Research Article

Effect of Cysteine Supplementation in Maturation Medium on Bovine Embryos Development

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Abstract | This research study investigated cysteine supplements and their effects upon *in vitro* maturation (IVM) media. Experimentally, cumulus-oocyte complexes (COCs) which were obtained from bovine ovaries underwent culturing for a period of 22 h in TCM-199 containing an additional 10% fetal bovine serum, streptomycin (100 µg/ml), penicillin (100 U/ml), 25 µg/ml FSH, 2 IU/ml hCG, 0.2 mM sodium pyruvate, 1 µg/ml 17-β-Estradiol, and either 0, 100, or 200 µM/ml cysteine where 0 served as the control. IVF-TALP medium was used to fertilize the mature oocytes which subsequently underwent culturing in a simple culture medium (KSOM). The results showed that percentage of matured oocytes, fertilized oocytes, 8-16 cells developments, blastocyst in 100 µM cysteine group (73.00%, 54.79%, 30.14%, and 17.81%, respectively) were higher than the control group (48.00%, 37.50%, 18.75%, and 10.42%, respectively) and 200 µM cysteine (respectively, 52.00%, 38.46%, 19.23%, and 9.62%) at a significance level of 0.05, but the difference between the 200 µM cysteine group and the control was not significant (p>0.05). The findings confirm that adding 100 µM cysteine to IVM media significantly increased the percentage of matured oocytes, fertilized oocytes, 8-16 cells developments and blastocyst stage.

Keywords | Bovine, Embryo, Cysteine, *In vitro* maturation, *In vitro* production

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INTRODUCTION

Over the past 10 years, the blastocyst from *in vitro* embryo production (IVEP) are used for the genetic improvement of herds (Stroebech et al., 2015) and also used to generate a nuclear transfer of embryonic stem cells (NT-ESCs) for stem-cell research (Wolf et al., 2017). Moreover, this method, based on the production of safe and healthy food, conservation genetics in endangered species (Folch et al., 2009; Lopez et al., 2020), and an animal model for studying human disease (Sui et al., 2018). However, the efficiency of IVEP obtained through IVM (*in vitro* maturation), IVF (*in vitro* fertilization) or IVC (*in vitro* culture) remains limited (Luciano et al., 2018). IVEP has developed over two decades. There are many factors of procedure success. One of the problem for embryonic development is oxidative stress (OS) since the quality of the oocytes may decrease as a consequence as meiotic

arrest is induced in the oocyte (Khazaei and Aghaz, 2017). The IVEP conditions usually increase reactive oxygen species (ROS). ROS production is increased during IVM because oxygen is employed by the oocytes for energy production via the process of mitochondrial oxidative phosphorylation. OS results from an imbalance between ROS and antioxidants. Thus, the enzymatic antioxidants comprise catalase (CAT) and superoxide dismutase stress (SOD) while the non-enzymatic antioxidants comprising glutathione (GSH), cysteamine, vitamin C, and vitamin E have been used to supplement the culture medium to reduce the intracellular level of ROS (Caamano et al., 1998; He et al., 2017). GSH functions protection against oxidative damage, raising embryo development competence (Sandal et al., 2018). Cysteine and cysteamine are precursors of GSH. There are many reports indicating that when antioxidants are introduced to the *in vitro* maturation of bovine oocyte to supplement the culture media, this caused the high intracellular GSH level and decreased ROS production, raising embryo quality and development (Lee and Fukui, 1996; Hosseini et al., 2009; Hansen and Harris, 2015). Moreover, it was demonstrated that addition of cysteine to IVM medium, increased the maturation rate of bovine oocytes (Rahim et al., 2011). Nabenishi et al. (2012) reported that the supplementation of cysteine to IVM mediumcaused increases of oocytes GSH content and inhibiting the production of oocyte ROS. Therefore, cysteine can be used as a tools to enhance the efficiency of bovine embryo *in vitro* system. This research sought to examine how the development of IVP of a bovine embryo is influenced by cysteine.

MATERIALS AND METHODS

Oocyte retrieval

The ovaries required for the study were gathered at the abattoir within 3 hours of the animal slaughter and placed in a solution of 0.9% NaCl at a temperature of 37°C for transportation to the laboratory. The recovery of bovine oocytes was achieved by slicing open the ovaries in Dulbecco's Phosphate Buffered Saline (DPBS) mixed with 2% fetal bovine serum (FBS), Pen/Strep solution (All supplied by Thermo Fisher Scientific Inc., Grand Island, USA), with streptomycin (100 µg/ml) and penicillin (100 U/ml).

