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Research article

The Effect of Antioxidant Cysteamine With Ascorbic Acid On In Vitro Fertilization in Cows

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ABSTRACT

Oxidative stress (OS) has been recognized as an effective reason for decreasing the outcome of assisted reproductive technology (ART). The supplementation of antioxidants in culture medium has been used widely to improve the in vitro fertilization (IVF) in many species. This study was conducted to evaluate the effect of cysteamine (CYS) or ascorbic acids (AA) (either in combination or individually) as antioxidants in all stages of bovine IVF, particularly on in vitro oocytes maturation, cleavage rate and subsequent development of blastocysts. We found that adding of 100 μM CYS or 200 mM AA significantly ($p<0.05$) improved the bovine oocytes maturation compered to control group, while using of CYS plus AA didn't improve the oocytes maturation compared to CYS or AA groups. The same dose of CYS or AA (either in combination or individually) didn't improve the cleavage rate but CYS and CYS plus AA significantly ($p<0.05$) induced the early cleavage (≤ 24 h after IVF) and formation of 2 cells embryos compered to AA and control groups. The supplementation of CYS or AA (either in combination or individually) to culture medium significantly ($p<0.05$) improved the blastocyst rate after IVF and also prolonged the viability of blastocysts till day ninth from IVF in CYS and CYS plus AA groups compared to AA and control groups. In conclusion, the supplementation of in vitro culture medium with antioxidant CYS or AA improve the bovine IVF and subsequent production of blastocyst compared to control group but the combination between them did not improve bovine IVF compared to control group.

Key Words: antioxidant, cysteamine, ascorbic acid, in vitro fertilization

INTRODUCTION

The assisted reproductive technologies (ART) are regarded as one of the modern technologies that are widely used for improvement the reproduction in human and animals. In addition, it has been used in treating many cases of infertility which may happen during the reproductive life [1].

The *in-vitro* embryos production is affected by many factors which could effectively contribute to the reduction the success rate of ART. Reactive oxygen species (ROS) is one of the harmful causes that could be generated during stages of *in vitro* embryo production especially during IVF [2, 3]. The production of ROS like hydrogen peroxide (H_2O_2) , superoxide anion (O_2^-), hydroxyl radical (OH-), singlet oxygen and alpha-oxygen during IVF has a close relationship with quality and viability of produced embryos [2-4]. During IVF process, of *in-vitro* produced embryos, H_2O_2 could be generated at a high concentrations leading to degeneration followed by apoptosis of these embryos [2-5].

In general, ROS can lead to harmful oxidative changes for the cellular structure followed by indirect fragmentation for DNA, lipid peroxidation, protein oxidation, and mitochondrial destruction; which may lead to regression in embryonic development [4, 6, 7].

In vivo, there are intrinsic mechanisms for protection the oocytes and embryos against the ROS. This include many types of antioxidants like hypotaurine, taurine and AA. The effect of these antioxidants is vital inside follicular, oviductal, and uterine fluid [4]. In addition, there are other self-protection mechanisms in embryos that involves enzymatic and non-enzymatic mechanisms. The enzymatic mechanisms includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and gamma glutamylcysteine synthetase (GCS) [2-7]. Whereas the non-enzymatic mechanism includes vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione which control the cellular redox balance and provide a protection against ROS and maintain a normal embryonic development [8, 9].

Many researchers suggested that the supplementation of antioxidants in the culture medium could regulate the cellular redox balance and provide appropriate condition to improve the embryonic development [10-13]. Several enzymatic and non- enzymatic antioxidants have been studied to improve embryonic development. Addition of SOD, CAT or thioredoxin (TRX) (enzymatic free radical scavenger) has been reported to enhance embryos development by

scavenging the free radicals and protecting the embryos from the oxidative stress and subsequently improve the embryonic development in mouse [14], pig [15] and cow [5]. Supplementation of vitamins like β-carotene, α-tocopherol, vitamin C and polyphenols to culture medium had a positive effect for development of *in vitro* produced embryos in mouse [16], pig [17] and cow [18].

