Effect of antioxidant supplementation on in vitro maturation of Camelus Dromedaries oocytes

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Abstract: Antioxidant supplementation during oocyte *in vitro* maturation (IVM) has been reported to improve the maturation rate of oocytes in a range of species; but no studies have been done in camels. The present study investigated the effect of some antioxidants on the maturation rate of *Camelus Dromedaries* oocytes. The ovaries were collected from slaughters house during December 2013 till May 2014. Excellent and good COCs were matured *in vitro* in TCM-199 medium contained fetal dromedary camel serum (FDCS) or fetal calf serum (FCS) supplemented with different antioxidants β-mercaptoethanol (βME), royal jelly (RJ), ascorbic acid (Vit C), selenium (Se) and melatonin (MEL) under controlled condition (38.5 °C in 5 % CO_2 for 24 hours in humidified air). Recovery rate was 8.1 oocytes/ovary and the maturation rate was significantly higher in βME and Se supplemented medium compared to control medium ($p \leq 0.05$), while no significant difference between the control group and other groups ($p \leq 0.05$) MEL, Vit C and RJ in medium enriched with FDCS while the medium enriched with FCS there was no any significant difference between the control group and the different antioxidant supplemented groups. In conclusion, culturing dromedary camel oocytes in TCM-199+FDCS maturation medium supplemented with different antioxidants used in the present study could have a beneficial impact on maturation rates in camels especially βME and Se while no effect in case of medium enriched with FCS.

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1. Introduction:

The female camel has a seasonal reproductive pattern that is primarily controlled by nutritional condition and management [1, 2]. The reproductive efficiency of camels under their natural pastoral conditions is low [3]. Abdel- Khalek *et al.* [4] found that the high number of oocytes/ ovary obtained as compared to the other previously reported on camel may be attributed to the pronounced differences in animal ages, reproductive status, site of the ovary, breeding season and/ or their interactions. The IVM conditions are simpler than *in vivo* maturation condition and limited materials are using for IVM process which can seriously affect the maturation status of oocyte [5]. Addition of useful materials such as gonadotropins, estradiol, growth factors [5] and antioxidants [6] are necessary for improvement of bovine oocytes IVM.

The developmental ability of oocytes matured in defined media still tends to be lower than that of oocytes matured in media supplemented with chemicals such as amino acids or antioxidants [7, 8]. And this maturation medium plays an important role in subsequent IVF and *in vitro* embryo development [9- 11].

The maturation media used for the camel have been adopted from ones used in other domestic animal species. However, the media and culture conditions for IVM of dromedary oocytes may have different requirements. Therefore, a detailed study on the maturation of the oocytes is a preliminary requirement for optimization of the IVP technique in this species [12]. Tissue Culture Medium (TCM) - 199 is the most widely used culture medium for IVM [13].

Downs *et al.* [14] suggested that it may be important to include serum in the IVM medium to prevent hardening of zona pellucida which could adversely affect fertilization. Also, serum provides a source of albumin that balances the osmolarity[15]. Kan and Yamane, [16] recorded another beneficial action of serum which is its antioxidant properties by reducing superoxide formation.

Oxidative stress is a major factor in IVF that affects the overall yield of viable embryos [17]. *In vitro* handling and culture conditions cause oocytes and embryos to oxidative stress (OS) resulting from events such as: exposure to light, elevated oxygen concentrations and unusual concentrations of metabolites and substrates [18]. An excessive amount of ROS (Reactive oxygen species) cause oxidative stress and may damage molecules and structures of

oocyte and granulose cells, ROS accelerate oocyte aging and deteriorate oocyte quality [19, 20]. Addition of antioxidant alone is not enough to protect from ROS. Besides, the choose of antioxidant and its concentrations are very critical. Bucak *et al.* [21] revealed that different antioxidants had different effects in different culture media. Higher implantation and clinical pregnancy rates are reported when antioxidant supplemented media was used, rather than standard media without antioxidants [22].

Although, there is no available literature to describe the effect of antioxidants on the maturation rates in *Camelus dromedaries* oocytes so this work was aimed to study the effect of some different antioxidants added to *in vitro* maturation medium.

2.Material and Methods

The present experiments were carried out at the laboratory of Reproductive Pharmacology, Department of Reproduction and Artificial Insemination, Veterinary Research Division, National Research Center, Giza, Egypt. It was conducted during the period from December, 2013 till May, 2014.

Unless stated otherwise, all chemicals and reagents were purchased from Sigma-chemical Company.

