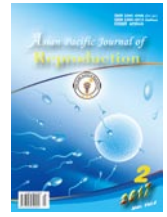


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Effect of using ascorbic acid and cysteamine supplementation on *in-vitro* development of buffalo embryosAl-shimaa Al-H H El-Naby¹, KarimaGh M Mahmoud^{2✉}, Gamal AM Sosa¹, Mahmoud EA Abouel-Roos¹, Youssef F Ahmed²¹Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, Egypt²Department of Animal Reproduction and A.I., National Research Center, Dokki, Giza, Egypt

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ABSTRACT

Objective: To improve *in vitro* embryo production in buffalo by supplementation of L-ascorbic acid during maturation and development (experiment 1) and combination with another antioxidant as cysteamine (experiment 2). **Methods:** Two experiments were performed, the first one aimed to evaluate the different concentrations (0, 25, 50, 100 µM) of L-ascorbic acid on embryo developmental rate of buffalo oocytes. The L-ascorbic acid was added to the maturation and culture media. In the second experiment, oocytes were cultured in media with two type of antioxidant (ascorbic acid + cysteamine) or ascorbic acid only. **Results:** There was a significant increase in cleavage rate at 25, 50 µM than 100 µM and control group. But, the blastocyst rate was higher at 50 µM ascorbic acid than other concentrations (0, 25, 100 µM). Supplementation of ascorbic acid and cysteamine to maturation and cultured media improved embryo development than ascorbic acid alone. **Conclusions:** Using of 50 µM L-ascorbic acid during *in vitro* maturation and development improve the developmental competence of buffalo oocytes, this effect was increase with the presence of cysteamine.

1. Introduction

Buffalo oocytes and embryos had abundance lipid content[1]. Therefore, they were likely to be more sensitive to oxidative damages lead to low embryo livability and production[2]. In efforts to curtail these damaging effects, the extrinsic protection of embryo mainly involved hypotaurine, taurine and ascorbic acid antioxidants. While embryo intrinsic protection involved enzymes as superoxide dismutase, glutathione peroxidase and gamma glutamyl-cysteine synthetase[3], and other extracellular small molecules scavengers like vitamins A, C, E and also sulphur compounds which including reduced glutathione, hypotaurine, taurine and cysteamine[4].

In the oocyte, a critical intracellular concentration of ascorbic acid would be necessary for normal cytoplasmic maturation[5] and

embryo developmental competence[6,7]. Ascorbic acid deficiency results in infertility in the female which associated with ovarian atrophy, follicular atresia, and the premature resumption of meiosis[8]. Vitamins C (L-ascorbic acid) and E (α-tocopherol and derivatives) were well-known antioxidants and were recorded to be the primary free radical scavengers in mammalian cell membranes[9]. So, the addition of ascorbic acid to the maturation medium maintained alpha-tocopherol levels during IVM, possibly because of ongoing functionality of vitamin E naturally present in the COC membrane[10]. A complex relationship existed between vitamin E and vitamin C. *In vitro*, vitamin C regenerated vitamin E molecules by tocopherol from tocopheroxyl radicals[11], that had

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undergone free radical attack and might also spare vitamin E from oxidation by reacting directly with free radicals[12].

Cysteamine might reduce cysteine to cystine that increased oocyte glutathione synthesis[13]. Cysteamine supplementation was recorded to improve nuclear maturation rates in buffalo[14] by enhancing synthesis of GSH[15] and improve male pronucleus formation[16]. Combination of ascorbic acid with vitamin E was previously reported to improve *in vitro* culture system, but no studies were reported on combination of ascorbic acid with cysteamine. So, this work aimed to improve *in vitro* embryo production in buffalo by supplementation of media with ascorbic acid with combination with cysteamine.

2. Materials and methods

2.1. Experimental designs

Experiment 1: Effect of L-ascorbic acid concentrations on buffalo embryo development. Oocytes were cultured in TCM-199 media with different concentration (0, 25, 50, 100 μ M) of ascorbic acid in CO₂ incubator at 38 °C during maturation and development.

Experiment 2: Evaluation the combination effect of L-ascorbic acid with cysteamine (double antioxidant substance) on cleavage and blastocyst rate. Group of oocytes were matured and cultured in media supplemented with combination of 50 μ M L-ascorbic acid and 50 μ M cysteamine. The second group of oocytes were matured, cultured in media with L-ascorbic acid but without cysteamine.