CYS is one of the thiol compounds that is successfully used in *in- vitro* oocytes maturation by acting as a scavenger for ROS and maintain the cellular redox balance for suitable conditions for embryo development [19, 20]. The supplementation of CYS in maturation medium provided an increase in the level of intracellular glutathione (GSH) in mature oocytes and improved embryo development rates [21]. GSH is a non-protein sulphydryl compound that protects the cells and oocytes from oxidative damage as well as it has an effect on amino acid transportation, DNA and protein synthesis and reduction of disulfides [22].

AA (vitamin C) is a water soluble vitamin which acts as antioxidant to scavenge the ROS and protects against the harmful effects of ROS and free radicals on cells. Many studies revealed the ability of ascorbic acid to protect the cellular membrane and other cellular structures from lipid peroxidation by regenerating the antioxidant form of vitamin E (α-tocopherol) by donating electrons to the α-tocopheroxyl radical. In addition, AA reduced coenzyme Q which interacts with α-tocopherol to create the antioxidant ability [23, 24). AA successfully used as antioxidant in IVF to improve oocytes maturation and blastocysts production [25, 26].

Limited studies were conducted to examine the effect of a combination of two antioxidants on IVF and embryo production, so we designed this study to investigate the effect of two well-known antioxidants, CYS and AA either alone or together on all stages of bovine IVF and their effects on maturation, cleavage and blastocyst rate.

MATERIALS AND METHODS

Media and reagents

All chemicals and media were obtained from Sigma Chemical company (St. Louis, MO, USA) and Gibco (Invitrogen Corporation, Grand Island, NY, USA), respectively. All the experiments were carried out in the central laboratory, university of Basrah, college of veterinary medicine.

Recovery and in vitro maturation of bovine oocytes

Immature bovine cumulus oocytes complexes were aspirated from 4-8 mm follicles of slaughterhouse ovaries by using 18 gauge needle connected with syringe. The recovered oocytes were transferred into tissue culture medium 199 (H-TCM199) supplemented with 10% fetal bovine serum (FBS). Oocytes with clear cytoplasm and with many layers of cumulus cells were selected for *in vitro* maturation, and incubated for 20 h according to [27] in tissue culture medium supplemented with 2.5 mM sodium pyruvate, 10 μg/ml LH, 10 μg/ml FSH, 1 μg/ml estradiol–17β, 100 ng/ml insulin-like growth factor (IGF), 100 ng/ml epidermal growth factor (EGF) and 10% FBS at 38.5 °C, 5% CO_2 and maximum humidity.

In vitro embryo production

Frozen bovine semen straws (HO Health, USA; 0.5ml) were thawed in room temperature. Sperm capacitation was induced by kept semen in Tyrode's albumin lactate pyruvate medium at 38.5 °C under 5% CO2 in maximum humidity for 30-40 min [28]. For in-vitro fertilization (IVF), approximately $5x10^3$ capacitated sperms were transferred for each matured oocyte into 50 μl drops of fertilization medium which is modified fert-TALP medium containing 0.2 mM penicillamine, 0.1 mM hypotaurine, 6 mg/ml bovine serum albumin (BSA) and 10μg/ml heparin for 18–20 h at 38.5 °C under 5% CO_2 in humidified air overlaid with mineral oil [29]. After that, the cumulus cells were denuded by vortexing the presumptive zygotes in TCM supplemented with 1mg/ml polyvinyl alcohol (PVA) and 6mg/ml BSA for 3 min. Thereafter, they were cultured into 20 μl drops of culture medium comprising modified synthetic oviduct fluid (mSOF) supplemented with 8 mg/ml BSA at 38.5 °C under 5% CO_2 overlaid with mineral oil for 7 days [28].

Experimental design

In order to study the effect of antioxidants in all stages of bovine IVF, 100 μM/ml CYS according to [27] (group 1) or 200 μM AA according to [25] (group 2) were used with maturation medium, IVF medium and *in vitro* culture medium to evaluate the effect of each one on oocyte maturation, cleavage and blastocyst rate respectively. These were compared with a combination of 100 μM/ml CYS plus 200 μM AA (group 3) and control group which was not supplemented with antioxidants. Oocyte maturation was recorded through the presence of cumulus expansion as well as the presence of first polar body. The cleavage rate was assessed according to the number of oocytes which cleaved and formed 2 cells embryo. The occurrence of the cleavage at ≤24 h (early cleavage) or >24 h (late cleavage) after IVF was also assessed according to classifications of [30]. Blastocyst rate was recorded according to the number of

blastocyst which produced from cleaved oocytes after 6-7 days incubation inside culture medium.