2.1. Collection of dromedary camel ovaries

Dromedary camel ovaries were collected from camels slaughtered in Nahia and El-Warraq abattoirs, Giza, Egypt. The ovaries were maintained in a thermos flask containing sterile normal saline (0.9% NaCl) supplemented with 100.000 IU/l penicillin (Misr Co. for Pharmaceutical products), 100 mg/l streptomycin (Amirya Pharm. Ind. Co.) at 30- 35℃. The ovaries were transported to the laboratory within 2-3 hrs after slaughtering of animals.

In the laboratory, ovaries were washed three times with warm saline (0.9% NaCl) containing antibiotics. Then all ovaries were quickly washed one time with ethanol (70%) then rewashed with fresh normal saline 3 times and hold in warm normal saline . Follicular fluids were aspirated using a 20- gauge needle attached to a 5-ml plastic syringe containing 2 ml M-PBS. The follicular fluid containing COCs were placed into 4- cm grinned Petri dishes filled with 2 ml M-PBS at 37℃ and held on a warm plate until oocyte searching could be conducted. To recover all the oocytes after aspiration, ovaries were sliced in a 6- cm Petri dish with 2 ml M-PBS at 37℃. After slicing completed ovaries were rinsed with 2 ml of M-PBS using a 5-ml plastic syringe and 20- gauge needle then ovary removed from the dish and discarded [23]. The oocytes were left to settle down in a Petri dish on a warm plate for 15 minutes. COCs with at least one to three layers of compact cumulus cells and a homogenous ooplasm were selected under a stereomicroscope for further experiment.

Recovery rate was calculated as follow:

$$\text{Recovery rate} = \frac{\text{Total no. of harvested oocytes}}{\text{Total no. of ovaries}}$$

2.2. Oocyte in vitro maturation:

Selected COCs were washed twice in M-PBS and once in maturation medium TCM-199 (Vacsera, Dokki, Giza), supplemented with 10 IU/ml Folligon ® (pregnant mare serum gonadotropin, Inter Vet international B.V.B oxmeer Holland), 15 µl/ml 17β-estradiol (Misr Com. for Pharm. Ind. S.A.E.), 5 µg/ml Garamycin (Memphis Co. Pharm. Cairo) and in the first experiment the medium enriched with heat-inactivated 10% fetal dromedary camel serum {FDCS} Desert Research Center, El-Matarya, Cairo, Egypt) and it act as control group 1. The other groups contain the same concentrations as above mentioned in addition to the different antioxidants added as following:

Group 2: 100 µM/ml βME, Sigma- Aldrich Chemie GmbH, CH-9471 Buchs. Germany

Group 3: 100 µg/ml RJ, purchased from local market

Group 4: 100 µg/ml Vit. C, El-Nasr Pharmaceutical Chemicals Co. - Abu-Zaabal, Egypt.

Group 5: 100 µg/ml Se, (Sodium selenite AR [Anhydrous]®, Na_2Seo_3,M.W. 172.94), ALPHA CHEMIKA. Mumbai-400002, INDIA.

Group 6: MEL 100 µM/ml, Sigma chemical Co. St. Louis. M063178, USA.

While in the second experiment we used the same composition as in the first experiment except we use the 10% fetal calf serum (FCS, Sigma, USA).

Each 5 – 10 COCs were cultured in Petri dish contains 50 µl droplets of the maturation medium and covered with paraffin oil at 38.5℃ in 5% CO_2 and 90% relative humidity for 24 hours.

2.3. Evaluation of maturation:

Maturation was assessed by determination of cumulus cells mass expansion or by the presence of first polar body (PB). Matured oocytes were classified according to Schellander *et al.* [24].

Maturation rate was calculated as follow:

$$\text{Maturation rate} = \frac{\text{Total no. of matured oocytes}}{\text{Total no. of cultured oocytes}} \text{X100}$$

2.4. Statistical analysis

Data are presented as percentages. At least, three replicates were carried out for each experimental group. Data were analyzed using practicing statistical

analysis program SPSS, version 16 according to the method of Armitage [25].

3. Results

1- Recovery of oocytes:-

The oocytes were harvested from ovarian follicles by using the aspiration followed by slicing methods, the average number of oocytes recovered from dromedary she camel ovarian follicles is tabulated in table (1), the average recovery rate was 8.1 oocyte / ovary. Figures (1-3) showed the matured oocytes.

