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Effects of Ascorbic Acid on Maturation Rate, Morphology, and Gene Expression of Vitrified *In Vitro* Matured Dromedary Camel Oocytes

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ABSTRACT

In vitro embryo generation, cryopreservation, and embryo transfer are examples of assisted reproductive technologies that can be used to improve camel genetic performance and fertility. The aim of this study was to investigate the impact of ascorbic acid supplementation to *in vitro* maturation media on the maturation rate, morphology, and gene expression of fresh and vitrified *in vitro* matured dromedary camel oocytes. In the current study, 810 oocytes of excellent and good quality were *in vitro* matured in maturation medium (TCM-199 + 10 ug/ml follicle stimulated hormone + 10% fetal calf serum + 100 IU/ml Pregnant mare serum + 50 µg/ml gentamycin) without any additives to act as a control group (C) and with 50 µg/ml ascorbic acid group (AA) and incubation in a CO2 incubator (38.5℃, 5% CO2, 20% O2 and 95% humidity) for 40 hours. *In vitro* matured dromedary camel oocytes with the first polar body (n = 210) in C group and AA group (n = 250) were placed in basic medium (BM) and then placed in vitrification solution1 (VS1) for one minute, followed by the transfer of oocytes to VS2 (double concentration of VS1, containing 20% Ethyl Glycol (EG) and +20% Dimethyl sulfoxide) for 30 seconds. Oocytes were then loaded into sterile 0.25 ml straws and stored in liquid nitrogen for 7-10 days. The normal fresh and vitrified /thawed *in vitro* matured dromedary camel oocytes were kept in RNA later at a -80°C freezer for gene expression analysis. The maturation rate of dromedary camel oocytes in the *in vitro* matured AA group was significantly higher than that of the C group. The percentage of normally recovered vitrified/thawed oocytes was higher in the *in vitro* matured with ascorbic acid (VAA) than in the control (VC) group. The expression pattern of the SOD1 gene and GDF9 gene was upregulated in fresh AA and VAA groups than in the fresh C and VC groups. The profile of the SOD1 gene was more abundant in the vitrified/thawed oocytes VAA group than in the VC group. All vitrified/thawed groups, whether control or ascorbic acid supplemented, had lower levels of SOD1, GDF9, and BMP15 expression, compared to the fresh groups. In conclusion, the supplementation of the maturation medium with ascorbic acid has an increased maturation rate, and normal morphology of vitrified/ thawed oocytes which was linked with upregulation of SOD1, GDF9 genes expression.

Keywords: Dromedary camel**,** Gene expression, *In vitro* maturation, Morphology, Vitrification

INTRODUCTION

Dromedary camels are among the most economically important animals. They are used for various purposes, including entertainment, transportation, racing competitions, and beauty pageants (Faraz, 2019). The limited reproductive patterns of female dromedary camels in natural conditions (induced ovulatory, seasonal breeding, infertility delays, prolonged calving period, poor expressions of estrus signs) have been highlighted as a problem (Bello and Bodinga, 2020). Therefore, progress has been made in using assisted reproductive technologies, including *in vitro* production, cryopreservation, and embryo transfer, to optimize the fertility rate and enhance the performance of genetics in camels (Tukur et al., 2020).

A crucial stage in producing mature oocytes capable of effective embryonic development is *in vitro* maturation (Hashimoto, 2009). Camel oocytes transferred from the germinal vesicle (GV) stage to the metaphase II (M II) stage. The main obstacle to oocyte maturation is reactive oxygen species (ROS) production because of an imbalance between free radical production and internal antioxidants in oocytes. This is especially true in camel oocytes because of the long maturation period of about 40 hours, which can severely damage cell membrane integrity and other critical cellular organelles, as well as genetic material (Abdelkhalek et al., 2017).

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Various approaches, such as the supplementation of external antioxidants to the maturation medium, are considered the essential defense factor against oxidative stress (Khattab et al., 2020). Several studies have indicated that supplementing the maturation medium with ascorbic acid (AA) as an antioxidant is effective in reducing or scavenging the negative effect of ROS production and increasing glutathione, which allows better nuclear maturation and subsequent embryo development in camels (Ashour et al., 2021; Kandil et al., 2022).

Vitrification is a promising cryopreservation method that enables the preservation of genetic material from germ cells (oocytes and embryos) to create gene banks and expand relevant databases for breeding and animal research programs (Arav and Natan, 2019). To solidify the cell into a glass-like state without of developing ice crystals, it is necessary to subject the oocyte to a high concentration of cryoprotectants (Penzias et al., 2021). Cryoprotectants and vitrification have been extensively used for humans (Fabbri, 2006) as well as other species, such as porcine (Zhou and Li, 2009), bovine (Hwang and Hochi, 2014) and goat (Purohit et al., 2012). Some recent studies on the verification of immature camel oocytes discovered that vitrification caused mechanical damage and reduces the potential for oocyte development (Moawad et al., 2019; Yaqout et al., 2022). Moreover, vitrification has adverse effects on mitochondrial functions, gene transcript, and camel oocyte development (Saadeldin et al., 2020; Moulavi et al., 2021).

The addition of antioxidants to the maturation medium, vitrification medium, or culture media has a good effect on the viability and development of the oocytes after thawing as well as the gene transcript (Castillo-Martín et al., 2014). Several enzymes eliminate the stress in the oocytes; therefore, superoxide oxygen anion (O2) is changed into hydrogen peroxide (H2O2) by the antioxidant enzyme Cu-Zn-Superoxide Dismutase (SOD1, Ighodaro, and Akinloye, 2018). The Transforming Growth Factor (TGF-family) of genes includes the genes for bone morphogenetic protein 15 (*BMP15*) and growth differentiation factor 9 (GDF9), which are crucial regulators for follicle development, oocyte maturation, cell proliferation, and differentiation, as well as lowering the quality of oocytes following vitrification (Paulini and Melo, 2011).

Given the importance of this issue, there are few studies on the impact of ascorbic acid (AA) supplementation on the viability and gene expression of vitrified/thawed mature dromedary camel oocytes, as well as its impact on the maturation medium. Therefore, the current study aimed to study the effect of supplementation of ascorbic acid (AA) to *in vitro* maturation medium on the viability and gene expression of vitrified/thawed *in vitro* matured dromedary camel oocytes.

