Effect of ascorbic acid supplementation on *in vitro* production of camel embryos cultured under oxidative stress

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Abstract:

The aim of the present study was to evaluate the effect of ascorbic acid supplementation to *in vitro* culture (IVC) medium on the development of dromedary camel embryos. Cumulus-oocyte complexes (COCs) of camels were recovered from ovaries collected from a local Egyptian abattoir. The quality of harvested COCs was evaluated morphologically and good quality COCs were used in the standard protocol of *in vitro* embryo production, including *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). The presumptive zygotes were incubated in culture media supplemented with different concentrations of ascorbic acid. The presumptive zygotes were allocated into four treatments of ascorbic acid, namely, culture medium without ascorbic acid (control: T1), 50 µg/ml (T2), 100 µg/ml (T3) and 150 µg/ml of ascorbic acid (T4) in 5% CO_2, at 38.5 ˚C and in a moist atmosphere for 6 days. Results showed that the cleavage rate of T4 group was significantly higher than the T1, T2 and T3 groups (27.5% vs 14.29%, 13.3% and 16.67%, respectively). All groups supplemented with ascorbic acid (T2, T3 & T4) were significantly higher in blastocyst rate compared to the control group (10%, 16.67%, 25% and 2.04%, respectively). Meanwhile, no significant difference was observed among ascorbic acid treatments. In the same context, the proportion of blocked embryos at morula stage was significantly higher in the control group (10.2%) than the groups supplemented with ascorbic acid (T2, T3 and T4) (3.33%, 0% and 2.5%). In conclusion, ascorbic acid supplementation in general enhanced the development rate of *in vitro* produced dromedary camel embryos and the highest concentration, 150 µg/ml of ascorbic acid, had a significant positive effect on both cleavage and blastocyst rates compared to the non-treated group. However, more research on ascorbic acid supplementation at higher concentrations is required, as the best results were obtained using the highest concentration (150 µg/ml).

Key words: *in vitro*, embryos, dromedary, culture medium, ascorbic acid.

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Introduction

Despite the advanced progress that has been made in procedures for *in vitro* maturation, fertilization and culture of oocytes, the percentage and quality of *in vitro* produced embryos are still less than for *in vivo* produced embryos. Comparing these two types of embryos, *in vivo* produced embryos that showed superiority in many traits, such as total cell numbers, lipid content, tolerance to cryopreservation and chromosomal abnormalities (Corrêa *et al.,* 2008). Plenteous evidence has been displayed over the last two decades, demonstrating that the developmental potential of embryos produced *in vitro* is ultimately dependent on the quality of the oocyte (Lonergan *et al.*, 2003; Sirard *et al.*, 2003; Rodriguez and Farin, 2004). However, there is more evidence that the quality of embryos is influenced by the culture environment (Van Soom *et al.*, 2002; Rizos *et al.*, 2003; Yuan *et al.*, 2003). In fact, many factors can affect the culture environment, such as the composition of media and protein supplementation (Khurana and Niemann, 2000), as well as the number of embryos in the culture drop and the levels of the gas atmosphere (Fukui *et al.*, 2000). Among those factors, oxidative stress (OS), which is induced by greater oxygen tension, has received special attention (Ali *et al.*, 2003; Bedaiwy *et al.*, 2004; Fatehi *et al.*, 2005). One of the most important differences between *in vivo* and *in vitro* environments is the oxygen level. Oxygen (O_2) concentration in air (20%), which is generally employed in an embryo culture system, is considerably greater than the O_2 tension in the oviduct and uterus (Fischer and Bavister, 1993). In various species, *in vitro* embryo development greatly increased when the oxygen level was reduced from 20% to 5% (Takahashi *et al.*, 2000; Yuan *et al.*, 2003; Kitagawa *et al.*, 2004). Those results proposed that high levels of O_2 tension amid the culture was hindering embryonic development and was likely due to the accumulation of reactive oxygen species (ROS). Indeed, the embryo undergoes OS when the production of ROS is higher than the cells' capacity to produce antioxidants (Burton and Jauniaux, 2011). However, the physiological antioxidants produced by embryos during *in vitro* culture are not sufficient to prevent OS (Ali *et al.*, 2003). Therefore, providing embryos with exogenous antioxidant may be necessary. Therefore, the aim of this study is to investigate the effect of supplementing *in vitro* culture media with different levels of ascorbic acid, as antioxidant, on the development of *in vitro* produced dromedary camel embryos under oxidative stress.