Table (1): The recovery rate of dromedary camel oocytes harvested from ovarian follicles:

Total no. of ovaries	Total no. of collected oocytes	Recovery rate
338	2744	8.1 oocytes/ ovary

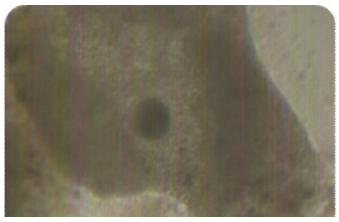

Fig.(1): Matured oocytes surrounded by highly expanded cumulus cells

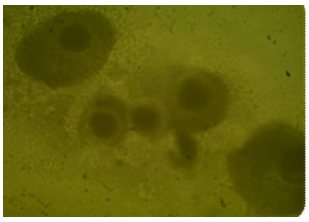

Fig (2): Matured oocytes showed different grades of oocytes surrounded by expanded cumulus cells.

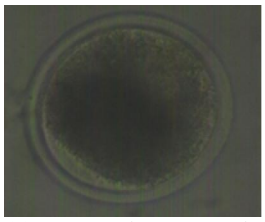

Fig (3): Matured oocytes showed extrusion of first polar

2- The effect of addition of different antioxidants to the TCM- 199 medium enriched with FDCS on the maturation rate of dromedary she camel oocytes:-

Looking on table (2), showed that there was a significant increase in the maturation rate of dromedary she camel oocytes matured *in vitro* in maturation medium containing β- mercaptoethanol and selenium with maturation rates (86.6±3.35%, $p \leq 0.05$), and 84.73±5.02%, $p \leq 0.05$, respectively) than the control group which have the lowest maturation rate (64.83±8.08%, $p \leq 0.05$), while there was no significant difference in the media contain melatonin, ascorbic acid and royal jelly with maturation rates (83.33±3.33%, $p \leq 0.05$), (81.93±3.67%, $p \leq 0.05$) and (75.00±8.94%, $p \leq 0.05$), respectively when compared to control or β-mercaptoethanol and selenium.

Table (2): The effect of addition of different antioxidants to the TCM- 199 medium enriched with FDCS on the maturation rate of dromedary she camel oocytes:-

Groups	Maturation Rate		
	Mean%	±SE	*P* value
Control	64.83[a] (30/46)	8.08	$p \leq 0.05$
βME	86.60[b] (52/60)	3.35	
RJ	75.00[a,b](44/60)	8.94	
Vit C	81.93[a,b](46/56)	3.67	
Se	84.73[b] (48/56)	5.02	
MEL	83.33[a,b](50/60)	3.33	

Means with different superscripts are significantly different at $P \leq 0.05$, βME: β- Mercaptoethanol, R J: Royal Jelly, Vit C: Ascorbic acid, Se: Selenium & MEL: Melatonin

3-The effect of addition of different antioxidants to the TCM- 199 medium enriched with FCS on the maturation rate of dromedary she camel oocytes:-

The present results showed that there was no significant differences in the maturation rate of dromedary she camel oocytes between the control group and medium contained different antioxidants but we found that the media contained royal jelly is the best group (77.60±1.45%, $p \leq 0.05$), than the control group without antioxidant and maturation rate (76.67±1.67%, $p \leq 0.05$), Ascorbic acid group with a maturation rate (71.53±10.85%, $p \leq 0.05$), β-mercaptoethanol group with a maturation rate (70.83±4.17%, $p \leq 0.05$), selenium group with a maturation rate (64.73±2.23%, $p \leq 0.05$) and the lowest group is media contain melatonin antioxidant with a maturation rate (63.33±3.33%, $p \leq 0.05$) as shown in table (3).

Table (3): The effect of addition of different antioxidants to the TCM- 199 medium enriched with FCS on the maturation rate of dromedary she camel oocytes

Groups	Maturation Rate		
	Mean%	±SE	*P* value
Control	76.67 (40/52)	1.67	$p \leq 0.05$
βME	70.83 (40/56)	4.17	
RJ	77.60 (48/62)	1.45	
Vit C	71.53 (50/70)	10.85	
Se	64.73 (48/58)	2.23	
MEL	63.33 (38/60)	3.33	

βME: β- Mercaptoethanol, R J: Royal Jelly, Vit C: Ascorbic acid, Se: Selenium & MEL: Melatonin

4. Discussion

By using the aspiration followed by slicing methods the average number of oocytes recovered from dromedary she camel ovarian follicles is tabulated in table (1), the average recovery rate was 8.1 oocyte / ovary. While Amal *et al.* [26] recorded that the average number of collected oocytes/ ovary in the breeding season was 10.69 and this was higher than the present results. Abdoon and Omaima [27] reported that the average number of collected oocytes was 11.31± 2.61 COCs/ ovary which collected by aspiration. However, low recovery rate was recorded by Khatir *et al.* [28], and this difference may be due to the effect of the diameter of collecting needle or the technique used. While others found that the recovery rate during breeding season is 6.6 oocytes/ ovary[29]. El-Harairy *et al.* [30] found that lower average number of follicles per dromedary camel ovary was 12.7/ovary, regardless site of the ovary (right or left) and reproductive status (pregnant or not). In the literature there are wide variations in number of oocytes per camel ovary, being 5.3 [31], 7.64 [32] and 6.67 [33].