MATERIALS AND METHODS

Ethical approval

This study was carried out according to standard protocols without causing discomfort or injury to the camel. Furthermore, the experimental procedure was approved by the Centre for Research and Community Service at National Research Centre, Dokki, Cairo, Egypt.

Unless otherwise noted, all the chemicals and media used in this experiment were bought from Sigma-Aldrich (St. Louis, MO, USA). The National Research Center in Cairo, Egypt's Embryo and Genetic Resources Conversation Bank, was the site of conducting the current investigation.

Collection of dromedary camel ovaries

Dromedary camel's Ovaries (n = 400) from 200 animals were taken from a slaughterhouse in El-Warraq abattoirs, Giza, Egypt, during the breeding season between December 2019 and 2020 to May 2020 and 2021. The animals were within the age range of 7-10 years and weighed 500-700 kg. The ovaries were kept in sterile normal saline solution (0.9% NaCl) with antibiotics (100 IU penicillin and 100 µg/ml streptomycin/ml) at 37°C for 1 hour before being transported to the lab. After removing extra tissues, the ovaries were washed in the laboratory three times in warm saline (0.9% NaCl). All ovaries were then promptly washed with ethanol (70%) and then with fresh normal saline and kept in a water bath (37°C). Camel oocytes were aspirated from follicles in diameter 2-8 mm using a 22-gauge needle connected to a 5-mL syringe. The aspiration medium was modified phosphate buffer saline (m-PBS) supplemented with 4 mg/ml of bovine serum albumin (BSA) and 50 µg /ml of gentamicin. The aspirated follicular fluid containing cumulus oocyte complex (COCs) was put into 15 mL conical tubes in a water bath at 37°C to allow COCs to settle for 20-25 minutes in the bottom of the conical tube. The aspirated COCs was ejected into a sterile dish (100 mm) for evaluation of the oocytes using Zeiss stereomicroscope (90 x).

Categorizing of oocytes

The oocytes were categorized into four groups of excellent, good, fair, and poor according to Kandil et al., (2014). Oocytes in the excellent group had five layers of compact cumulus cells surrounding their evenly granulated, homogeneous cytoplasm. Those in the good group had uniformly granulated, homogenous cytoplasm and 3–4 layers of compact cumulus cells. Oocytes with fragmented cytoplasm and partially surrounded by COCs were categorized as fair.

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Finally, denuded oocytes in the poor group had no granulation. Excellent and good-quality oocytes were used for further experiments.

Oocytes *in vitro* maturation

The maturation medium is composed of TCM-199 was used, supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 µg/ml gentamycin, 10 µg/ml FSH (Foltrobin V, Bioniche, Canada), and 100 IU/ml pregnant mare serum (sterilized using syringe filter 0.22 um, thermos fisher). Selected excellent and good COCs (n = 810) oocytes with round and homogenous cytoplasm were washed twice in modified phosphate buffer saline (M-PBS) and three times in the maturation medium. Maturation medium without any additives acted as the control group (C) containing 420 oocytes. Another group (AA) matured in maturation media + 50 µg/ml ascorbic acid, containing 390 oocytes. Each group of oocytes was cultured in 500 µL of *in vitro* maturation medium in Nunc™ 4-well dishes (Thermo Fisher, Waltham, MA, USA) for 40 hours at 38.5°C in a 5% CO2 incubator with 95% humidity. Thirteen biological replicates were used to evaluate the *in vitro* maturation of camel oocytes. The first polar body (M II) in the perivitelline space indicated that the oocytes reached nuclear maturity. The first polar body detection was carried out using a 20X inverted microscope.

Maturation rate = (Number of matured oocytes (M II)/ Number of Excellent and Good quality oocytes) × 100.

Vitrification/thawing procedures

Vitrification procedure

In vitro matured dromedary camel oocytes with the first polar body in the control group (n = 210) and AA group (n = 250) were placed in BM (9.5 ml TCM 199 + 0.5 ml FCS + 50 µg/ml gentamicin). Oocytes were equilibrated for 1 minute, then moved to vitrification solution one (VS1, 10% Ethyl Glycol (EG) + 10% Dimethyl sulfoxide (DMSO), followed by vitrification solution two (VS2, 20% EG + 20% DMSO) for 30 seconds. Oocytes were loaded in holding media (BM + 0.5 Mol sucrose).

Oocytes loading

Oocytes were loaded into 0.25 ml French straw using a micro-classic pipette (Karl Hecht No. 558). The oocytes in the vitrification solution separated from the holding medium by two air bubbles from two sides, The loaded straw was sealed, and groups of around 25-30 oocytes were loaded into the straw. After 10 seconds of exposure to liquid nitrogen (LN2) vapor, the straw was submerged in LN2 and stored for 7 days (Ismail et al., 2022).

Oocytes thawing

Oocytes vitrified in straws were submerged in a water bath at 37°C for 10-15 seconds. Oocytes that had been vitrified and warmed were immediately transferred to a new medium comprising BM + 0.5 Mol (M) sucrose. Using a three-step process and a one-minute equilibration period in each solution, sucrose was successively diluted to concentrations of 0.5, 0.33, 0.17,0 M in BM. After that, three fresh BM washes were performed on the oocytes (Ismail et al., 2022).

