Antioxidant supplementation effects on *in vitro* maturation and fertilization of dromedary camel oocytes

Amira Khaled Khattab[1,2], Nehal Ali Abu Elnaga[2], Hesham Attia Shedeed[1], Ahmed Mohamed Kamel[1*], Mervat Ahmed Bekhit Abd Rabu[3] and Khalid Ahmed El-Bahrawy[1]

[1]Animal and Poultry Production Division, Desert Research Center, 11753, Cairo, Egypt; [2]Department of Zoology, Faculty of Science, El-Azhar University, Egypt; [3]Department of Biology, College of Science, Jouf University, P.O. Box 2014, Sakaka, Kingdom of Saudi Arabia.

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Abstract

The current investigation was conducted to evaluate the effects of antioxidant addition (ascorbic acid, cysteine, and a combination of both) to the *in vitro* maturation medium of dromedary camel oocytes on maturation rate, as well as to investigate maturation medium antioxidant supplementation on *in vitro* fertilization of oocytes. The oocytes were retrieved by slicing ovaries collected from a local slaughterhouse. Recovered oocytes were examined before and after *in vitro* maturation (IVM) culture for grading, investigating cytoplasmic and nuclear maturation. Only good-quality oocytes (with more than one layer of cumulus cells and homogeneous dark cytoplasm) were selected (n=656) and allocated into four groups, namely: the control group (G1) was cultured in an IVM medium free of antioxidant; Group 2 (G2) was supplemented with 100 µg/ml ascorbic acid; Group 3 (G3) was supplemented with 6.9 µM cysteine; and the fourth group (G4) was supplemented with a mix of 100 µg/ml ascorbic acid and 6.9 µM cysteine. All groups were incubated at 38.5 ºC, 5% CO_2 and 95% humidity for 40 hours. In the *in vitro* fertilization experiment, cumulus oocytes complexes (COCs) were inseminated with frozen-thawed semen ($3x10^6$ spermatozoa/ml) then cultured in the fert-TALP medium at 38.5 ºC, 5% CO2 and 95% humidity for 18 hours. The mean recovery rate was 8.83 oocytes per ovary. After IVM, the maturation rate due to expansion in cysteine (89.62%) was significantly higher than the control group (78.98%), the ascorbic acid group (76.04%) and the mix group (72.64%). However, according to the polar body extrusion percentage, the results revealed that the maturation medium treated with ascorbic acid (29.17%) was significantly higher than the cysteine group (20.75%), the G4 mix group (17.83%) and the control group (10.45%). The results of the fertilization trial showed that there was no significant difference between the ascorbic acid group (9.72%) and the control group (5.97%). In conclusion, the treatment of *in vitro* maturation medium with 6.9 µM cysteine improved cumulus cells expansion, while ascorbic acid supplementation (100 µg/ml) improved the first polar body extrusion. However, the combination of both had a negative effect on both expansion and polar body.

Keywords: antioxidant, ascorbic acid, cysteine, dromedary, IVF, IVM, oocytes.

