001).

Experiment 2: The effect of different additives to the TCM-199 medium on oocyte maturation

The results showed that the rate of maturation to the second metaphase stage (M-II) in TCM-199 medium with different additives [3mg/ml bovine serum albumin (BSA), 20% male serum (MS), oviductal secretion and cells (OSC) and 0.5mg/ml Vitamin E (VE)] are summarized in Table 2.

Table 2. *In vitro* maturation of she-camel oocytes cultured in TCM-199 with different additives for 36 h.

Additives	Total COCs	Viable oocytes		GVBD[a]		MI[a]		MII[a]		Degenerted	
	Number	Number	%	Number	%	Number	%	Number	%	Number	%
Control	160	130	81	16	12	30	23	84	65	30	19
BSA	157	134	85	4	3	10	8	120	89	23	15
MS	151	122	81	22	18	28	23	72	59	29	19
OSC	133	111	83	6	6	18	16	87	78	22	17
VE	143	113	79	12	11	19	17	82	72	30	21

[a] Proportion of the viable oocytes.
Chi-square value = 40.2, significantly higher (P<0.001).

There was no difference (P<0.05) in the proportion of oocytes, which were viable for *in vitro* maturation of 36 h (79-85%) using four different additives to the culture medium (TCM-199). The respective proportion (P<0.001) of COC's that reached M-II stages in the four additives were 65% (control), 89% (BSA), 59% (MS), 78% (OSC) and 72% (VE), showing that BSA and OSC were the best additives for *in vitro* maturation of dromedary oocytes.

DISCUSSION

The present study conducted to monitor the changes in cumulus cells of camel COCs during IVM depending on type of maturation media and the effect of adding different additives to the maturation media. In the current work, aspiration of the ovarian follicles was employed to isolate the COCs. However, several studies have presented different methods for recovering COCs from ovaries of farm animals. The number and the quality of COCs are influenced by the method of COC recovery ***(13,14)***. Previous data on techniques of recovery and evaluation of camel COCs are available only from the work of other authors ***(3)***. Nowadays, it is important to have an economic source of embryos, either to be able improve livestock genetics and also to carry out research involving new genetic technology. Today, using IVF in the laboratory, it is possible to obtain embryos from immature oocytes, in each stage of maturation utilizing different culture media.

In the first experiment, two different media (TCM and Ham's) were compared to find out the most suitable base media for nuclear maturation of oocytes in this species. Tissue culture medium-199 and Ham's medium are complex media, and there is little specific rationale for employing any of these media for oocyte maturation, but there is little doubt regarding the current effectiveness of these media for the specific purposes in other domestic as well as laboratory animal ***(15,16)***. Since many different media are available for oocyte culture, it is not always clear which medium is most appropriate for a particular species. The results of our experiment show that a higher nuclear maturation rate can be achieved when COCs are cultured in TCM-199 compared with Ham's these results are agreement with previous study ***(17)***. However, a higher nuclear maturation rate achieved when COCs are cultured in TCM-199 compared with CRI or CMRL ***(18)***. Moreover, FF has a less effect on the maturation rate, and had an inhibitory effect on *in vitro* oocyte maturation ***(19)***. Also the fractionation of FF

revealed inhibitory factors such as proteic, nucleotide molecules, small peptide and purine e.g. adenosine and hypoxanthin *(20)*.

Our results show that BSA significantly improves the oocyte maturation, while BSA had an inhibitory action on maturation of cow oocytes *(21)*. Adding of VE significantly improves maturation rate. These results are in agreement with other reported *(22)* which found that VE supplementation significantly more expanded and hatched bovine blastocyst than the basic medium. Moreover, OSC significantly improves the oocyte maturation (78% vs. 65% in the basic medium). Synthetic oviductal fluid (SOF) was superior to TCM-199 for both cleavage and development to blastocysts *(23)*. The male serum had an inhibitory action on maturation of camel oocytes, as well as, increases the proportion of oocytes that arrested at GV stage. It was suggested that MS contains factors which suppress the resumption of meiosis in oocytes.