Morphological evaluation

Oocytes that had been recovered were examined under an inverted microscope. The number of retrieved oocytes in the control group (n = 160) and AA group (n = 200) were evaluated. Oocytes with morphological modifications of membrane damage, swelling, degeneration, or leakage of cellular material, ruptured zona pellucida or vitelline membrane, and fragmented cytoplasm in control (n = 60) and AA groups (n = 50) were counted as abnormal. The numbers of normal morphology and surviving oocytes in the control group and the AA group were 100 and 150, respectively. The percentage of recovered vitrified oocytes= $\frac{\text{number of recovered oocytes after thawing}}{\text{total number of vitrified oocytes}} \times 100$

Gene expression of fresh and vitrified-thawed *in vitro* matured camel oocytes

RNA extraction and cDNA synthesis

Oocytes in the fresh group (90 oocytes for control and 65 for AA groups) and oocytes in the vitrified/ thawed group (110 for control and 150 for AA groups) were kept in RNA later in cryogenic vials (Corning Incorporated, Corning, NY, USA) and directly put into a -80°C freezer for later analysis. Following the kit's instructions, total RNA isolation was performed using a PicoPureTM RNA isolation kit (Arcturus, Thermo Fisher, Lithuania). All oocyte groups were mixed with extraction buffer and incubated for 30 minutes at 42°C in Thermo Block. The complete lysis was loaded into a pre-conditioned spin column and centrifuged for 2 minutes at 1000 rpm to allow the RNA to bind to the spin column, followed by 13250 rpm/30 seconds. DNA was removed using a column RNase-free DNase kit (Qiagen GmbH, Hilden, Germany). The column was washed twice using two different wash buffers (WB1 and WB2). Finally, RNA was eluted with 12 µl of RNase-free water. The extracted and quality of RNA were evaluated by NanoDrop 2000 (Thermo Fisher, USA) with 260/280 and 260/230 values ≥1.8. The QuantiTect Reverse Transcription (Qiagen, Germany) kit was used to

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perform cDNA, which was first adjusted to reach the same RNA concentration by adding RNase-free water. RNA sample of 14.0 µ1 was added to 4 µ1 5X RT buffer, 1 µ1 RT primer mix, and 1 µ1 Quantiscript reverse transcriptase. The mixture was incubated at 25°C for 10 minutes, 37°C for 120 min, 85°C for 5 minutes, and held at 4°C. The cDNA samples were stored at -20°C until real-time PCR quantification.

Real-time polymerase chain reaction

The real-time relative quantitative PCR was performed by QuantStudio RT-PCR (Applied Biosystems, USA) using Maxima SYBR Green QPCR Master Mix (Thermofisher, Lithuania). The reactions were 2 µl cDNA, 1µl each of forward (5 mM) and reverse primers (5 mM), 8.5 µl nuclease-free water, and 12.5 µl SYBR Green PCR Master Mix in a total reaction volume of 25 µl (96-well plates). RT-PCR was performed on the thermal cycler with the condition of 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute, and 72 °C for 40 seconds. The PCR reactions were run in triplicates, and *GAPDH* was used as a reference gene. The fold change and relative quantity of the target transcripts (SOD1, BMP15, GDF9) were calculated using the 2−ΔΔCt method (Schmittgen and Livak, 2008). Reactions without reverse transcriptase or cDNA template were used as negative controls, which resulted in no amplification. PCR primers were designed using the NCBI (Table 1).

Table 1. Primers sequences and GenBank accession numbers for dromedary camel

Gene name	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size (bp)	Accession no.	References resources
GAPDH	AGGTCGGAGTGAACGGATTC	GGAAGATGGTGATGGCCTTT	219	XM_010990867.2	MODEL Camelus dromedarius (Arabian camel)
CuZn-SOD (SOD1)	TGCAGGCCCTCACTTTAATC	CTGCCCAAGTCATCTGGTTT	216	JF758876.1	MODEL Camelus dromedarius (Arabian camel)
BMP15	GCCACTACTTTGCCCCTGAT	GGGGTGCAATGATCCAGTGA	167	XM_010998065.1	MODEL Camelus dromedarius (Arabian camel)
GDF9	CCATCAGTGGACCTGCTGTT	CACTGAGGGGTCGAGCTTTT	131	XM_010981399.1	MODEL Camelus dromedarius (Arabian camel)

bp: Base pair, no: Number

Statistical analysis

Data were expressed as mean ± standard error (SE). Statistical analyses were performed using SPSS version 16.0 and the ANOVA test (one-way analysis of variance). The significance of differences ($p < 0.01$) was tested using LSD.

RESULTS

Cytoplasmic and nuclear maturation of dromedary camel oocytes

The present study showed no significant difference in the cumulus expansion rate of camel oocytes matured *in vitro* in a maturation medium containing 50 µg/ml AA and control group (55.38% and 60.71%, respectively). The extrusion of the first polar body significantly ($p < 0.01$) increased (80.77%,315/390) in camel oocytes matured *in vitro* in the maturation medium containing 50 µg/ml AA, compared to the control group (71.43%, 300/420) as shown in Table 2 and Figure 1.

Viability and morphology of vitrified /thawed mature dromedary camel oocytes

The results indicated that after thawing, the number of recovered *in vitro* matured dromedary camel oocytes in maturation medium containing 50 µg/ml AA was higher than that matured in a control medium (80% (200/250) vs. 76.19% (160/210), respectively, $p < 0.01$). This was reflected in the normal morphological rate, which significantly increased in oocytes matured *in vitro* in a maturation medium containing 50 µg/ml ascorbic acid ($p < 0.01$, 75 %), compared to that matured oocytes in the control group (62.5%, Table 3). The morphological abnormalities were higher in the vitrified warmed matured camel oocytes in the control group ($p < 0.01$, 37.5%), compared to mature camel oocytes supplemented *in vitro* maturation with 50 µg /ml ascorbic acid (25%). Cytoplasm shrinkage and fragmentation was observed as the most frequent abnormalities were observed in the vitrified/thawed control group less (50% and 33%,