***Corresponding author**: Dr Ahmed Mohamed Kamel; ahmed_kamel_drc@yahoo.com

Introduction

One of the most important biotechnologies in livestock breeding presently is *in vitro* embryo production (IVP). However, it is still low with only 30-40% of oocytes that are able to develop into blastocysts. Several factors can affect IVP efficiency and participate in the existing differences between *in vivo* and *in vitro* produced embryos. This could possibly be because the *in vitro* environment cannot mimic the *in vivo* environment, resulting in embryos with altered morphology and gene expression. There is also some evidence that shows IVP can cause some disorders during gestation and in the offspring (Abd El-Aziz *et al.,* 2016). Basically, *in vitro* oocyte maturation is the most critical step for IVP-derived embryos (Ferreira *et al*., 2009 & Abd-Allah, 2011). In 2010, Badr and Abdel-Malak reported that IVF technology in the dromedary will improve understanding of the fundamental mechanisms of fertilization and early embryonic development and promote the development of other technologies, including cryopreservation of oocytes and embryos as well as embryo sexing and somatic cloning and transgenesis. Earlier, in 2001, Guérin *et al*. reported that the oxidative stress caused during IVF manipulation is a major factor that affects the overall yield of viable embryos. *In vitro* handling and culture conditions, such as exposure to light, elevated oxygen concentrations and unusual concentrations of metabolites and substrates, can cause oxidative stress to the oocytes and embryos (Agarwal *et al.,* 2006). Therefore, antioxidants, such as vitamin C (ascorbic acid), which help in the neutralization of free radicals and thereby prevent their oxidizing actions, can be added to the culture medium to improve oocyte maturation and fertilization rates (Nishikimi and Yagi, 1996). Further studies have shown that the addition of Thiol containing precursors of glutathione (GSH), such as cysteine (CySH), cysteamine, β-mercaptoethanol or use of a cysteine-rich medium (TCM 199 or Waymouth MB 75211), increased GSH content of oocytes after maturation and also caused a higher intracellular GSH level which improved embryo development and quality (de Matos *et al.,* 2002 & de Matos and Furnus, 2000). The present study was conducted to investigate the effects of adding antioxidants to the maturation medium on *in vitro* maturation and *in vitro* fertilization rates of dromedary camel oocytes.

Materials and methods

The present study has been conducted at the Embryology Manipulation Unit (EMU), Department of Animal and Poultry Physiology, Division of Animal and Poultry Production, Desert Research Center (DRC), Cairo, Egypt. The experimental work was carried out during the camel breeding season (December to April) for two successive years (2017/2018 and 2018/2019).

Chemicals and media

Unless otherwise mentioned, all the chemicals and media constituents were purchased from Sigma-Aldrich Chemicals, Germany. All media were prepared daily from a stock solution of each compound and sterilized before use by passing through a Millipore filter 0.22 µm in diameter fitted on a 10 ml syringe (Russo *et al.,* 2014).

Biological material

Camel (*Camelus dromedarius*) ovaries (n=177) of unknown reproductive history as the source of oocytes were collected from El-bassatin slaughterhouse located in Cairo in a thermos flask containing warm saline solution (NSS, 0.9% NaCl, 35 to 37 ºC) supplemented with antibiotic antimycotic (AA) (100 IU penicillin and 100 µg streptomycin/ml) and transported to the laboratory within 2-3 hrs. Frozen semen was obtained from an artificial insemination and embryo transfer lab – Maryout Research Station – Desert Research Center, Alexandria, Egypt.

Ovaries manipulation, oocyte retrieving and in vitro maturation

As soon as the camel ovaries reached the EMU, all excessive tissues were discarded, and the ovaries were washed three times with pre-warmed (30 ºC) NSS. Afterwards, all ovaries were washed once with ethanol (70%) to remove any contamination on the ovarian surface, followed by a final wash using pre-warmed (30 ºC) phosphate buffer saline (PBS) supplemented with antibiotics (100 IU penicillin and 100 µg streptomycin/ml**)** according to Russo *et al.* (2014). After that, the ovaries were preserved in glass gars containing PBS and kept in a 30 ºC adjusted water bath (El-Sayed *et al*., 2015).