Statistical analysis

The data of this study were analysed by one-way ANOVA model of SPSS version 20 (SPSS Science, Chicago, IL, USA). Differences were compared by Tukey's multiple-comparison post hoc test. All data were presented as means ± SEM and the differences were considered as significant at $P < 0.05$.

RESULTS

The presence of CYS plus AA in maturation medium showed a significant ($p<0.05$) increase in the number of matured oocyte (81.72±1.62%) compared to control groups (61.03±4.77%) (Table 1). The presence of combination of two antioxidants (CYS plus AA) didn't show any improvement in the maturation rate compared to using one antioxidant (CYS or AA) (Table 1).

The addition CYS plus AA in the IVF medium had no clear effect on cleavage rate (71.05±2.87%) compared to control group (74.46±3.98%) (Table 1). There was no difference in the cleavage rate when antioxidants (CYS and AA) were used individually or together compared with control group (Table 1).

Supplementation of antioxidants significantly ($p<0.05$) improved the blastocyst rate in all groups (cysteamine, AA and CYS plus AA) (24.48±1.93, 23.91±4.65 and 24.07±2.74%) respectively compared to control group 8.57±0.68% (Table 1).

Table 1: Maturation, cleavage and blastocyst rates after supplementation of antioxidants (CYS and AA) together or individually in maturation, IVF and culture medium

Groups	Oocytes no.	Maturation rate no. (%±SEM)	Cleavage rate no. (%±SEM)	Blastocyst rate no. (%±SEM)
Cysteamine	89	72 (80.89±2.17)^a	49 (68.05±3.81)^a	12 (24.48±1.93)^a
Ascorbic acid	86	67 (77.90±2.72)^a	46 (68.65±1.21)^a	11 (23.91±4.65)^a
Cysteamine + Ascorbic acid	93	76 (81.72±1.62)^a	54 (71.05±2.87)^a	13 (24.07±2.74)^a
Control	77	47 (61.03±4.77)^b	35 (74.46±3.98)^a	3 (8.57±0.68)^b

Data are presented as number (percentage ± SEM). ^{ab} Different letters within each column indicate significant difference ($p < 0.05$)

The results showed that the incidence of cleavage at ≤ 24 h from IVF (early cleavage) was significantly ($p<0.05$) greater in CYS and CYS plus AA groups (63.37±2.13, 61.18±1.79%) respectively compared with AA and control groups (45.53±3.43, 42.60±1.83%) respectively (Table 2, Figure 1).

The current study revealed that the early cleavage (≤ 24 h post IVF) significantly had a tendency to form blastocyst in all groups (cysteamine, ascorbic acid, CYS plus AA and control) 29.29± 2.02, 33.13± 2.57, 30.45± 3.28 and 20.55± 2.42% respectively compared to late cleavage (> 24 h after IVF) 16.98± 1.65, 15.74± 3.91, 14.48± 1.20 and 0.0% respectively (Figure 2).

Table 2: Early and late cleavage rates after supplementation of antioxidants (CYS and AA) together or individually in *in-vitro* fertilization medium

Groups	Cleavage rate no. (%±SEM)	Early cleavage no. (%±SEM)	Late cleavage no. (%±SEM)
Cysteamine	49 (68.05±3.81)^a	31 (63.37±2.13)^a	18 (36.63±2.13)^a
Ascorbic acid	46 (68.65±1.21)^a	21 (45.53±3.43)^b	25 (54.47±3.43)^b
Cysteamine + Ascorbic acid	54 (71.05±2.87)^a	33 (61.18±1.79)^a	21 (38.82±1.79)^a
Control	35 (74.46±3.98)^a	15 (42.60±1.83)^b	20 (57.40±1.83)^b

Data are presented as number (percentage ± SEM). ^{ab} Different letters within each column indicate significant difference ($p < 0.05$).