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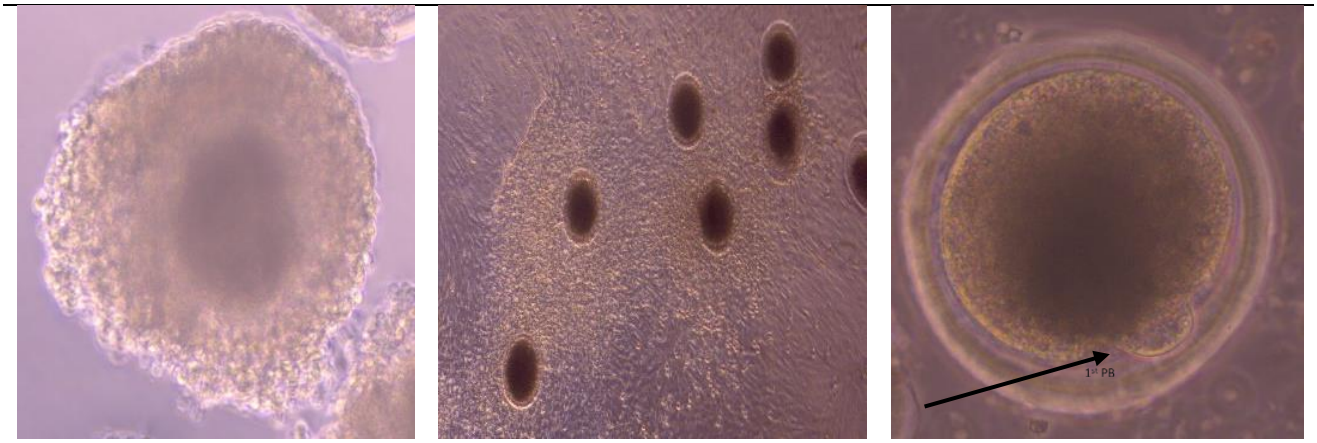
respectively) than vitrified/thawed supplemented oocytes in AA group (10% and 10%, respectively). As shown in Table 4 and Figure 2, zone breaking and cellular content leakage were lower in the vitrified/ thawed control group (8.33%, and 8.33%, respectively) than AA group (60% and 20%, respectively).

Table 2. The Effect of ascorbic acid supplementation to the maturation media on cumulus expansion, polar body extrusion of the dromedary camel oocytes

Group	COC (n)	Maturation rate		
		Cumulus expansion rate (%)	(GII and GIII)	Polar body extrusion (%)
Control	420	60.71%[a]		71.43%[a]
Ascorbic acid	390	55.38%[a]		80.77%[b]

n: Number of Oocytes, SE: Standard error, COC: Cumulus-oocyte complex; [a,b] Values with different superscripts within a column are significantly different at $p < 0.01$



Cumulus oocyte complex of camel oocytes (A) | Mature camel oocytes judged by the expansion of cumulus cells (B) | Extrusion of the first polar body of mature camel oocytes (C)

Figure 1. Steps of dromedary camel oocytes maturation using inverted microscope 20X (Zeiss). A: Cumulus oocyte complex, B: Mature camel oocytes judged by the expansion of cumulus cells, C: Extrusion of the first polar body

Table 3. Effects of the ascorbic acid on the viability and morphology of the vitrified/ thawed mature dromedary camel oocytes

Group	No. of oocytes	No. of recovered oocytes	Morphologically normal oocytes %	Morphologically abnormal oocytes %
Vitrified/thawed control	210	160	62.5%[b]	37.5% [a]
Vitrified/thawed ascorbic acid	250	200	75% [a]	25% [b]

n: Number of Oocytes; [a, b] Values with different superscripts within a column are significantly different at $p < 0.01$

Table 4. Effects of the ascorbic acid on types of the abnormal mature dromedary camel oocytes after vitrification/ thawing (percentage=%)

Group	No. of abnormal oocytes	Types of oocytes abnormal after vitrification/ thawing %			
		Zone breaking	Leakage of cellular content	Shrinking cytoplasm	Fragmented cytoplasm
Vitrified/ thawed control	60	8.33%[b]	8.33%[b]	50%[a]	33.33%[a]
Vitrified/ thawed ascorbic acid	50	60% [a]	20%[a]	10 %[b]	10%[b]

[a,b] Values with different superscripts within a column are significantly different at $p < 0.01$

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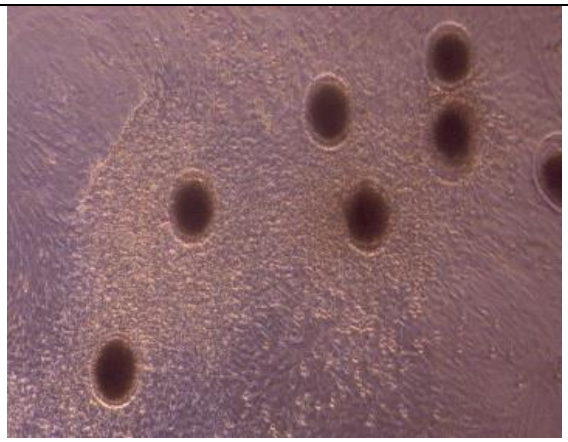
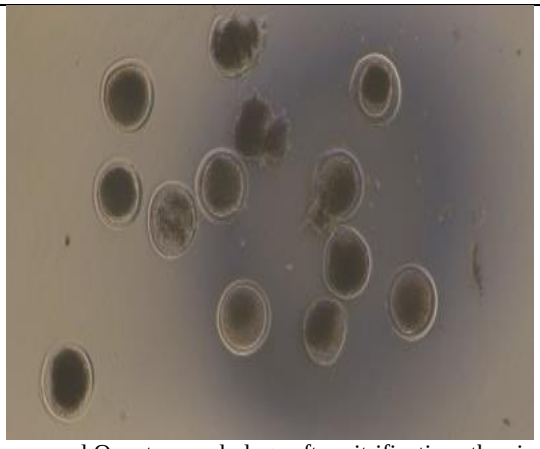
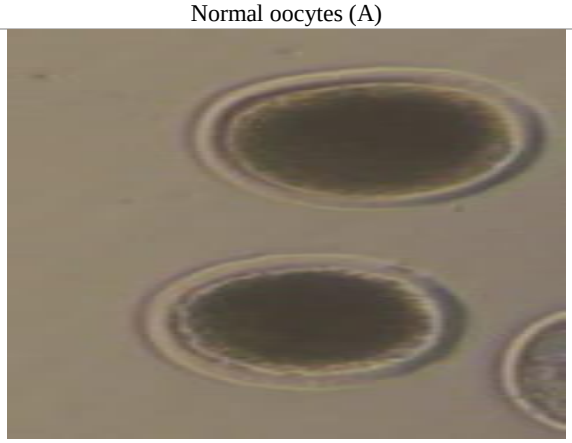