2.2. Chemicals

Tissue culture medium (TCM-199) and fetal calf serum were purchased from Gibico company. Cysteamine (M-6500, Sigma), Ascorbic acid (A-5960, Sigma) and chemicals used *in vitro* fertilization were from Sigma Company.

2.3. Oocytes recovery and maturation

Ovaries were collected from slaughtered buffalo, then they were aspirated to a syringe of PBS with 0.3% bovine serum albumin and 100 μ g/mL streptomycin sulfate and 100 IU/mL penicillin. Oocyte maturation was carried out according to Mahmoud[17]. The oocytes were cultured in maturation medium consisted of TCM-199, 10% calf serum, L-ascorbic acid, cysteamine and gentamycin (50 μ g/mL). Then oocytes were incubated at 38.5 °C, with 5% CO₂ in air, and 95% humidity for 24 h.

2.4. In vitro fertilization and culture

Fertilization was performed according to Niwa and Ohgoda[18]. Two straws of frozen semen were thawed in a water bath at 35-37 °C for 1 min. The spermatozoa were washed at 800 g for 10 min in BO

medium[19] free from BSA containing 10 μ g/mL heparin and 2.5 mM caffeine by centrifugation. The pellets were diluted with BO medium with 20 mg/mL bovine serum albumin to reach the concentration of spermatozoa to 12.5 \times 10⁶ sperm/mL. Oocytes were washed in BO containing with 10 mg/mL BSA before introducing into sperm suspension. The spermatozoa and oocytes were co-cultured for 4-5 h in 5% CO₂, 38.5 °C and 95% humidity. Then, the oocytes were washed several times in TCM-199 to remove attached spermatozoa and incubated in TCM-199 with 10% serum. Cleavage was assessed after 3 d of culture and the morula and blastocyst stages was determined on day 5 and 7, respectively.

2.5. Statistical analysis

Data in the first experiment were statistically analyzed by ANOVA using SPSS for Windows version 16.0. Means were compared by Duncan's Multiple Range Test. The *t*-test was performed to compare between means in the second experiment. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Effect of L- ascorbic acid concentrations on buffalo embryo development

The mean proportion of buffalo oocytes matured, cultured in media with different concentration (0, 25, 50, 100 μ M) of ascorbic acid was given in Table 1. There was a significant ($P < 0.01$) increase in cleavage rate of 25 μ M and 50 μ M of ascorbic acid than control and 100 μ M groups. While the embryo cleavage rate was not significantly differ between 25 μ M and 50 μ M of ascorbic acid groups. The mean number of cleavage rate was higher ($P < 0.01$) in 50 μ M and ($P < 0.05$) in 25 μ M than 100 μ M of ascorbic acid supplementation group.

The blastocyst rate after exposure to different concentrations (0, 25, 50, 100 μ M) of ascorbic acid was evaluated in Table 1. The percentage of blastocyst rate was significantly higher in 25 μ M ($P < 0.01$), 50 μ M ($P < 0.001$), 100 μ M ($P < 0.05$) ascorbic acid groups than control. The highest blastocyst yield was observed at 50 μ M ascorbic acid treatment. The blastocyst rate was nearly similar in 25 μ M and 100 μ M ascorbic acid supplementation.

Table 1

Effect of different concentrations of ascorbic acid supplementation on buffalo embryo development (mean \pm SE).

Ascorbic acid concentration	Total No. of embryo	Cleavage No. (%)	Blastocyst No. (%)
Control (0)	82	25 (56.03 \pm 3.12)	4 (4.87 \pm 0.06)
25 μ M	98	64 (66.13 \pm 1.60) ^{ab}	8 (9.50 \pm 1.50) ^a
50 μ M	144	97 (67.65 \pm 0.82) ^{ab}	18 (12.81 \pm 0.87) ^{abc}
100 μ M	132	77 (58.23 \pm 1.05)	12 (8.88 \pm 0.84) ^a

^a $P < 0.05$, compared to control; ^b $P < 0.05$, compared to 100 μ M; ^c $P < 0.05$, compared to 25 μ M.

3.2. The effect of combination between ascorbic acid and cysteamine on developmental competence of buffalo oocytes

Data regarding the combination effect between ascorbic acid (50 μM) and cysteamine (50 μM) on cleavage and blastocyst rate was illustrated as mean \pm SE. The total number of embryo for ascorbic acid with cysteamine was 80 while for the ascorbic acid without cysteamine was 92. There was significant ($P<0.05$) increase in embryo cleavage rate of media supplemented with ascorbic acid [52, (65.20% \pm 0.98%)] and cysteamine than media supplemented with ascorbic acid only [53, (56.81% \pm 2.19%)]. Also, blastocyst rate was higher significantly ($P<0.01$) in ascorbic acid [10, (12.31% \pm 0.64%)] and cysteamine combination than in ascorbic acid only [7, (7.20% \pm 0.75%)]. So the combination between two antioxidant substances gave better embryonic developmental rate than single supplementation.