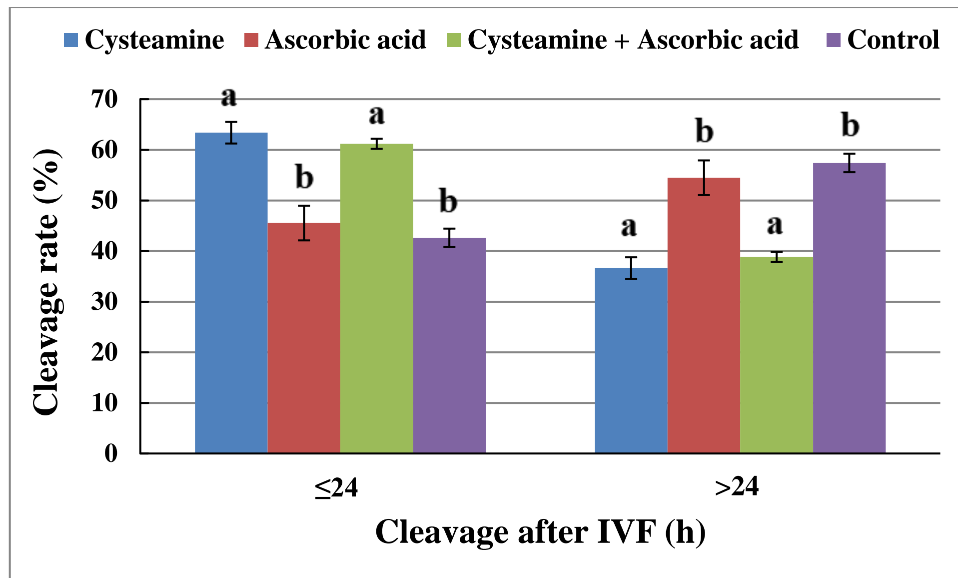

Fig.1: Early and late cleavage rates after supplementation of antioxidants (CYS and AA) together or individually in IVF medium

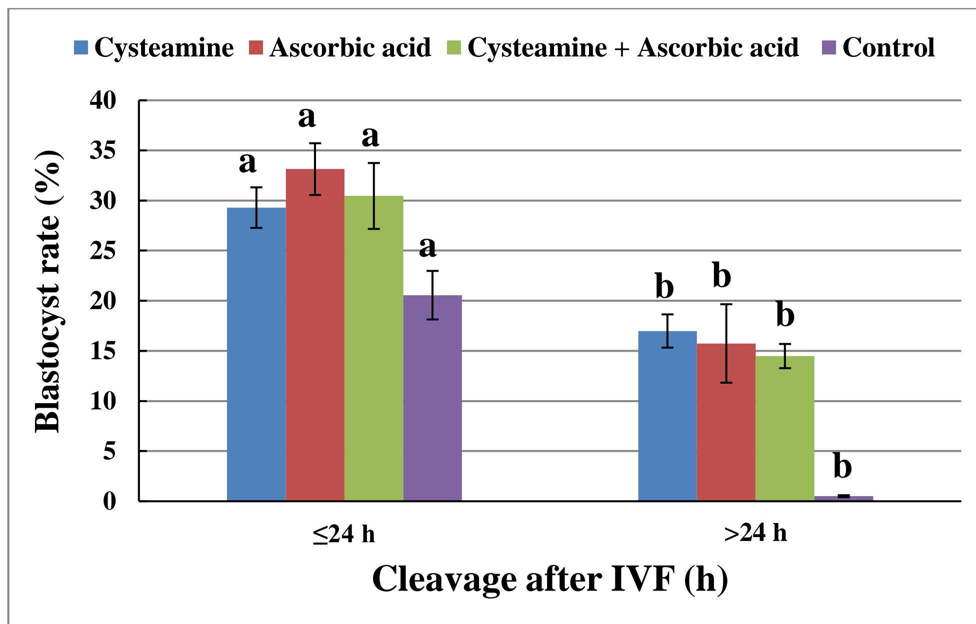

Fig.2: Blastocyst rate in early and late cleavage after supplementation of antioxidants (CYS and ascorbic acid) together or individually in IVF medium

Moreover, this study revealed the ability of CYS plus AA and CYS groups significantly ($p < 0.05$) to maintain the viability of blastocysts till the day ninth from IVF compared to AA and control groups which showed their ability to maintain the viability of blastocysts till day 6th- 7th from IVF (Figure 5).