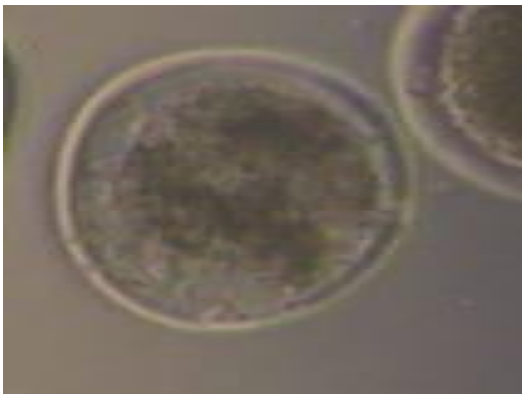
Normal oocytes (A)



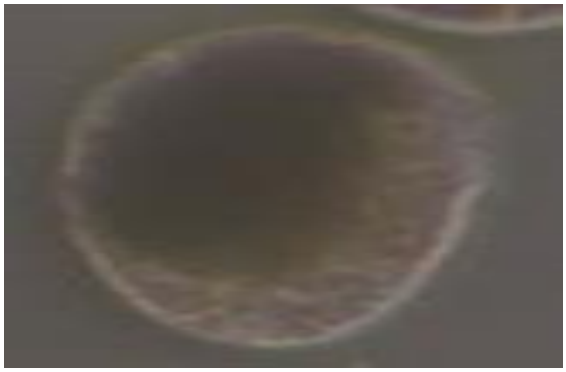
Mature camel Oocyte morphology after vitrification- thawing (B)



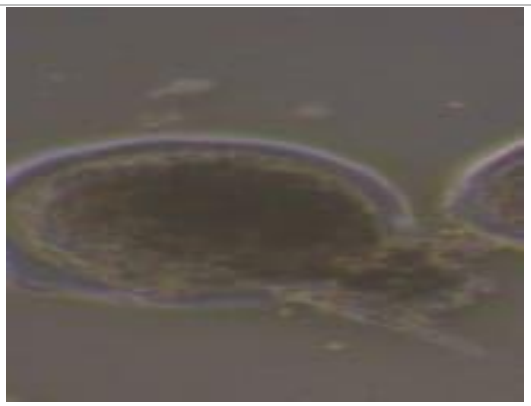
Normal vitrified-thawed mature camel oocyte with spherical shape (C)



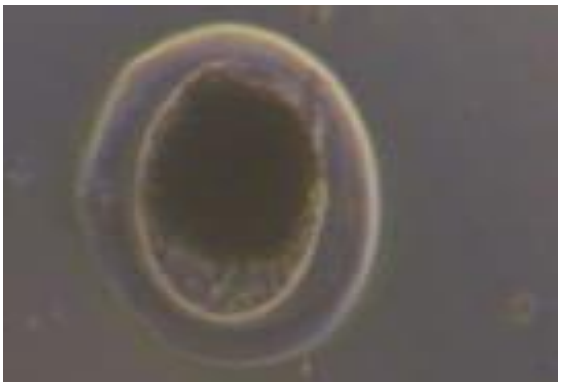
Abnormal vitrified-thawed mature camel oocyte with heterogeneous cytoplasm (D)



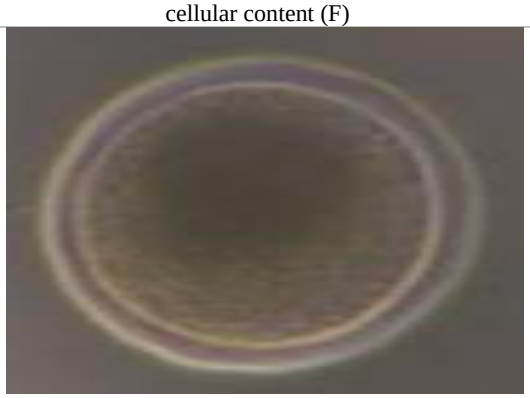
Abnormal vitrified-thawed mature camel oocyte with zone breaking (E)



Abnormal vitrified-thawed mature camel oocyte with leakage in cellular content (F)



Abnormal vitrified-thawed mature camel oocyte with shrined (G)



Abnormal vitrified-thawed mature camel oocyte with shrined fragmented cytoplasm (H)

Figure 2. Normal dromedary camel cumulus-oocyte complex COCs (A). Oocyte morphology after vitrification- thawing (B), Normal vitrified-thawed dromedary mature camel oocyte with spherical shape (C), Abnormal vitrified-thawed mature dromedary camel oocyte with heterogeneous cytoplasm (D), Abnormal vitrified-thawed mature dromedary camel oocyte with zone breaking (E), Abnormal vitrified-thawed mature dromedary camel oocyte with leakage in cellular content (F), Abnormal vitrified-thawed mature dromedary camel oocyte with shrined cytoplasm (G), Abnormal vitrified-thawed mature camel dromedary oocyte with fragmented cytoplasm (H). The pictures were taken with an inverted microscope (Zeiss) 20X.

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SOD1 Gene expression of fresh and vitrified-thawed mature camel oocytes

The effect of AA on the relative expression SOD1 gene on the fresh group (control vs. supplemented with 50 µg/ml AA) and the vitrified-thawed group (control vs. supplemented with 50 µg/ml AA) in the mature camel oocytes were investigated. The gene expression of *SOD1* in mature camel oocytes was significantly ($p < 0.01$) higher in fresh oocytes supplemented with AA (2.05 ± 0.01) when compared with the control group (1.0 ± 0.05). Moreover, SOD1 gene expression was significantly ($p < 0.01$) higher in vitrified /thawed oocytes supplemented with AA (0.54 ± 0.02) when compared with the vitrified/ thawed oocytes control group (0.22 ± 0.04). As shown in Table 5 and Figure 3, the gene expression of SOD1 in mature camel oocytes supplemented with AA in either fresh or vitrified/ thawed group was significantly double upregulated, compared to mature dromedary camel oocytes in the control group, either fresh or vitrified/ thawed ($p < 0.01$).

