

The Influence of Meiotic Spindle Configuration by Cysteamine during *in vitro* Maturation of Mouse Oocytes

Amaneh Mohammadi Roushandeh¹ and Mehryar Habibi Roudkenar^{*2}

¹Dept. of Anatomy, Medicine Faculty, Medical University of Tabriz, Tabriz; ²Research Center, Iranian Blood Transfusion Organization, Tehran, Iran

Received 5 April 2008; revised 25 August 2008; accepted 2 September 2008

ABSTRACT

Background: The aim of this study was to assess effects of cysteamine as an anti-oxidant on rate of *in vitro* maturation of oocyte and determination of its effects on spindle size and shape. **Methods:** Pre-mature mice were primed with pregnant mare stimulating gonadotrophin (PMSG) and germinal vesicle (GV) stage oocytes were obtained 48 h after. The oocytes were cultured in tissue culture medium (TCM 199) with 50, 100, 200 and 500 μ M cysteamine. Experiments also included a group of ovulated oocytes (*in vivo* matured) after priming with PMSG and human chorionic gonadotrophin. For spindle immuno-cytochemistry of metaphase II (MII), oocytes α and β tubulin antibody were performed. Chromosome staining was performed with Hoechst. **Results:** Our results showed that the rate of GV breakdown (GVBD) and MII increased in all cysteamine groups except in group cultured with 500 μ M cysteamine. Immuno-cytochemistry analysis showed that spindle area in all *in vitro* Maturation oocytes increased when compared to *in vivo* oocytes. Spindle shape and size (spindle area) in 100 μ M cysteamine in comparison to *in vivo* group was insignificant ($P>0.05$). **Conclusion:** Our results revealed that cysteamine improved IVM rate in a dose dependant manner. The size and shape of spindle in the presence of given concentration of cysteamine was similar to *in vivo*. Therefore, cysteamine improved microtubule organization, rate of GVBD and MII development. *Iran. Biomed. J.* 13 (2): 73-78, 2009

Keywords: Cysteamine, *in vitro* maturation, Microtubule, Anti-oxidant, Meiosis

INTRODUCTION

The formation of reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2) is a normal process that occurs in the cell when there is a deviation of electrons to oxygen (O_2) during electron transfer reactions [1]. There are some evidences that ROS may be beneficial at some steps of reproduction to permit successful gamete interaction [2]. However, an increasing number of *in vitro* studies have demonstrated the detrimental effects of ROS on reproduction. The main detrimental effects include reduced sperm motility and axonemal protein phosphorylation [3], *in vitro* two-cell block of embryos [4], and reduced embryo development [1,

5]. *In vitro* oocyte or embryo culture results in higher O_2 concentrations than in *in vivo* environments, leading to increased ROS levels [6]. ROS such as O_2^- are able to diffuse and pass through cell membranes and alter most types of cellular molecules such as lipids, proteins and nucleic acids. This can affect the early development of mouse, hamster, and bovine embryos [7-9]. Living organisms possess natural protective equivalents known as ROS scavengers (anti-oxidants) that counteract the negative effects of ROS. These anti-oxidants include enzymes such as superoxide dismutase, which will eliminate O_2^- , catalase and selenium-dependent glutathione (GSH) peroxidase, which will transform H_2O_2 into H_2O and O_2 , as well as lipid- and water-soluble anti-oxidants such as vitamins C and E and uric acid [9]. However, during

*Corresponding Author; Tel. (+98-912) 694 4566; Fax: (+98-21) 8860 1599; E-mail: Roudkenar@ibto.ir. **Abbreviations:** PMSG, pregnant mare stimulating gonadotrophin; GV, germinal vesicle; TCM 199, tissue culture medium; hCG, human chorionic gonadotrophin; MII, metaphase II; GVBD, germinal vesicle breakdown (GVBD); IVM, *in vitro* maturation; ROS, reactive oxygen species; GSH, glutathione; RT, room temperature.

in vitro oocyte and embryo culture, the levels of anti-oxidants are lower than *in vivo* because the oocytes or embryos are divorced from the donor body and do not benefit from the maternal anti-oxidant protection. The addition of an anti-oxidant to the culture medium, therefore, may be important for *in vitro* oocyte maturation and embryo development.