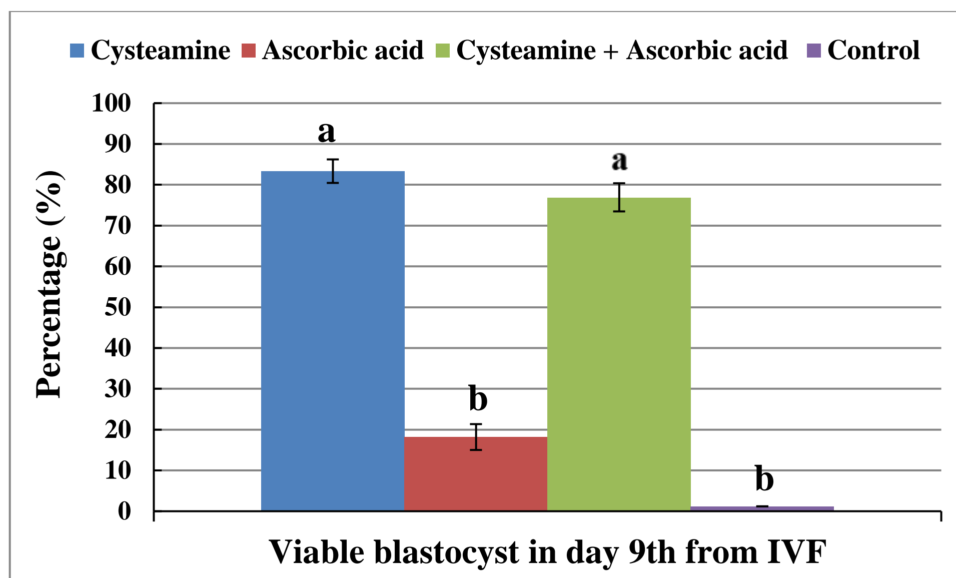

Fig.3: Blastocyst viability till day 9th post IVF after supplementation of antioxidants (CYS plus AA, CYS and AA) in in-vitro culture medium

DISCUSION

Several studies have been done to improve the *in vitro* production of embryos in human and animals. Many researchers proved that the supplementation of antioxidant in one or more steps during IVF had a positive role for improvement of the *in vitro* oocyte maturation and consequently the embryo production [3, 5, 10, 11, 15].

In the current study, we showed that using of antioxidants (cysteamine, AA and CYS plus AA) during *in vitro* oocyte maturation improved production of mature oocytes compared to control group. Previous studies have shown that the ROS produced during *in vitro* maturation had a negative effect on oocytes maturation [5,10, 11]. The formation of free radicals during in vitro oocytes maturation is induced by autoxidation of glucose which lead to a biochemical modifications for the oocytes leading to degeneration [31-33]. The exposure of oocytes to high concentration of glucose affect the expression of g-glutamylcysteine synthetase, which impairs the intracellular synthesis of glutathione which is important for scavenge the ROS (34, 35).

CYS is successfully used as a ROS scavenger in the *in vitro* maturation medium to improve oocytes maturation rates [19-22], which is consistent with our results. The addition of CYS in maturation medium induced an increase in the level of GSH in mature oocytes [21]. GSH plays an important role to protect the oocytes from oxidative damage, transportation of amino acid, DNA and protein synthesis and reduction of disulphide bonds [22]. As well as cysteamine, AA is commonly used as antioxidant to protect the oocytes from the effect of ROS [23-26], which is consistent with our results. AA showed an ability to protect the oocyte structures from lipid peroxidation by regenerating the antioxidant form of α-tocopherol through donating electrons to the α-tocopheroxyl radical, and its ability to reduced coenzyme Q which interacts with α-tocopherol to create the antioxidant ability [23, 24].

In vitro bovine oocytes maturation with combination of CYS plus AA failed to improve the maturation rate compared with using them individually. The presence of either CYS or AA was enough to scavenge the free radicals and protect the oocytes from the harmful effect of oxidative stress that is produced during *in vitro* maturation. These findings are inconsistent with [36, 37] that showed a positive relationship between the combination of antioxidants and the maturation rates. These differences in results may be related to the type of antioxidants as well as the synergistic effect among these antioxidants.