GDF9 gene expression of the fresh group and vitrified-thawed group of the mature camel oocytes

The relative expression GDF9 *gene* for fresh mature oocytes supplemented with AA was significantly higher (2.05 ± 0.21) than the fresh mature control (1.0 ± 0.01, $p < 0.01$). In addition, the expression of *GDF9* in vitrified mature oocytes supplemented with AA was significantly higher (0.75 ± 0.07), than the vitrified mature control group (0.65 ± 0.24, $p < 0.01$), as shown in Table 6 and Figure 4.

***BMP15* gene expression in the fresh group and a vitrified-thawed group of the mature camel oocytes**

There was no significant change in gene expression of *BMP15* in fresh mature camel oocytes in control (1.0±0.01) and fresh mature camel oocytes supplemented with AA (1.11 ± 0.03). Similarly, there was no significant change in the expression of *BMP15* in vitrified / thawed mature camel oocytes in control (0.25 ± 0.01) and vitrified / thawed mature camel oocytes supplemented with AA (0.24 ± 0.07) as shown in Table 7 and Figure 5. There was significant ($p < 0.01$) upregulation on relative BMP15 gene expression in fresh (C and AA group) when compared with vitrified/thawed *in vitro* matured camel oocytes (VC and VAA).

Table 5. The Effect of ascorbic acid on the SOD1 gene on the fresh group and vitrified-thawed group of the mature dromedary camel oocytes

Groups	SOD1 gene (Fold change)
Fresh mature camel oocytes	1.0 ± 0.01^{b}
Fresh mature camel oocytes supplemented with ascorbic acid	2.05 ± 0.05^{a}
Vitrified mature camel oocytes	0.22 ± 0.02^{c}
Vitrified mature camel oocytes supplemented with ascorbic acid	0.54 ± 0.04^{d}

[a,b,c,d] Values with different superscripts within a column are significantly different at $p < 0.01$

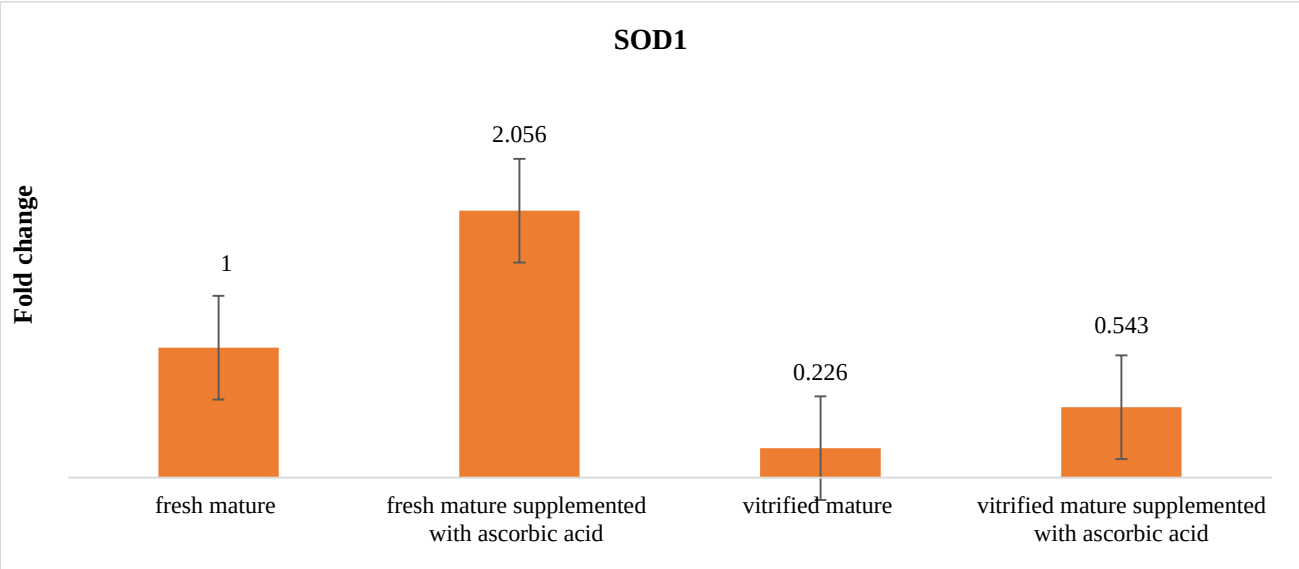

Figure 3. The effects of ascorbic acid on the SOD1 gene on the fresh group and a vitrified-thawed group of the mature camel oocytes

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Table 6. The effects of ascorbic acid on the GDF9 gene on the fresh group and vitrified-thawed group of mature camel oocytes

Groups	GDF9 gene (Fold change)
Fresh mature camel oocytes	1.0 ± 0.01^{b}
Fresh mature camel oocytes supplemented with ascorbic acid	2.05 ± 0.07^{a}
Vitrified mature camel oocytes	0.65 ± 0.21^{c}
Vitrified mature camel oocytes supplemented with ascorbic acid	0.75 ± 0.24^{d}

[a,b,c,d] Values with different superscripts within a column are significantly different at $p < 0.01$