Cumulus oocytes complexes (COCs) were harvested by slicing ovaries in a 90 mm petri dish and rinsing with warm (30 ºC) phosphate-buffered saline (PBS) supplemented with 50 µg/ ml gentamicin (El-Sayed *et al*., 2015). A total number of 1632 of oocytes were collected. The recovered solution containing COCs was examined under a stereomicroscope (GX microscope, UK, Range: 8x to 50x) to pick out, evaluate and classify as good quality (more than one layer of cumulus cells and homogeneous dark cytoplasm) according to the criteria described by Kandil *et al.* (2014). Selected oocytes (Plate.1, A) were washed twice with TCM 199 supplemented with 25 mM HEPES, 5% FBS, then finally washed in IVM medium with/without antioxidant according to the experimental group. Collected oocytes were distributed into four groups: (G1) control group cultured in IVM medium consisting of tissue culture medium-199 (TCM-199) supplemented with 15% (v/v) heat-treated (56 ºC for 30 min) fetal bovine serum (FBS), 40 IU PMSG, 1 µg/ml estradiol (E2), 0.25 mg/ml Na^+ pyruvate and 20 ng/ml epidermal growth factor (EGF) with no antioxidant supplementation; (G2) supplemented with 100 µg/ml ascorbic acid; (G3) supplemented with 6.9 µM cysteine; and (G4) supplemented with a mix (100 µg/ml ascorbic acid and 6.9 µM cysteine). All media were sterilized using 0.22 µm Millipore syringe filters and incubated for at least two hours according to Russo *et al.* (2014).

Oocytes were cultured in drops of maturation media (10 to 15 oocytes/ 100 µl drop) in a 35 mm petri dish, covered with mineral oil and incubated in a CO_2 incubator for 40 hrs at 38.5 ºC under 5% CO_2 and 95% relative humidity (RH). Cumulus expansion was examined under a stereo- microscope (GX microscope, UK, Range: 8x to 50x) after the incubation period. The percentage of oocytes with expanded cumulus (Plate.1, B) was calculated and recorded, based on the criteria of Amer and Moosa (2009). Oocytes were denuded by gentle pipetting

with 80 IU of hyaluronidase under the either immature or mature with polar body (pb), in each group were washed twice in HEPES-TCM 199 and examined under an stereomicroscope. Cumulus-free oocytes, inverted microscope to detect polar bodies (Plate.1, C).

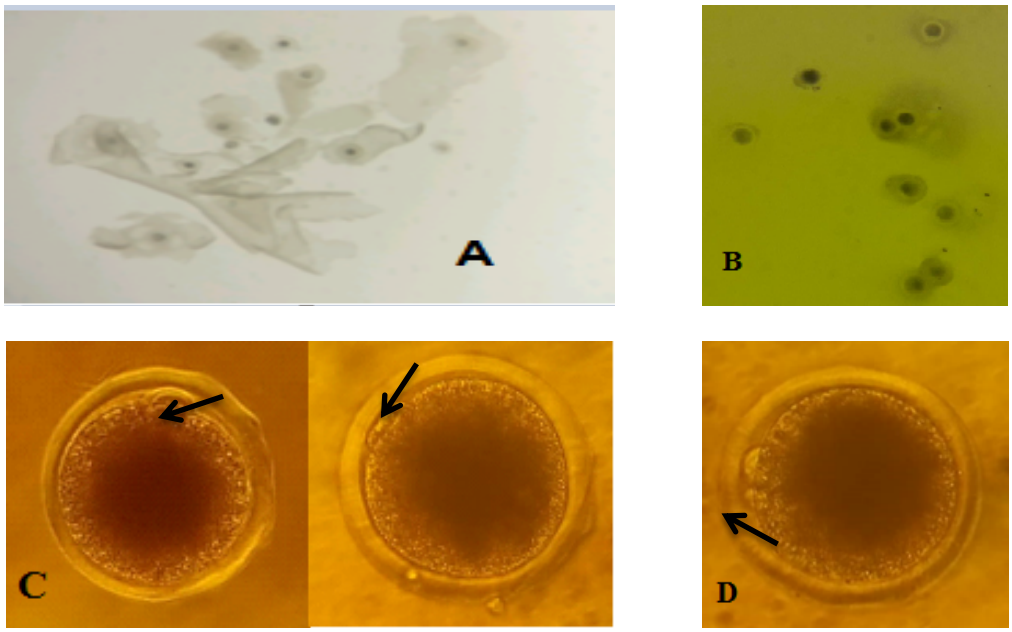

Plate 1. Developmental stages of *in vitro* oocyte maturation showing: (A) immature oocytes with the condensed cumulus cells 30X, (B) matured oocytes with expanded (black arrows) 400 cumulus cells 50X, (C) denuded matured oocyte with 1st polar body (black arrow) 400X. X, (D) matured oocyte extruded the 2nd polar body.