The results of this study revealed that the supplementation of CYS and AA individually or as a combination of in IVF medium didn't improve the cleavage rate and the formation of 2 cells embryos. These results conflicted with [5, 10]. This result may be related to the interference between the antioxidant and sperm capacitation. One of the main process for fertilization is sperm capacitation. This process involves morphological and biochemical changes in the plasma membrane of sperm, which are associated with the removal of cholesterol, thus leading to an increase in membrane fluidity, calcium influx and cAMP levels [38, 39]. Another report demonstrated that ROS play a role in the fertilizing capacity in spermatozoa [40], and

demonstrated that O^{-}_2 is required for the capacitation process and that a low concentration of H_2O_2 participates in enzymatic and membrane modifications, leading to the induction of the acrosome reaction in cryopreserved bovine spermatozoa [41].

At the same time this study demonstrated a significant increasing in the incidence of early cleavage (≤ 24 h) from IVF in CYS and CYS plus ascorbic acid groups compared to ascorbic acid and control group . Previous studies on *in vitro* produced embryos indicated a clear relationship between time of cleavage and embryo development [42, 43]. Accordingly, early cleaved oocytes (≤ 24 h from IVF) had higher developmental competence compared with intermediate (24-36 h post IVF) and late cleaved (36-44 h post IVF) groups. These findings are in consistence with our results which showed highly blastocyst rates from early cleaved oocytes. A report showed 3 important genes [isocitrate dehydrogenase (IDH), YY1- E4TF1/hGABP-associated factor-1 (YEAF1) and histone 2A (H2A)] which had significantly higher expression in early cleaved than late cleaved oocytes [30]. The antioxidant especially CYS in our study may be triggering these genes and seducing the incidence of early cleavage.

The results of the present study demonstrated that enrichment of *in vitro* culture medium with antioxidants CYS or AA, individually or as a combination, improved the embryo output by increased blastocyst yields in compared with control group. These results came in agreement with the previous studies which demonstrated that supplementation of *in vitro* culture medium with antioxidants (CYS or AA) improves embryo production [21-26]. At the same time the results of this study revealed the uselessness of using a combination between CYS and AA as antioxidants to improve blastocyst rate compared of using them individually. Free radical scavenger has been proven to be beneficial for embryo culturing by scavenging ROS and serving embryos development through inducing normal division after cleavage. Previous studies revealed the role of ROS scavenger to reduce the incidence of apoptotic cells within preimplantation embryos, which could be the case in this study, by prolonging the viability of blastocysts until the day ninth after IVF compared to the blastocyst of control group, which stayed viable till day sixth to seventh from IVF. The production of ROS during IVF has a close relationship with quality and viability of produced embryos [2-4]. The presence of H2O2 during IVF was found to be in high concentration in *in-vitro* produced embryos and subsequently promotes apoptosis followed by degeneration in these embryos [2-5]. This prolongation in the viability of blastocysts may be related to the effect of antioxidants on the expression of genes which are responsible for embryonic development and decreases the incidence of apoptosis and degeneration [44].

REFERENCES

1. Rodriguez-Martinez H, Assisted reproductive techniques for cattle breedingin developing countries: a critical appraisal of their value and limitations, Reproduction in Domestic Animals, 2012; 47 (1): 21–26.
2. Kitagawa Y, Suzuki K, Yoneda A, Watanabe T, Effects of oxygen concentration and antioxidants on the *in vitro* developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos, Theriogenology, 2004; 62( ): 1186–1197.
3. Johnson MH, Nasr-Esfahani MH, Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos *in vitro*?, Bioessays, 1994; 16(1): 31–38.
4. Guérin P, El Mouatassim S, Menezo Y, Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings, Human Reproduction Update, 2001; 7(2): 175–189.
5. Ali AA, Bilodeau JF, Sirard MA, Antioxidant requirements for bovine oocytes varies during *in vitro* maturation, fertilization and development, Theriogenology, 2003; 59 (3-4): 939-949.
6. Sikka SC, Role of oxidative stress and antioxidants in andrology and assisted reproductive technology, Journal of Andrology, 2004; 25(1): 5-18.
7. Goto Y, Noda Y, Mori T, Nakano M, Increased generation of reactive oxygen species in embryos cultured *in vitro*, Free Radical Biology and Medicine, 1993; 15(1): 69-75.
8. Aruoma OI, Spencer JP, Mahmood N, Protection against oxidative damage and cell death by the natural antioxidant ergothioneine, Food and Chemical Toxicology, 1999; 37(11): 1043-1053.
9. Cornell JS, Meister A, Glutathione and gamma-glutamyl cycle enzymes in crypt and villus tip cells of rat jejunal mucosa, Proceedings of the National Academy of Sciences, 1976; 73(2): 420-422.
10. Choe C, Shin YW, Kim EJ, et al., Synergistic effects of glutathione and β-mercaptoethanol treatment during *in vitro* maturation of porcine oocytes on early embryonic development in a culture system supplemented with Lcysteine,

Journal of Reproduction and Development, 2010; 56(6): 575-582.