Various thiol compounds especially cysteine and cysteamine are commonly added to IVM media to support GSH synthesis, and to improve the developmental competence of oocytes. Low molecular weight compounds such as β -mercaptoethanol and cysteamine enhance cysteine mediated GSH synthesis in bovine embryos [10-12]. The addition of cysteamine in maturation medium enhanced the GSH synthesis and improved oocyte maturation by protecting the oocytes from oxidative stress and probably by acting on delicate process of cytoplasmic maturation [13-15]. But, thiol compounds like cysteamine when added to culture medium could have different effects depending on the concentration used, the species and type of oocyte in the study [16-18]. In this study, the effects of different concentrations of cysteamine on IVM rate of mouse oocytes have been investigated.

The use of spindle analysis as a parameter for assessment of oocyte quality derived from studies conducted on oocytes of various mammalian species [19-21]. The technique has recently been utilized to assess the effects of toxicants and drugs [20]. ROS can affect microtubule organization and spindle formation, and culture conditions can alter spindle shape and size [21-23]. Supplemented of culture media with anti-oxidants could act as ROS scavenger and improve cytoskeleton organization in oocyte. Thiol compounds such as cysteamine are proved that induce GSH synthesis in oocytes but there is not any report about its role on spindle organization in *in vitro* matured oocytes.

This study was conducted to determine whether cysteamine, as an anti-oxidant, affect on spindle shape and size (spindle area) and could improve rate of *in vitro* maturation of mouse oocytes.

MATERIALS AND METHODS

Reagents. All materials were purchased from Sigma Company (USA).

Collection of germinal vesicle (GV) oocytes. Mice were primed with 5 IU of pregnant mare stimulating gonadotrophin (PMSG). Forty-five to

fifty h later, the animals were scarified by cervical dislocation and the ovary was placed in tissue culture medium (TCM 199) with 10% FBS and 100 IU penicillin/streptomycin. Cumulus oocyte complexes were retrieved with two 27-gauge needles directly from the follicles under a stereomicroscope by rupture of ovary.

In vitro maturation. IVM medium consisted of TCM 199 supplemented with 10% fetal bovine serum, 75 mIU follicular stimulating hormone, 10 ng/ml epidermal growth factor and 50, 100, 200 or 500 μ M cysteamine. Control group was cultured without cysteamine. GV (n = 15-20) oocytes were transferred to a 50- μ l droplet, covered with mineral oil and cultured in 5% CO₂ at 37°C.

Assessment of *in vitro* maturation of oocytes. After culturing GV oocytes for 20 h, GV breakdown (GVBD) and metaphase II (MII) oocytes were analyzed. GV in GVBD was disappeared and MII oocyte was indicated by the presence of the first polar body.

Collection of *in vivo* MII oocytes. To obtain *in vivo* oocytes, 4-week-old female ICR mice were primed with 5 IU of PMSG and 48 h later, with 5 IU of human chorionic gonadotrophin (hCG). About 14-15 h post-hCG, MII oocytes, released from the oviductal ampullae, were collected into TCM 199 supplemented with 0.23 mM sodium pyruvate, 10% fetal bovine serum and 100 IU penicillin/streptomycin (collection medium). Cumulus cells were removed by brief treatment with hyaluronidase at room temperature (RT).