In vitro maturation (IVM)

The IVM medium comprised TCM 199 medium and Earle's salts, L-glutamine, and $NaHCO_3$ (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) with the addition of 10% (v/v) FBS and Pen/Strep solution (Thermo Fisher Scientific Inc., Grand Island, USA), streptomycin (100 µg/ml), penicillin (100 U/ml), 2 IU/ml of hCG (Chorulon, Intervet, Netherlands), 25 µg/ml of FSH (Folltropin® - V, Bioniche Animal Health (A/Asia) Pty. Ltd., Australia), 0.2 mM sodium pyruvate, and 1 µg/ml 17-β-Estradiol (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Cumulus-oocyte complexes (COCs) were cultured in the medium for IVM and the designated levels of cysteine (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were 0 (Control), 100 µM and 200 µM. COCs which had at least three cumulus cell layers were then rinsed thrice in IVM medium, cultured in 100 µl drops (12-15 COCs/drop) under mineral oil (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 22 h under a humidified atmosphere of 5% carbon dioxide at a temperature of 38.5°C.

In vitro fertilization (IVF)

Sperm preparation for IVF, frozen semen was thawed and prepared for sperm capacitation. 100 ul of semen added into 1 ml sperm-TALP medium which comprised 100 mM NaCl, 25 mM $NaHCO_3$, 3.1 mM KCl, 0.29 mM NaH_2PO_4, 21.60 mM Lactic Acid, 10 mM Hepes, 1 ul/ml Phenol red, 0.40 mM $MgCl_2.6H_2O$, 2.1 mM $CaCl_2.2H_2O$, 6 mg/ml BSA (All supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 0.25 mM sodium pyruvate. The motile sperm were selected using the swim-up technique. The sperms were incubated with sperm-TALP medium in a 1.5 ml Eppendorf tube for 1 h at 38.5 °C in 5% CO_2 for swim-up. Then the top 0.8 ml from each tube, pooled sperm inside a 15 ml plastic centrifuge tube prior to centrifugation for 5 min at 1,500 rpm twice. The final sperm pellet concentration in fertilization microdroplets was 2 X 10^6 sperm/ml. Once the oocytes had been cultured for 22 h in IVM media, 12-15 expanded bovine cumulus-oocyte complexes were washed three times then transfer into a fertilization drop (500 ul) of IVF-TALP containing 114 mM NaCl, 3.2 mM KCl, 25 mM $NaHCO_3$, 0.34 mM NaH_2PO_4, 10 mM Lactic Acid, 1 ul/ml Phenol red, 2 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2.6H_2O$, 6 mg EFAF BSA (All supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and 0.25 mM sodium pyruvate, 3 IU/ml Heparin (Leo Pharmaceutical Products, Ballerup, Denmark), 100 U/ml penicillin, 100 µg/ml streptomycin. Incubation of the sperm and oocytes was subsequently conducted for a period of 18-20 h at a temperature of 38.5°C under an atmosphere comprising 5% carbon dioxide.

In vitro culture (IVC)

After 18-20 h of incubation, fertilized oocytes were removed cumulus cells by repeated pipetting of oocytes. Then, washed 3 times and cultured in KSOM medium containing 2.5 mM KCl, 95.0 mM NaCl, 0.35 mM KH_2PO_4, 0.20 mM $MgSO_47H_2O$, 0.20 mM glucose, 10.00 mM Lactic Acid, 0.20 mM sodium pyruvate, 25.0 mM $NaHCO_3$, 1.71 mM $CaCl_22H_20$, 1.0 mM L-glutamine, 0.01 mM EDTA, 1.0

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mg/ml BSA, 100 µg/ml streptomycin, 100 U/ml penicillin, 5 ul/ml MEM non-essential solution 100x, 10 ul/ml BME essential solution 50x, and 10% FBS (All supplied by Sigma Aldrich, Merck KGaA, Darmstadt, Germany). Embryos were placed in 50 ul drop (5 embryos/drop) in a Petri dish and covered with mineral oil and cultured in an incubator under humid conditions in a 5% carbon dioxide atmosphere at a temperature of 38.5°C. In addition, the embryos underwent culturing and the medium was replaced at each 48 h until 8-cells then cultured in KSOM medium containing 1.5 mM glucose. At 7-9 days after culturing, the blastocysts rate was evaluated.