The differences between the results might be due to the differences in age, health, nutritional, site of ovary, reproductive status and genetic status of the oocyte donors obtained from a slaughterhouse source. No study focused on antioxidant effects of IVM in dromedary camel is available in the literature to compare the present results. Therefore, the effects of antioxidants used in the present study in the view of IVM could not be objectively discussed in relation to camels and compare to other investigations in details.

There was a significant increase in the maturation rate of dromedary she camel oocytes *in vitro* matured in maturation media (TCM-199+ FDCS) containing βME than the control group while no significant difference in the medium enriched with FCS. Hossein *et al.* [34] found that various thiol compounds especially cysteine and cysteamine are commonly added to IVM media especially (TCM-199) help to support GSH synthesis, and to improve the developmental competence of oocytes by improving the cytoplasmic maturation after 72hr culture of canine oocytes and it resemble the present result. Luberda [35] and Roushandeh *et al.* [36] found that GSH synthesis in oocytes may be stimulated by different low molecular weight thiol compounds such as sulphuric amino acids: cysteine and cystine, cysteamine or βME. βME and cysteamine reduce cystine to cysteine and promote the uptake of cysteine enhancing glutathione synthesis and this may explain why βME has a good effect on the maturation rate of oocytes. Supplementation the IVM medium (TCM-199 + FBS) of bovine oocytes with the thiol compounds especially βME increase the GSH synthesis and improved embryo development and quality, producing more embryos reaching the blastocyst stage on day 6 than embryos matured in unsupplemented medium. This phenomenon has been observed in oocytes matured *in vitro* and fertilized *in vitro*. The mechanism of action through which βME exerts its effect on embryo development is not-yet known. It is possible that βME is itself an antioxidant and removes free radicals. Alternatively, it has been proposed that βME may act by increasing intracellular GSH [37].

The present result may agree with Songsasen and Apimeteetumrong [38] who showed that supplementation of the IVM medium (TCM-199) with βME, GSH stimulator, increased the proportion of oocytes exhibiting synchronous pronuclei formation in swamp buffalo and blastocysts produced from these oocytes developed faster.

The different results explain that the effect of βME on maturation rate of oocytes may be related to the type of medium and serum added to it. However, these effects appear to be dependent on species and antioxidants [39].

There was no significant variations in the maturation rate of dromedary she camel oocytes in vitro matured in maturation media containing RJ. Abd-Allah [40] showed that the use of different concentrations from RJ combined with serum supplements has beneficial effect on IVM. Increase the proportion of oocytes showing full expanded cumulus cells, viability and IVM of ovine oocytes for all the supplements dependent effect of RJ supplementation. Therefore, the higher maturation rate ($p<0.05$) (ranged between 30% to 55% from control to media contain 0.5% RJ) *in vitro* of sheep oocytes in TCM-199+ FCS media may be attributed to some factors in RJ composition such as gonadotropins and essential amino acids that stimulate DNA and RNA synthesis and enhance cell division in addition, increase the level of intracellular cAMP, the activity of the hyaluronic acid synthesis enzyme system and induce cumulus expansion in intact complexes. Our results is parallel to that results but the discrepancy may be due to type of serum, type and source of RJ used and animal species.

Vit C is an important water soluble antioxidant that reduces sulfhydryls, scavengers free radicals and protects against endogenous oxidative DNA damage [41]. Also, it may become a pro-oxidant when free transition metals are present [42]. Addition of ascorbic acid to embryo culture significantly affects embryo development.

The present study show that there was no significant difference in the maturation rate of dromedary she camel oocytes *in vitro* matured in medium containing Vit C and the control group. The present results were in contrast to [43] who indicated

that the combination of Vit E+ Vit C in TCM-199 has a positive effect on oocyte nuclear maturation as indicated by cumulus oophorus expansion. However, polar body formation as a sign of nuclear maturation was not influenced in a beneficial manner. Although, antioxidant combinations have been proven to have a positive effect on swine oocyte maturation [44] the same can't be said for the ovine female gamete. This could be the result of differences between lipid compositions in the gametes of the different species but also because of specific interactions between fatty acids and the antioxidants.