4. Discussion

In our study, the percentage of embryo cleavage rate significantly increased at 25 μM and 50 μM of L-ascorbic acid than 100 μM and control groups. Moreover, blastocyst rate was highly significant at 50 μM than other concentrations 25 μM , 100 μM and control groups. It was shown that the optimum concentration of vitamin C was 50 $\mu\text{g}/\text{mL}$ during IVM of porcine oocytes[20], this concentration not only improved blastocyst rates, total cell numbers but also, reduced apoptotic indices and the high concentration (100 $\mu\text{g}/\text{mL}$) of vitamin C induced high apoptotic index. Miclea[21] and Huang[22] mentioned that the treatment with 50 $\mu\text{g}/\text{mL}$ vitamin C resulted in increasing pregnancy rate and enhancing the development somatic cell nuclear transfer embryos in pigs. Moreover, Castillo-Martin[23] mentioned that L-ascorbic acid supplementation in culture and/or vitrification media, enhanced survival rates of porcine blastocyst.

The effect of ascorbic acid and its concentration as a supplement in *in-vitro* maturation and *in vitro* culture mediums were recorded previously. The beneficial effect of vitamin C addition might be ascribed to an improved the culture condition and oocytes cytoplasmic maturation by reduction the intra cellular oxidative status for embryo development[7,20]. Moreover, Eppig[24] and Wang[6] mentioned that the addition of ascorbic acid into the culture media prevented follicular apoptosis in rat and mouse follicles, and also improved the blastocyst production.

It was suggested that vitamin C at high concentration might act as a pro-oxidant that had deleteriously effect on oocyte maturation and cumulus cell viability[25]. In sheep, Natarajan[26] stated that there was a relation between the oxygen levels in culture environment and

concentration of vitamin C to achieve optimal embryo development. It was observed that 50 μM L-ascorbic acid in embryo culture medium at 5% oxygen levels and 100 μM of ascorbic acid at 20% oxygen levels were resulted in a significant increasing the morula and blastocysts rates compared to control. While supplementation of embryo culture media with 400 μM , 500 μM L-ascorbic acid resulted in significant retardation for the development of ovine embryos.

In contrast to our results, Cordova[27] stated that the presence of L-ascorbic acid to *in-vitro* maturation of prepubertal bovine oocytes exerted no effect on maturation rates and significantly reduced the proportion of embryos developing to blastocyst stage. Also, the presence of alpha-tocopherol or ascorbic acid in the maturation medium of bovine oocytes failed to modify nuclear maturation rate and obtained blastocysts[28,29]. The same results were observed in rat[8] and porcine[5] oocytes. Also, in dromedary she camel, Elsayed[30] stated that there was no significant difference in the maturation rate of *in-vitro* matured oocytes in medium containing vitamin C and the control group.

To our knowledge, this work was the first investigation to study the positive effect of combination between L-ascorbic acid and cysteamine added to maturation media. Our study was proved that benefits effect of using two antioxidant substances during oocytes maturation and culture. The cleavage rate was significantly ($P<0.05$) increased by using 50 μM ascorbic acid and 50 μM cysteamine combinations than using ascorbic acid only. Ocampo and Ocampo[31] mentioned that 50 μM cysteamine supported the maturation, fertilization, subsequent embryonic development *in vitro*.

Some studies reported the synergistic relationship between vitamin C and vitamin E in culture medium. It was expected that the combination of vitamin E and C improved embryonic developmental rate more than vitamin E alone[11,32]. This was disagreement with Olson and Seidel Jr.[12] who stated that co-supplementation of α -tocopherol or L-ascorbic acid at 100 μM had deleterious effect on bovine culture system. It may be due to the higher concentration used in their work. In contrast, Saikhun[33] stated that culture medium supplemented with either α -tocopherol or L-ascorbic acid at a concentration of 250 μM increased the quality of IVF buffalo derived embryos, blastocyst rate and blastocyst cell number.

In conclusion, 50 μM L-ascorbic acid was the best concentration for *in vitro* produced buffalo embryos. Higher cleavage and blastocyst rate were achieved by using combination of ascorbic acid and cysteamine.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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