Statistical analysis

Evaluation of the effects of cysteine in maturation medium was carried out using Duncan's New Multiple Range Test (DMRT), and analysis of variance (ANOVA) was performed using SAS software. The statistical significance level of 0.05 was employed for all analyses.

RESULTS AND DISCUSSION

The examination of different levels of cysteine showed that percentage of matured oocytes, fertilized oocytes, 8-16 cells developments, blastocyst in 100 µM cysteine treatment were higher than control group. Between treatments, percentage of matured oocytes, fertilized oocytes, 8-16 cells developments, blastocyst in 200 µM cysteine treatment were lower than 100 µM cysteine treatment at a significance level of 0.05, but difference between the 200 µM cysteine treatment and the control group was not found to be significant (Table 1). Figure 1 presents the first polar body, the zygote with two polar bodies, 8-cells, 16-cells, early blastocyst, and blastocyst observed after IVC.

IVEP of bovine embryos has become a technology which has been used for genomic selection and systems biology (Stroebech et al., 2015), but this technology is still being improved. IVM of oocytes is the first step that develop to the blastocyst stage. According to the reports, multiple factors contribute to the maturation of oocytes such as media compositions; growth factors, estradiol, gonadotropin, and antioxidants (Vahedi et al., 2009; Balasubramanian and Rho, 2007). The major factor affecting IVM maybe OS. OS arises whenever an imbalance between oxidants and antioxidants. Under normal conditions, ROS is kept at a normal balance by a defense system consisting of enzymatic

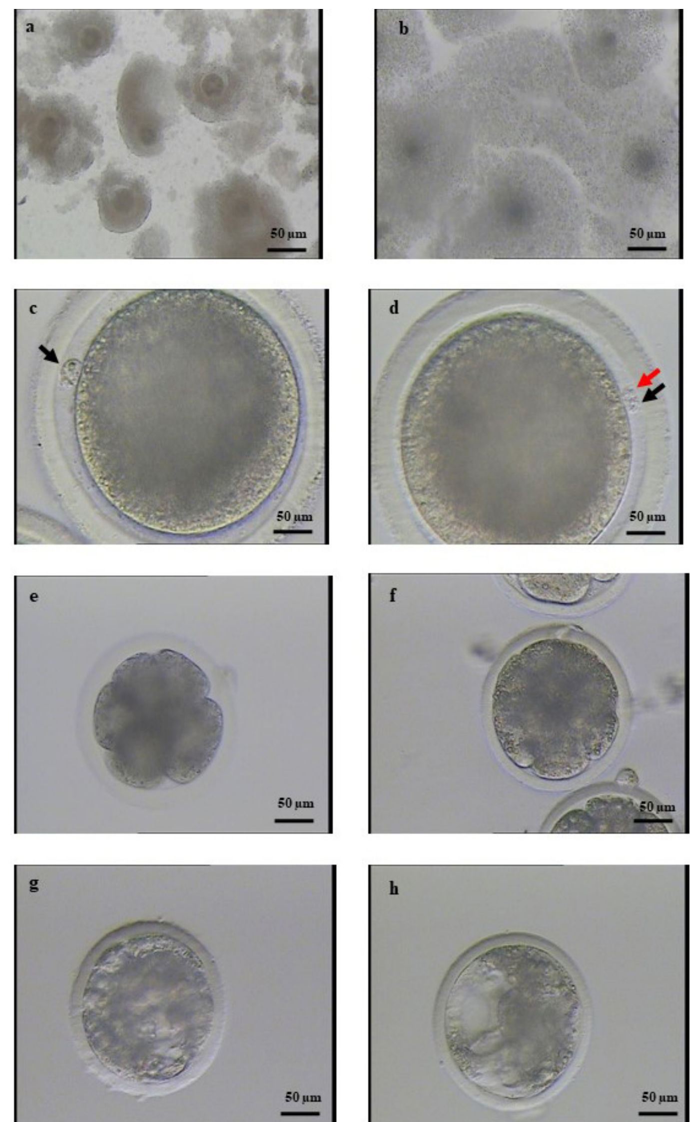

Figure 1: Representative image of embryonic development from bovine oocytes using cysteine supplementation during IVM. (a) Immature cumulus-oocyte complexes; (b) Cumulus expansion at 22 h after culturing in IVM medium; (c) Matured oocyte with a polar corpuscle (black arrow); (d) Zygote with two polar bodies (red and black arrows); (e) 8-cells embryo at 72 h after IVC; (f) 16-cells embryo at 96 h after IVC; (g) Early blastocyst at 156 h after IVC; (h) blastocyst at 168 h after IVC.