Materials and methods

Experimental site

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.

Ethical approval

This experiment was performed according to all ethics and animal rights (Desert Research Center). As much as possible, this work considered all rules and regulations in conformity with the European Union directive for the protection of experimental animals (2010/63/EU).

Chemicals and media

Unless otherwise mentioned, all the chemicals and media constituents were purchased from Sigma-Aldrich Chemicals, Germany. All media were prepared overnight 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 as described by Russo *et al.* (2014).

Biological material

Ovaries of dromedary camels (*Camelus dromedarius*) were collected from animals, with unknown reproductive histories, that were slaughtered in El-Bassatin slaughterhouse located in Cairo. The ovaries were kept in a thermos flask container containing warm saline solution (NSS, 0.9% NaCl), at 35 to 37 ºC and supplemented with antibiotic antimycotic (AA) (100 IU penicillin and 100 µg streptomycin/ml) and transported to the laboratory within 2-3 h of the animals' slaughter. Cryopreserved semen used in this experiment was obtained from the Artificial Insemination and Embryo Transfer Lab - Marriott Research Station - Desert Research Center, Alexandria, Egypt in 0.5 ml straws.

In vitro maturation

Cumulus-oocyte complexes were harvested from the ovaries' surfaces by slicing in a 9 cm petri dish followed by rinsing with warm (30 ºC) phosphate-buffered saline (PBS) supplemented with 50 µg/ ml gentamicin (Ashour et al., 2020; El-Sayed et al., 2015). The recovered solution containing COCs was examined under a stereomicroscope (GX microscope, UK, Range: 8x to 50x) to pick out and evaluate COCs. Cumulus-oocyte complexes were morphologically evaluated according to Ashour et al. (2020) and El-Sayed et al. (2015).

A total number of 328 grade A oocytes (more than 5 layers of cumulus cells and homogeneous dark cytoplasm) were collected and distributed into the four experimental groups. Selected oocytes were washed twice with tissue culture medium-199 (TCM-199) supplemented with 25 mM HEPES, 5% fetal bovine serum (FBS) and finally washed in IVM medium consisting of TCM-199 supplemented with 15% (v/v) heat-treated FBS (56 ºC for 30 min), 40 IU pregnant mare serum gonadotropin (PMSG), 1 µg/ml estradiol (E2), 0.25 mg/ml Na^+ pyruvate, 20 ng/ml epidermal growth factor (EGF) and 50 µg/ml gentamicin. Afterward, COCs were incubated in drops of pre-incubated (at least two hours before used) maturation media (15 to 25 oocytes/ 100 µl drop) in a 35 mm petri dish, covered with mineral oil and incubated in a CO_2 incubator for 30 hours at 38.5 ºC under 5% CO_2, 20% O_2, 75% N_2 and 95% relative humidity (Ashour et al., 2020; El-Sayed et al., 2015).

In vitro fertilization

Semen preparation

Two straws were thawed at 37˚C for 40 seconds (El-Bahrawy, 2017). Thereafter, the straws were wiped with 70% ethyl alcohol before being opened. The frozen thawed semen were over layered with 5 ml of sperm washing medium (Sperm TALP medium). Afterwards, sperm were centrifuged twice at 300xg for 5 min each. The final pellet was re-suspended in appropriate volume (depending on the sperm concentration after washing, measured by a hemocytometer slide) of pre-warmed fertilization medium (Fert. TALP medium). Finally, the sperm concentration was adjusted to $3x10^6$ sperm/ml (Khattab et al., 2020).

In vitro fertilization

Matured COCs were washed twice with washing medium (TCM-199 supplemented with 25 mM HEPES, 5% FBS), then washed three times with fertilization medium, followed by co-culture with the prepared sperm in a four-well plate (400 µl sperm suspension fertilization medium covered with 400 µl mineral oil per well) in a CO_2 incubator at 38.5 ˚C in a moist atmosphere of 5% CO_2, 20% O_2 and 75% N_2 for 18h, according to Khattab et al. (2020).