In vitro fertilization

Two 0.5 ml straws of frozen camel semen from one male were thawed, 37 ºC for 40 seconds (El-Bahrawy 2017), and wiped with 70% ethyl alcohol before being opened, and the contents were layered on top of 5 ml of sperm washing medium (Sperm TALP medium) in a 15 ml falcon tube, and then centrifuged twice at 300 X g for 5 min each. The final pellet was re-suspended in an appropriate volume (depending on the sperm concentration after washing) of pre-warmed fertilization medium (Fert. TALP medium), then the final sperm concentration was adjusted to $3x10^6$/ml live sperm (60% total motility) (El-Sayed *et al*., 2015). Three drops of 100 µl sperm suspension (approximately 300,000 sperm per drop) were placed in a 35 mm petri dish and covered with pre-warmed mineral oil. Matured oocytes were washed three times in the fertilization medium before 10–15 oocytes were placed in each of the sperm drops and cultured in a CO_2 incubator (Khatir *et al*., 2007). Eighteen hours after fertilization, oocytes were denuded from cumulus cells by gentle pipetting in fertilization medium, then observed with a Leitz Fluovert FU (Leica Microsystems, Wetzlar, Germany). The fertilization rate

was calculated based on the observation of the second polar body.

Statistical analysis

Data of the maturation rate (due to expansion and polar body) and fertilization rate were statistically analyzed by a Chi square test using the SAS program, 2004 (Statistical Analysis System, SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Maturation rate according to expansion rate

As presented in Table (1), a total number of 656 good quality oocytes were allocated into four groups, namely: control, ascorbic acid, cysteine, and a mix of ascorbic acid and cysteine (157, 192, 106 and 201, respectively). The maturation rate % due to expansion in the cysteine group was significantly higher than for the control, ascorbic acid and mix groups. There was no significant difference between both the control group (G1) and the ascorbic acid group (G2). In addition, there was no significance difference between the ascorbic acid group and the mix group. On the other hand, the G1 surpassed significantly ($P<0.005$) the mix group (G4). The treatment of maturation media with cysteine 6.9 µM showed a very high expansion rate as compared to the control group. This result surpassed what had been reported by Rahim *et al*. (2011), who found that supplementation of TCM 199 media with 100 or 500 µM/ml of cysteine showed an expansion rate of 53.33% vs. 42.22%, respectively, while his control group had an expansion rate of 33.33% in bovine oocytes. Also, the results were higher than that previously reported by Khalil *et al.* (2014), as they reported that supplementation of maturation media of buffalo oocytes with tocopherol, ascorbic acid and retinol yielded higher percentages of expansion rate of cumulus cells (77.2, 69.4 and 69.2) as compared to control medium (60.2%).

Table 1. Maturation rate according to expansion rate.

Treatment	Total number of oocytes	No. Expanded oocytes	Expansion rate%	Chi square value
Control	157	124	78.98[b]	12.18
Ascorbic	192	146	76.04[bc]	
Cysteine	106	95	89.62[a]	
Mix	201	146	72.64[c]	

[a,b,c]: Superscripts to be compared statistically within the same column. Values with different letter superscripts are significantly different ($P\leq0.05$).