11. de Matos DG, Furnus CC, The importance of having high glutathione (GSH) level after bovine *in vitro* maturation on embryo development effect of beta- mercaptoethanol, cysteine and cysteine, Theriogenology, 2000; 53(3): 761–771.
12. Franzoni RC, Galeta F, Laurenza I, et al., An *in vitro* study on the free radical scavenging capacity of ergothioneine: Comparison with reduced glutathione, uric acid and trolox, Biomedicine and Pharmacotherapy, 2006; 60(8): 453-457.
13. Gasparrini B, Boccia L, Marchandise J, et al, Enrichment of *in vitro* maturation medium for buffalo (Bubalus bubalis) oocytes with thiol compounds: effects of cystine on glutathione synthesis and embryo development, Theriogenology, 2006; 65(2): 275-287.
14. Natsuyama S, Noda Y, Yamashita M, Nagahama Y, Mori T, Superoxide dismutase and thioredoxin restore defective p34cdc2 kinase activation in mouse two-cell block, Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1993; 1176(1-2): 90-94.
15. Ozawa M, Nagai T, Fahrudin M, et al., Addition of glutathione or thioredoxin to culture medium reduces intracellular redox status of porcine IVM/IVF embryos, resulting in improved development to the blastocyst stage, Molecular Reproduction and Development: Incorporating Gamete Research, 2006; 73(8): 998- 1007
16. Wang X, Falcone T, Attaran M, Goldberg JM, Agarwal A, Sharma RK, Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate, Fertility and Sterility, 2002; 78(6): 1272-1277.
17. Huang Y, Tang X, Xie W, et al., Vitamin C enhances *in vitro* and in vivo development of porcine somatic cell nuclear transfer embryos. Biochemical and Biophysical Research Communications, 2011; 411(2): 397-401.
18. Olson SE, Seidel GE, Culture of *in vitro*-produced bovine embryos with vitamin E improves development *in vitro* and after transfer to recipients, Biology of Reproduction, 2000; 62(2): 248–252.
19. Gasparrini B, Neglia G, Di Palo R, Campanile G, Zicarelli L, Effect of cysteamine during *in vitro* maturation on buffalo embryo development, Theriogenology, 2000; 54(9): 1537-1542.
20. Gasparrini B, Sayoud H, Neglia G, de Matos D, Donnay I, Zicarelli L, Glutathione synthesis during *in vitro* maturation of buffalo (Bubalus bubalis) oocytes: effects of cysteamine on embryo development, Theriogenology, 2003; 60(5): 943-952.
21. de Matos DG, Furnus CC, Moses DF, Baldassarre H, Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured *in vitro*, Molecular Reproduction and Development, 1995; 42(4): 432-436.
22. Rodríguez-González E, López-Bejar M, Izquierdo D, Paramio MT, Developmental competence of prepubertal goat oocytes selected with brilliant cresyl blue and matured with cysteamine supplementation, Reproduction Nutrition Development, 2003; 43(2): 179-187.
23. Beyer RE, The role of ascorbate in antioxidant protection of biomembranes: interaction with vitamin E and coenzyme Q, Journal of Bioenergetics and Biomembranes, 1994; 26(4): 349-358.
24. May JM, Is ascorbic acid an antioxidant for the plasma membrane?, The FASEB journal, 1999; 13(9): 995-1006.
25. Hossein MS, Hashem MA, Jeong YW, et al., Temporal effects of alpha-tocopherol and L-ascorbic acid on *in vitro* fertilized porcine embryo development, Animal Reproduction Science, 2007; 100(1-2): 107-117.
26. Öztürkler Y, Yildiz S, Güngör O, Pancarci SM, Kaçar C, Ari UÇ, The Effects of L-Ergothioneine and L-Ascorbic Acid on the *In vitro* Maturation (IVM) and Embryonic Development (IVC) of Sheep Oocytes. Development, 2010; 1(1): 5-14.
27. Alsalim H, Jafarpour F, Tanhaei Vash N, Nasr-Esfahani MH, Niasari-Naslaji A, Effect of DNA and histone methyl transferase inhibitors on outcomes of buffalo–bovine interspecies somatic cell nuclear transfer, Cellular Reprogramming, 2018; 20(4): 256-267.
28. Pezhman M, Hosseini SM, Ostadhosseini S, Varnosfaderani SR, Sefid F, Nasr-Esfahani MH, Cathepsin B inhibitor improves developmental competency and cryo-tolerance of *in vitro* ovine embryos, BMC Developmental Biology, 2017; 17(1): 1-9.
29. Hosseini SM, Forouzanfar M, Hajian M, et al., Antioxidant supplementation of culture medium during embryo development and/or after vitrification warming; which is the most important?, Journal of Assisted Reproduction and Genetics, 2009; 26(6): 355-364.
30. Dode MA, Dufort I, Massicotte L, Sirard MA, Quantitative expression of candidate genes for developmental competence in bovine two-cell embryos, Molecular Reproduction and Development: Incorporating Gamete Research, 2006; 73(3): 288-297.
31. Hashimoto S, Minami N, Yamada M, Imai H, Excessive concentration of glucose during in vitro maturation impairs the developmental competence of bovine oocytes after in vitro fertilization: relevance to intracellular reactive oxygen species and glutathione contents,