Denudation of cumulus-oocyte complexes

After co-incubation, fertilized oocytes were washed three times with washing medium then incubated for two minutes in 200 µl drop of 80 IU/ml Hyaluronidase (Moura et al., 2017) in FertiCult flushing medium (Fertipro©, Beernerm, Belgium). This step was followed with repeat pipetting in washing medium to remove cumulus cells and any sperm attached. The denuded oocytes were checked under a stereomicroscope.

In vitro culture

Immediately after denudation, the presumptive zygotes were washed with in vitro culture medium consisting of TCM-199 (Öztürkler et al., 2010) supplemented with 1X MEM essential amino acids, 1X MEM nonessential amino acids, 5% FBS, 50 µg/ ml gentamicin and different concentrations of ascorbic acid according to the experimental group. Embryos were incubated in a CO_2 incubator at 38.5 °C in a moist atmosphere of 5% CO_2, 20% O_2 and 75% N_2 in air for 6 days. The in vitro culture medium of dromedary camel embryos was supplemented with three different concentrations of ascorbic acid: 50 µg/ml (T2), 100 µg/ml (T3) and 150 µg/ml (T4), in addition to a control group (T1) without ascorbic acid supplementation.

Statistical analysis

Data from this experiment were analyzed by Chi square analysis using SAS/STAT® 9.2 program (SAS, 2008). The analyzed data were expressed as mean ± standard error (SE) of means (SEM). Comparisons were significantly different if $P<0.05$.

Results

The highest concentration of ascorbic acid (T4: 150 µg/ml) had a positive significant ($P < 0.05$) effect on cleavage rate (27.5%) compared with the control and the other ascorbic acid supplemented groups. On the other hand, the low concentration of ascorbic acid in the T2 group had no significant difference on cleavage rate (13.33%) when compared with the control group (14.29%) as well as the T3 group (16.67%) (Table 1).

Table 1. Effect of supplementing *in vitro* culture (IVC) medium of dromedary camel embryos with ascorbic acid on cleavage rate.

Ascorbic acid conc.	No. Oocytes	No. Cleavage	Cleavage rate (%)
Control (T1)	98	14	14.29 ± 10.7^{b}
50 µg (T2)	90	12	13.33 ± 10.7^{b}
100 µg (T3)	60	10	16.67 ± 10.7^{b}
150 µg (T4)	80	22	27.5 ± 10.7^{a}

^{a,b,c}: Superscripts to be compared statistically within the same column. Values within a column with different letter superscripts are significantly different ($P \leq 0.05$).

Supplementing the in vitro culture medium with different levels (50, 100, 150 µg/ml) of ascorbic acid as antioxidant affected ($P<0.05$) the development of dromedary camel embryos to blastocyst stage. The percentage of blastocysts increased by increasing the level of ascorbic acid (10, 16.6 and 25%) respectively, in comparison with the control group (2%), as shown in Figure 1. In addition, the proportion of blocked embryos at morula stage was significantly higher in the control group (10.2%) than in all of the ascorbic treated groups: T2 (3.33%), T3 (0%) and T4 (2.5%). However, there was no significant difference between the ascorbic groups (Figure 2).

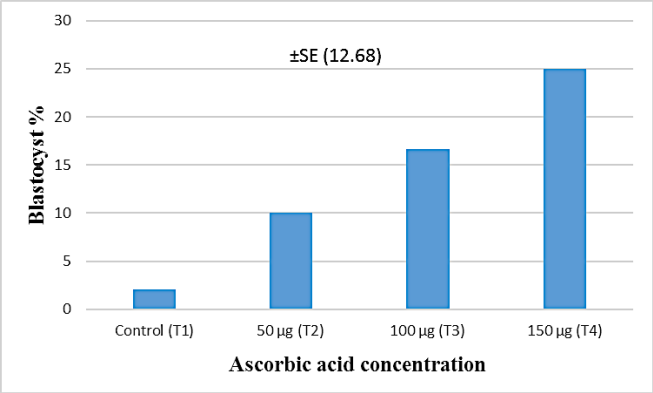

Figure 1. Effect of supplementing IVC medium of dromedary camel embryos with ascorbic acid on blastocyst rate.