It may be concluded that TCM-199 is superior to Ham's medium for the *in vitro* nuclear maturation of the dromedary oocytes, and FF did not improve the maturation percentage. Moreover, adding of BSA, OSC or VE to the TCM-199 improves the dromedary oocyte maturation.

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الملخص العربى

تأثير نوع البيئات الغذائية والأضافات المختلفة لها على الأنضاج المعملى لبويضات نوق الجمال

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أجريت هذه الدراسة لمعرفة تأثير نوع الوسط الغذائى والأضافات المختلفة لها على الأنضاج المعملى لبويضات نوق الجمال. أجماليا، استخدم فى هذه الدراسة أثنان وثمانون نوقة تتصف بصحة جيدة وعند ٨-١٠ سنوات من العمر. تم أجراء تجربتان فى هذه الدراسة. التجربة الأولى تم اجراؤها لتقييم مقدرة بويضات نوق الجمال (n=533) على الأنضاج المعملى خارج الجسم فى أنواع مختلفة من البيئات الغذائية وكذلك لمعرفة أنسب الطرق لتحديد حيوية البويضات الصالحة للأنضاج وذلك بفحص تمدد الخلايا الحبيبية للبويضات وظهور الجسم القطبى الأول فى الفراغ السيتوبلازمى. تم امتصاص البويضات من مبايض النوق المجمعة من السلخانة، وتلك البويضات التى تتميز باكثر من طبقتان أو ثلاث من الخلايا الحبيبية وسائل سيتوبلازمى متجانس تم أختيارها وزرعها فى ٥٠ ميكروليتر من أربع أنواع مختلفة من الأوساط الغذائية وهى كالآتى: ميديا-١٩٩؛ هامس-ف١٢؛ ميديا-١٩٩+٢٠% سائل جريبى و هامس-ف١٢+٢٠% سائل جريبى وذلك فى أطباق بترى ذو قطر ٣٥ ملليتر. تم تغطية الأوساط الغذائية بطبقة من الزيت المعدنى الدافىء ووضعت فى حضانة ثانى أكسيد الكربون عند ٣٩ درجة حرارية و ٥% ثانى أكسيد الكربون و ٩٠-٩٥% درجة رطوبة وذلك لمدة ٣٦ ساعة. تم قياس معدل انضاج البويضات بواسطة تمدد الخلايا الحبيبية وظهور الجسم القطبى الأول فى الفراغ السيتوبلازمى وكذلك عن طريق وصول البويضة الى مرحلة الميتافيس-٢ من الأنقسام الخلوى. فى التجربة الثانية تم استخدام ٧٤٤ بويضة وذلك لمعرفة تأثير الأضافات المختلفة الى الميديا-١٩٩ (٣ ملليجرام/مل من الألبيومين البقرى – ٢٠% من بلازما ذكور لجمال – خلايا وسائل القناة المبيضية – ٠,٥ ملليجرام/مل من فيتامين هـ) على تمدد الخلايا الحبيبية وأنضاج بويضات نوق الجمال معمليا.

لقد أظهرت النتائج فى التجربة الأولى، أن الميديا-١٩٩ تمتاز معنويا عن الهامس-ف١٢ لأنضاج بويضات نوق الجمال معمليا خارج الجسم، كذلك اضافة السائل الجريبى ليس له أى تأثير على النسبة المئوية لنضوج البويضات. أما التجربة الثانية فأثبتت أن أضافة الألبيومي البقرى - خلايا وسائل القناة المبيضية - فيتامين هـ له تأثير فعال على أنضاج بويضات نوق الجمال معمليا. ويستخلص من هذه الدراسة؛ أن الميديا-١٩٩، الألبيومين البقرى ، خلايا وسائل القناة المبيضية كأضافات الى الأوساط الغذائية تحسن البيئة الغذائية لأنضاج بويضات نوق الجمال خارج الجسم.