Vit C addition did not interfere with cumulus expansion and oocyte maturation [45] who found that adding Vit C to the maturation medium diminishes the number of oocytes that show no signs of maturation. Also, [46] found that the addition of Vit C to the maturation and embryo culture media resulted in a significant increase in the number of embryos.

Córdova *et al.* [47] who found that the addition of Vit C to the oocyte maturation media exerted no effect on the maturation rates. Also, [48] found the same results in murine and bovine oocytes.

Nadri *et al.* [49] has been suggested that Vit C at high concentration may act as a pro-oxidant that affect deleteriously on oocyte maturation and cumulus cell viability. In fact, the lower concentrations of Vit C (80 and 250 µM/ml) promote cell growth

In the present study there was a significant increase in the maturation rate of dromedary she camel oocytes in vitro matured in maturation media (TCM-199+ FDCS) containing Se than the control group and no difference in maturation medium (TCM-199+FCS).

The present results are in parallel with Smiljaković *et al.* [50] who showed that Se has a very good effect for decreasing the amount of coliform bacteria, especially with addition of Bacellus Cereus into the *in vitro* culture (TCM-199) of oocytes, the pathways are still unclear, and how Se travels and gives signals into the oocyte from plasma membrane to nucleus.

Jeong et al. [51] suggested that ITS supplementation during IVM (TCM-199) improved cytoplasmic maturation which is indicated by the higher intracellular glutathione concentration of oocytes and lower polyspermic fertilization rate. Our results may be in agree with Abedelahi et al. [52] who showed that during IVM of oocytes cultured in TCM-199+FBS supplemented media containing 10ng/ml sodium selenite (SS) were shown larger diameter and it affects on growth and survival of oocytes in dose dependent and it acts as proliferative and antioxidant factor.

Cavilla *et al.* [53] showed that SS promoted human oocyte growth during IVM. It was shown that SS prevents cell damage by reduce ROS and inhibits lipid peroxidation [54, 55].

There was no significant difference in the maturation rate of dromedary she camel oocytes in vitro matured in maturation media containing MEL than the control group. Our results is disagreeing with Tsantarliotou et al. [56] who demonstrated that MEL supplementation during IVM in TCM-199+15% bovine serum (BS), did not affect bovine IVF at concentrations of 10 µM, 100 µM and 1mM as indicated by the similar cleavage rates recorded in all experimental groups. In their work neither beneficial nor toxic effects were observed in the range of concentrations tested that was chosen on the basis of the few reports available in literature for other species.

In swine an increased maturation rate was recorded following MEL enrichment of the IVM medium [57] and these results agree with our results. It is reported that 1µM MEL reduce cumulus cells apoptosis by activating its receptors on cumulus cells [58]. However, our results is parallel to Farahavar *et al.* [59] who found that cumulus cells expansion did not show significant difference in compare to control (TCM-199+2%FBS). It has been reported that type of medium and supplements which is used for IVM may influence rate of cumulus cell expansion [60].

The addition of MEL to IVM and IVF media can improve *in vitro* oocyte maturation and cleavage rates in bovines [61], mice [58] and humans [62]. Results of [63, 64] suggest that the effect of MEL on oocyte maturation is dose-dependent. Although, MEL toxicity is reported to be extremely low, oocyte maturation in female mice was significantly impaired by MEL concentrations of 10^{-3} or higher. In the early stages of maturation, MEL has an even greater effect on the IVM of oocytes [61].

Takada *et al.* [65] indicated that MEL during IVM protects the bovine cumulus cells from DNA damage and it may explain the results but this effect did not influence embryo development *in vitro*.

Rezk [66] revealed that supplementation of maturation medium (TCM-199) with 10% FDCS significantly ($P<0.001$) showed the highest frequency distribution of oocytes (59.5%) at metaphase II (M II).

These results were in disagreement with Wani and Wernery [67, 68] who found the protein sources as FCS, BSA and estrus camel serum (ECS) in TCM-199 can be used in the maturation medium for dromedary oocytes without any effect on the proportion of oocytes reaching metaphaseII stage.

In conclusion the different antioxidants which were added to the maturation medium could have a beneficial impact on maturation rates in camel oocytes. But, the differences in the results may be due to the difference in the sera used. As it was the first time use antioxidant in camel IVM. So, further study needed for

more investigation of the effect of antioxidants in the IVM in camels.

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