The variation of the aforementioned results may be also related to the different animal species. However, the higher rate of oocyte maturation in the cysteine group than in the other groups may be due to the higher level of GSH in this group as indicated by Rahim *et al*. (2011), as cysteine is a precursor of glutathione. There are different mechanisms for controlling cellular reactive oxygen species (ROS) levels such as GSH and superoxide dismutase. Glutathione is a non-protein sulphydryl compound in cells. It serves as a reservoir for CySH and plays an important role in protecting mammalian cells from oxidative stress and its intracellular synthesis is very important in oocyte cytoplasmic maturation (Gordon, 2003; Gasparrini *et al.,* 2008). Considering these facts, the efficiency of GSH synthesis in COCs should be at its best when the environment is rich in cysteine, because both oocytes and the attached cumulus cells can uptake cysteine by the system alanine–serine– cysteine transport system (Nagai, 2001). It is likely that the cysteine induced GSH synthesis occurs during the first hours of IVM, before the oxidation of amino acid (Gasparrini *et al.,* 2006). Consequently, cysteine which is a thiol compound can be used to enhance the efficiency of *in vitro* matured oocytes.

The present result agrees with Elsayed *et al.* (2015) who found that there was no significant difference in the maturation rate according to cumulus cell expansion of dromedary she-camel oocytes *in vitro* matured in medium containing ascorbic acid when compared to the control group, with a mean of 81.93% vs 64.83%. The present study showed that supplementing the maturation medium of dromedary camel oocytes with a combination of 6.9 µM cysteine and 100 µg/ml ascorbic acid had a negative significant effect on oocyte maturation when compared to the control group. Contrarily, the results completely disagree with Miclea *et al.* (2012**)** who indicated that the combination of α-tocopherol and ascorbic acid in TCM-199 has a positive effect on nuclear maturation of oocytes as indicated by expansion of cumulus cells. Also, El-Naby *et al.* (2017) found that the combination between two antioxidant substances (ascorbic acid and cysteamine) gave a better embryonic developmental rate than ascorbic acid only. In contrast, Dalvit *et al.* (2005) found that the addition of α -tocopherol and/or ascorbic acid to the maturation medium failed to alter the IVF percentage, suggesting that the employed natural antioxidants exert no immediate effects on cytoplasmic maturation of the bovine oocyte. In agreement, it has been observed that the addition of β-mercaptoethanol, ascorbic acid or superoxide dismutase to the maturation medium fails to modify bovine IVF percentages (Blondin *et al.*, 1997).

In contrast, Sovernigo *et al.* (2017) reported that the sum of reduction in ROS levels and an increase in GSH levels would be important for oocyte developmental competence. However, as demonstrated in the present study, none of these antioxidants (ascorbic acid or cysteine) affected both parameters concurrently: increased GSH levels and reduction in ROS levels. A possible alternative to increase GSH levels and reduce the levels of ROS concurrently would be the interaction of antioxidants that act at different points on the antioxidant pathway.

Maturation rate according to polar body extrusion

According to the polar body extrusion percentage, it was found that maturation medium treated with ascorbic acid alone (29.17%) was significantly higher (P≤0.01) than medium treated with cysteine (20.75%) or control medium (17.83%) and the combination of ascorbic acid and cysteine (10.45%). Although the cysteine group was higher than the control (20.75% vs 17.83%, respectively), there was no significant difference. On the other hand, the control group was significantly higher than the mixed group (17.83% vs 10.45%, respectively) as shown in Table (2). These results are the same trend as described by Khanday *et al.* (2019) who concluded that ascorbic acid supplementation improved the developmental competence of goat oocytes during heat stress and demonstrated significantly higher maturation rates due to polar body extrusion (64%) when compared to a non-supplemented group (40%). On the other hand, results of the present study are in disagreement with Miclea *et al*. (2011), who reported that there was no significant difference in nuclear maturation of swine oocytes as indicated by polar body extrusion between an ascorbic acid group and a control group (57.33% vs 54.61%). The low percentage of polar body extrusion (10.45%) represented in the mix group (G4) completely agree with Miclea *et al.* (2012**),** who indicated that supplementing the *in vitro* maturation medium of sheep oocytes with a combination of α-tocopherol and ascorbic acid reduced the percentage of polar body extrusion. From our point of view, this could be the result of lipid composition in the oocytes and a specific interaction between fatty acids and the two antioxidants. In addition, the current result may be due to the role of ascorbic acid in preventing free radicals from initiating peroxidative tissue damage. It could protect the oocytes from oxidative stress, by increasing the intracellular glutathione (GSH) content (Tatemoto *et al.,* 2000) which protects cell membranes from pro-oxidants and decreased ROS accumulation in MII oocytes which allowed a better ooplasmic maturation as suggested by Kere *et al*. (2013). It can, therefore, be assumed that the supply of ascorbic acid in oocyte maturation media increases the GSH content, which protects the cumulus cell membranes from ROS and increases the levels of maturation activities of oocytes, thereby, preventing the apoptosis of cumulus cells as well as oocytes (Khanday *et al.,* 2019).