Molecular Reproduction and Development: Incorporating Gamete Research, 2000; 56(4): 520-526.
32. Trachtman H, Futterweit S, Bienkowski RS, Taurine prevents glucose-induced lipid peroxidation and increased collagen production in cultured rat mesangial cells, Biochemical and Biophysical Research Communications, 1993; 191(2): 759-765.
33. Donnini D, Zambito AM, Perrella G, Ambesi-Impiombato FS, Curcio F, Glucose may induce cell death through a free radical-mediated mechanism. Biochemical and Biophysical Research Communications, 1996; 219(2): 412-417.
34. Urata Y, Yamamoto H, Goto S, et al., Long exposure to high glucose concentration impairs the responsive expression of γ-glutamylcysteine synthetase by interleukin-1β and tumor necrosis factor-α in mouse endothelial cells, Journal of Biological Chemistry, 1996; 271(25), 15146-15152.
35. Meister A, Selective modification of glutathione metabolism. Science, 1983; 220(4596): 472-477.
36. Miclea I, Pacală N, Hettig A, Zăhan M, Miclea V, Alpha-tocopherol and ascorbic acid combinations influence the maturation of sheep oocytes, Scientific Papers Animal Science and Biotechnologies, 2012; 45(1): 310-313.
37. Truong T, Gardner DK, Antioxidants improve IVF outcome and subsequent embryo development in the mouse. Human Reproduction, 2017; 32(12): 2404-2413.
38. Langlais J, Roberts KD, A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa, Gamete research, 1985; 12(2): 183-224.
39. White DR, Aitken RJ, Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility, Gamete Research, 1989; 22(2): 163-177.
40. O'Flaherty CM, Beorlegui NB, Beconi MT, Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction, Theriogenology, 1999; 52(2): 289-301.
41. O'Flaherty CM, Beorlegui N, Beconi MT, Participation of superoxide anion in the capacitation of cryopreserved bovine sperm, International Journal of Andrology, 2003; 26(2): 109-114.
42. Lequarre AS, Marchandise J, Moreau B, Massip A, Donnay I, Cell cycle duration at the time of maternal zygotic transition for *in vitro* produced bovine embryos: Effect of oxygen tension and transcription inhibition, Biology of Reproduction, 2003; 69(5): 1707–1713.
43. Dinnyes A, Lonergan P, Fair T, Boland MP, Yang X, Timing of the first cleavage post-insemination affects cryosurvival of *in vitro*–produced bovine blastocysts, Molecular Reproduction and Development: Incorporating Gamete Research, 1999; 53(3): 318–324.
44. Sefid F, Ostadhosseini S, Hosseini SM, Zadegan FG, Pezhman M, Esfahani, MH, Vitamin K2 improves developmental competency and cryo-tolerance of *in vitro* derived ovine blastocyst, Cryobiology, 2017; 77(1): 34-40.