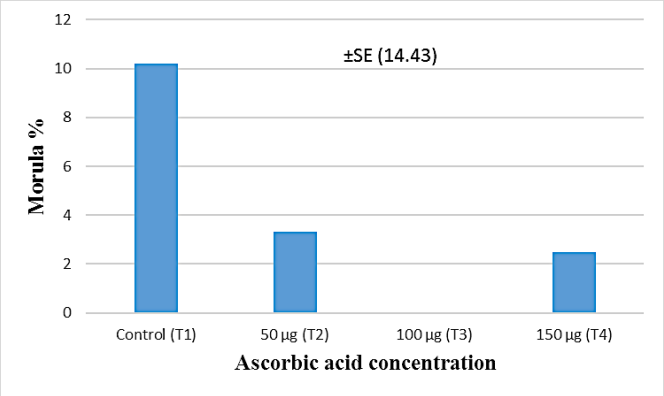

Figure 2. Effect of supplementing IVC medium of dromedary camel embryos with ascorbic acid on morula stage rate.

Discussion

To the best of our knowledge, this work is the first investigation to study the effect of ascorbic acid supplementation on the development of in vitro produced camel embryos to blastocyst stage. Our study demonstrated that exposing dromedary camel embryos to ascorbic acid enhanced the embryonic development rate to blastocyst stage, especially at the highest concentration used in this study (150 µg/ml) compared to the control group. Indeed, ROS are produced in vitro by the embryos themselves and the amount of ROS varies according to the stage of development (Guerin et al., 2001). Moreover, Wang et al. (2002) reported that during in vitro fertilization ROS-induced embryo damage occurred due to dead sperm around the oocytes in addition to deleterious effects of ROS during the in vitro maturation process. Ascorbic acid is a simple low molecular weight carbohydrate-like molecule that plays important roles in various enzymatic processes (Crha et al., 2003). Ascorbic acid is known for its powerful antioxidant properties since it acts in reducing or scavenging free radicals and ROS (Laganà et al., 2017). In vitro studies in different animal species tested the efficacy of ascorbic acid in improving the developmental competence of preimplantation embryos. Our results are in accordance with the conclusion that supplementing the culture medium with ascorbic acid can improve the development rates of in vitro produced embryos. These results are in agreement with a study conducted by Wang et al. (2002) who reported that exposure of mouse embryos to ROS for extended periods resulted in embryo toxicity, and incubating the embryos with ascorbic acid (50 µM) significantly increased

the blastocyst development rate. In the same context, Kere et al. (2013) reported that supplementing in vitro maturation and in vitro culture media of porcine embryos with 50 µg/ml ascorbic acid improved the cleavage rate, the blastocyst rate and total cell numbers per blastocyst. These results could be due to the supplementation of ascorbic acid during in vitro maturation, which led to an increase in intracellular glutathione (GSH) levels and reduced reactive oxygen species (ROS). Moreover, in buffalo, El-Naby et al. (2017) reported that the addition of ascorbic acid to in vitro maturation and in vitro culture media enhanced the developmental competence of buffalo embryos. However, these results are in contrast with Nohalez et al. (2018) who reported that ascorbic acid supplementation (50 µg/mL) had no significant effects on the maturation, fertilization, and development parameters of porcine embryos, when added to in vitro fertilization and embryo culture media. In spite of that, they reported that ascorbic acid enhanced vitrification survival of in vitro developed blastocysts.

Conclusion

Supplementing in vitro culture media of dromedary camel embryos with ascorbic acid (50, 100 and 150 µg/mL) enhanced the in vitro development of embryos to blastocyst stage in addition to decreasing the proportion of blocked embryos at morula stage. However, as the best results were obtained using the highest concentration (150 µg/ml), more research on ascorbic acid supplementation at higher concentrations is required in dromedary camels.

Conflict of interest

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

Authors' contributions

Mr. Ahmed Mohamed Kamel performed all laboratory work, data analysis and manuscript writing. Dr. Gamal Ashour Hassan designed the work plan and revised the manuscript. Dr. Nasser Ghanem helped in laboratory work and revising the manuscript. Dr. Ashraf Abd El-Halim El-Sayed helped in work plan design and revised the manuscript. Dr. Khalid Ahmed El Bahrawy helped in work facilities, work plan design and revising the manuscript.

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