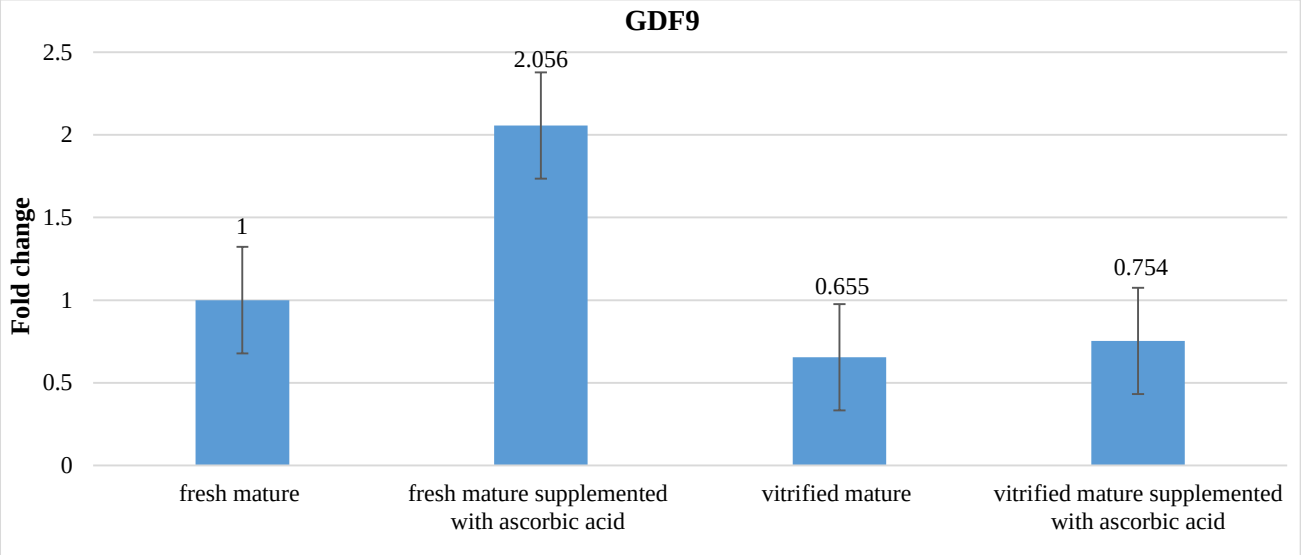

Figure 4. The effect of ascorbic acid on the GDF9 gene on the fresh group and a vitrified-thawed group of the mature camel oocytes

Table 7. The Effect of ascorbic acid on the BMP15 gene on the fresh group (control and supplemented with ascorbic acid) and vitrified-thawed group (control and supplemented with ascorbic acid) mature dromedary camel oocytes

Groups	BMP15 gene (Fold change)
Fresh mature	1.0 ± 0.01^{a}
Fresh mature supplemented with ascorbic acid	1.11 ± 0.03^{a}
Vitrified mature	0.24 ± 0.07^{b}
Vitrified mature supplemented with ascorbic acid	0.25 ± 0.01^{b}

[a,b] Values with different superscripts within a column are significantly different at $p < 0.01$

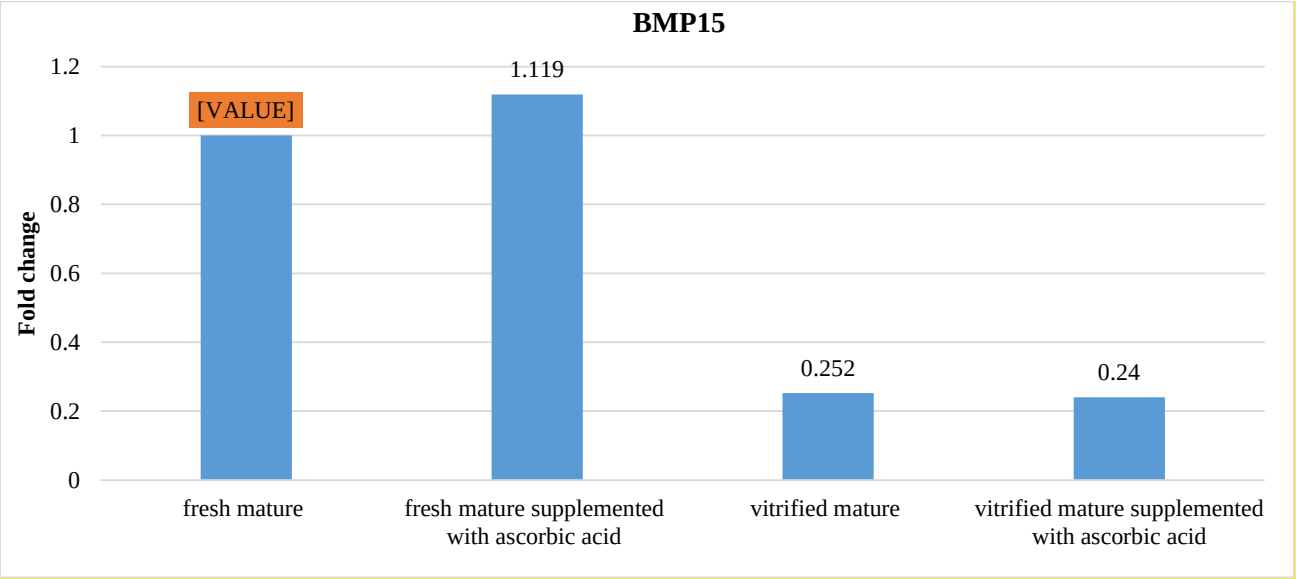

Figure 5. The effect of ascorbic acid on BMB15 gene expression of the fresh group and vitrified-thawed group of the mature camel oocytes

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DISCUSSION

Due to its participation in the first line of antioxidant defense, AA plays a critical function in the prevention of oxidative damage to proteins and lipid membranes (Njus et al., 2020). Since vitamin C is a water-soluble molecule, it can work both within and outside of cells, neutralizing free radicals and reducing the production of ROS formation (Pehlivan, 2017). The results of the current study revealed a significant increase in the maturation rate of dromedary camel oocytes *in vitro* matured in a maturation medium containing 50 µg AA when compared with control group. These findings are in line with those of Kere et al. (2013) who found that adding 50 µg/ml of a ascorbic acid to porcine oocytes in maturation medium or culture medium and reduced apoptotic index and ROS content, improved the blastocyst and increase the number of cells overall. This supports the idea that AA is a potent antioxidant due to its ability to sustain redox status in different species and promote cell growth, proliferation of mammalian cells and decrease apoptosis (Moussa et al., 2020).

According to Sovernigo et al. (2017), bovine oocytes with considerably elevated intracellular glutathione had lower levels of oxidative stress, greater developmental competence, and a higher rate of blastocyst formation in bovines. Ascorbic acid dramatically improved the nuclear maturation of canine oocytes in metaphase I and M II at a concentration of 250 M (26.98% against 6.00%). However, AA (50 µg/ml) has no appreciable influence on maturation, fertilization, or embryo development parameters in porcine oocytes (Nohalez et al., 2018). All these variations in results could be attributed to genetic material, oocyte age, dosage effects on membrane integrity, and differences in species.