Table 1: Maturation of bovine oocytes and embryonic development following various dosages of cysteine supplementation during *in vitro* maturation

Group	Number of oocytes examined	Matured oocytes (% mean ± S.E)	Fertilized oocytes (% mean ± S.E)	8-16 cells (% mean ± S.E)	Blastocyst (% mean ± S.E)
Control	100	48 (48.00±0.31)[b]	18 (37.50±1.92)[b]	9 (18.75±2.81)[b]	5 (10.42±1.00)[b]
100 (uM)	100	73 (73.00±3.33)[a]	40 (54.79±1.10)[a]	22 (30.14±2.78)[a]	13 (17.81±0.69)[a]
200 (uM)	100	52 (52.00±1.00)[b]	20 (38.46±0.75)[b]	10 (19.23±0.38)[b]	5 (9.62±1.68)[b]

[a,b] the values bearing different superscript with in a column differed significantly ($p<0.05$).

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and non-enzymatic antioxidants. But the *in vitro* system may lack defenses (Combelles, 2009). Therefore, there are many reports that the addition of antioxidants in the medium can lead to the improved development of bovine embryos when produced *in vitro*. In the present study, the effect of cysteine on bovine IVP. The results of the maturation rate of oocytes cultured with 100 μMand 200 μM cysteine were 73% and 52%, respectively. These results are similar to a previous study that culture with 100 μMand 500 μM cysteine. The matured oocytes were 42.22% and 53.33%, respectively (Rahim et al., 2011). Lee and Fukui (1996) demonstrated that glutamine, glycine, and alanine enhanced bovine embryonic development and blastocyst cell number. Moreover, in our study, the addition of 100 μM cysteine to the culture medium resulted in significant improvements to the rate of maturation as well as the development of the embryo and the blastocyst cell numbers, at a statistical significance level of 0.05. As shown in Table 1, fertilization rates were observed in 54.79±1.10% by 72 h after IVF cells, 8–16 cells were checked in 30.14±2.78% by 96 h after IVF, and 17.81±0.69% developed to blastocyst stage after 216 h of culture. But the addition of 200 μM cysteine to the culture medium did not improve fertilization rate and embryonic development. According to many reports and the results in this study showed that amino acids may play an important role with increased GSH synthesis then reduce the intracellular level of ROS. It may affect oocyte cytoplasmic maturation and embryonic development (Gordan, 2003; Gasparrini et al., 2006). However, the mechanism of amino acids affecting embryonic development requires more study to gain a better understanding.

CONCLUSIONS AND RECOMMENDATIONS

Our results showed that where the IVM media is augmented by the introduction of cysteine, this could result in a percentage increase in matured oocytes, fertilized oocytes, 8–16 cell development and blastocyst stage. These results support the evidence that ROS production is increased during *in vitro* mammalian embryo production. Moreover, the addition of 100 μM of cysteine supplementation in IVM media was shown to deliver efficiency improvements in the *in vitro* production of bovine embryos.

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NOVELTY STATEMENT

Our work demonstrated that when antioxidants are introduced to the *in vitro* maturation of the bovine oocytes to supplement the culture medium, this improved embryonic development. Thus, cysteine has great potential for use in prevention of oxidative stress during *in vitro* culture.

AUTHOR'S CONTRIBUTION

All the authors contributed to the manuscript. CA, SG, JJ, SD and JY designed research methodology, data collection, statistical analysis, manuscript writing. WS, ST and PK, DB and CL all approved the final document.

Ethical consideration

Approval for the techniques employed during the experiments in this study was granted by the Committee on the Ethics of Animal Experiments of Kalasin University (Approval Number: KSU-AE-005/2022).

Conflict of interest

The authors have declared no conflict of interest.

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