Table 2. Maturation rate according to polar body percentage:

Treatment	Total number of oocytes	No. oocytes with Polar body	Maturation rate%	Chi square value
Control	157	28	17.83^{c}	22.41
Ascorbic	192	56	29.17^{a}	
Cysteine	106	22	20.75^{bc}	
Mix	201	21	10.45^{d}	

^{a,b,c,d}: Superscripts to be compared statistically within the same column. Values with different letter superscripts are significantly different (P≤0.01).

Fertilization experiment

There was a higher fertilization rate (9.72%) for dromedary camel oocytes treated with ascorbic acid in the maturation stage when compared with oocytes from the antioxidant free control group (5.97%). However, this difference was not statistically significant (P≤0.05) as illustrated in Table (3). Previously, Castillo-Martín *et al.* (2014) mentioned that L-ascorbic acid supplementation in culture and/or vitrification media enhanced the survival rates of porcine blastocyst. Our finding in the present study was in contrast with Tatemoto *et al.* (2001) who found that the addition of ascorbic acid to maturation and embryo culture media resulted in a significant increase in the number of embryos. However, Miclea *et al.* (2009) and Huang *et al.* (2011) mentioned that treatment with 50 µg/mL ascorbic acid resulted in increasing the pregnancy rate and enhancing the development of somatic cell nuclear transfer embryos in pigs. Moreover, Eppig *et al*. (2000) and Wang *et al*. (2001) mentioned that the addition of ascorbic acid into the culture media improved blastocyst production. The beneficial effect of ascorbic acid addition might be due to an improved culture condition and oocytes cytoplasmic maturation by reducing the intra cellular oxidative status for embryo development (Suzuki *et al.*, 2007 and Kere *et al*., 2013).

Table 3. Effect of using ascorbic acid supplementation on fertilization rate in *in vitro* production of dromedary camel embryos

Treatment	Total number of oocytes	No. fertilized oocytes	Fertilization rate %	Chi square value
Control	67	4	5.97[a]	0.67
Ascorbic	72	7	9.72[a]	

[a]: No significant differences (P>0.05).

Conclusion

The treatment of *in vitro* maturation medium with 6.9 µM cysteine improved cumulus cells expansion, while ascorbic acid supplementation (100 µg/ml) improved the first polar body extrusion. However, the combination of both had a negative effect on both expansion and the polar body.

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Competing interests

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publications of this article. The authors declare that they have no competing interests.

Authors' contributions

Mrs. Amira Khaled Khattab did all laboratory work and all analysis, and manuscript writing. Prof. Dr. Nehal Ali Abu Elnaga designed the experiment and article revision. Dr. Hesham Attia Shedeed helped in securing biological material, collecting data, and the tabulation of experimental data. Dr. Ahmed Mohamed Kamel facilitated the laboratory activities, the tabulation of experimental data, and manuscript writing. Dr. Mervat Ahmed Bekhit Abd Rabu provided some research supplies and assisted with laboratory work, while Prof. Dr. Khalid Ahmed El Bahrawy designed the experiment, supervised the work and manuscript writing and the final revision. All authors have read and approved the final manuscript.

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