Cryopreservation is the process of preservation of living cells (germ cells, oocytes, sperm, and embryos) and tissues at extremely low temperatures. To preserve the genetic resource of the superior genetic animals in gene banks or for future use in *in vitro* fertilization and embryo transfer (Pegg, 2015). The development of the oocyte has been severely influenced by vitrification. The results of the current investigation showed that AA significantly improved the recovery rate of vitrified/thawed mature camel oocytes and reduced the appearance of abnormal oocyte morphology in comparison to the control. These findings are in agreement with Sonowal et al. (2017) who reported that the addition of a specific dose (100 M) of Vitamin C to *in vitro* maturation medium when used with immature bovine oocytes, increased the growth of vitrified-thawed bovine oocytes in terms of cumulus cell expansion and polar body formation after maturation. However, Chaves et al. (2017) found that immature COCs were more resistant to the effects of cryoprotectants than mature COCs and this result was confirmed by superior embryo growth (cleavage) after vitrification. According to Al-Soudy et al. (2016), mature camel oocytes were more resistant to cryo-injuries than immature oocytes and produced a high percentage of normal oocytes, which could be useful for future *in vitro* fertilization and camel improvement initiatives. Additionally, Castillo-Martín et al. (2015) revealed that L-ascorbic acid addition to culture and/or vitrification media increases porcine blastocyst survival rates. Nohalez et al. (2018) demonstrated that AA addition to vitrification and warming conditions increased the survival of *in vitro*-produced porcine blastocysts by reducing ROS production. Because vitrification has negative on morphology by increasing the frequency of defects including zona shattering, an increase in perivitelline space, leakage of cellular content, and cytoplasm fragmentation and non-symmetrical oocyte shape, it has a negative impact on oocyte morphology.

These abnormalities can be attributed to cooling and warming, which damaged the cytoskeleton and cause cellular degeneration (Allworth and Albertini, 1993). Osmotic stress is thought to be another factor that can induce damage in oocytes and alter their volume and negatively impact their viability (Mullen et al., 2007). This might be because camel oocyte cytoplasm is extremely susceptible to cryopreservation. In addition, there are many cryoprotectants (20% DMSO, 20% EG) in vitrification medium that could negatively affect oocyte viability. Furthermore, Yassen et al. (2020) reported that the survival rate, morphological characteristics, and ultrastructural quality were all improved when vitamin C or zinc chloride was added to the vitrification medium in bovine oocytes. These findings are consistent with the role of ascorbic acid in the reduction of abnormalities observed in the present results.

The results of this study indicate that SOD1 gene expression had dramatically increased in the groups supplemented with ascorbic acid (AA) either in fresh or vitrified/thawed oocytes. This is agreement with Fang et al. (2022), that reported upregulation of the antioxidant gene (SOD1) in porcine oocytes that in vitro matured in medium supplemented with ascorbic acid. According to the findings of the current study, vitrification causes lower expression in all groups whether control or AA-supplemented, than the fresh group. This suggests that vitrification can modify the mRNA content of the oocyte and change the expression of genes related to stress. This finding is consistent with Park and Kim (2014) who found an increase in the expression pattern of the SOD1 gene in fresh when compared with vitrified canine oocytes. Similarly, Habibi et al. (2010) found that expression of the SOD 1 gene was significantly up-regulated in vitrified IVM oocyte when compared with fresh IVM in immature human oocytes. On the contrary, Turathum et al. (2010) detected no variations in SOD1 gene expression in fresh or vitrified canine oocytes using real-time polymerase chain reaction (RT-PCR). Castillo-Martín et al. (2014), reported that addition of ascorbic acid during culture and vitrification/ warmed upregulated the expression of GPX1 and SOD1 genes in porcine blastocyst.

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GDF9 and BMP15 are important regulators of follicular development, oocyte maturation, and embryo quality. The results of this investigation showed that in the fresh and vitrified groups, AA supplementation significantly increase the relative expression of GDF9 when compared with to control groups and had no effect on the expression of BMP15 in control or other AA-supplemented oocytes. Roshan et al. (2021), reported that the addition of L-ascorbic acid to the *in vitro* maturation medium of porcine oocytes at both concentrations (50- and 100 M doses of L-ascorbic acid) did not significantly affect GDF9 and BMP15 genes expression. However, Yu et al. (2018) found that L-ascorbic acid (Vitamin C) can enhance meiotic maturation and developmental competence and increase BMP15 mRNA levels in porcine cells. In the current study the GDF9 and BMP15 genes relative expression pattern in fresh group was significantly increased than the vitrified /thawed group even in VC or VAA group. This results in agreement with Azari et al., (2017), who found that, bovine oocytes in the control group showed the highest expression level of GDF9 and BMP15 in compared to the vitrification groups. Unlike Di Pietro et al., (2010) revealed that the vitrification protocol keeps unaltered the human oocyte molecular profile including BMP15 and GDF9 does not cause messenger RNA degradation. These results deference might be due to the various breeds, vitrification protocol, variation in cryoprotectants and ascorbic acid concentrations employed during the *in vitro* maturation of oocytes.

CONCLUSION

The addition of ascorbic acid in *in vitro* maturation media improves the maturation rate and sustained the normal morphology of vitrified/thawed dromedary camel oocytes. Moreover, ascorbic acid upregulates SOD1 and GDF9 gene expression either in fresh or vitrified/thawed *in vitro* matured dromedary camel oocytes that supported the viability after thawing.

DECLARATIONS

Competing interests

The authors confirm that they do not have any conflicts of interest.

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Authors' contribution

Omaima Kandil designed the experiment, and supported all the equipment, chemicals, primers, and kits, Fatma Aboelwafa brought the samples and did lab work, Esraa Ismael did the gene expression and data analysis, Sahar Kandeel supplied the experiments with ascorbic acid and some chemicals for vitrification, Naser Ghanem support experiment with some primers and share in statistical analysis, Omaima Kandil, Fatma aboelwafa and Abd Elkader Gamal El-Dean write the manuscript and analysis of data. All authors confirmed the final analyzed data and the last revised article before publication in the present journal.

Ethical considerations

Ethical considerations (e.g., plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been made by the authors.

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