Immuno-fluorescence analysis. *In vivo* and IVM oocytes matured under different experimental conditions (control, 50, 100, 200 and 500 μ M cysteamine) were labeled for α and β tubulin. MII oocytes (n = 50) were fixed with methanol at RT for 10 min. Oocytes were stored in 0.01% PBS-Triton X-100 blocking solution at 4°C. MII oocytes were then incubated in the presence of mouse monoclonal anti- α and β tubulin (1:100) followed by donkey anti-mouse IgG (1:40) at RT for 40 min. Finally, the oocytes were labeled with Hoechst 33342 (1 ng/ml) in blocking solution at RT for 10 min and mounted on slides. The two poles of spindle and the width and length of the spindle were measured in all groups according to Hu *et al.* [24]. The center of the two outermost microtubule organizing centers at each spindle pole were used to determine spindle

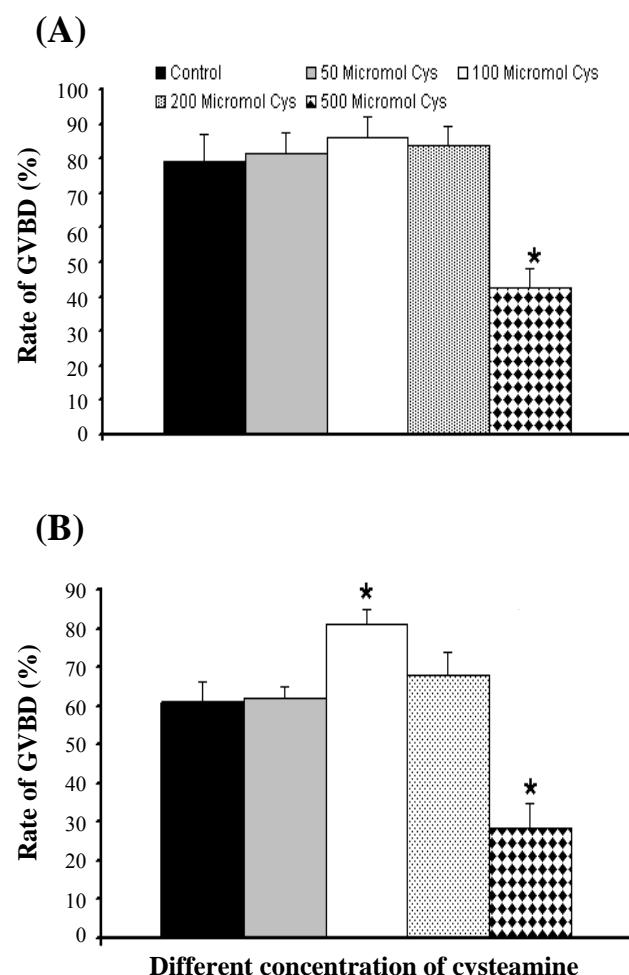


Fig. 1. (A) Rate of GVBD and (B) MII in different concentration of cysteamine, * $P<0.05$; cys, cysteamine.

width at poles. Measurements were carried out using a micrometer on the fluorescent microscope. Taking the middle of the imaginary line presented by the two polar plates as end points, the length of the spindle was determined. Spindle width was measured at the spindle equator. Spindle area was

calculated according to Sanfins *et al.* formula [20].

Statistical analysis. Statistical analyses of data from five replicate trials were carried out with INSTAT view. Comparisons among groups of oocytes were performed using Chi-square and ANOVA with the Tukey-Kramer multiple comparison test as a post test. All values are expressed as the Mean \pm SD. A probability of $P<0.05$ was considered statistically significant.

RESULTS

Effects of different doses of cysteamine on rate of IVM. Figure 1A shows the rate of GVBD development in the presence of different doses of cysteamine in TCM 199. In all cysteamine groups except with concentration of 500 µM, the rate of GVBD was improved. Rate of GVBD in 100 µM cysteamine was significantly higher than the control group (86% versus 72%, $P<0.5$). Rate of GVBD development in group with 500 µM cysteamine was significantly lower than control group (72% versus 45.2%, $P<0.5$). Figure 1B indicates the rate of MII development in the presence of cysteamine. Rate of MII was higher in the presence of cysteamine when compared to control group except with 500 µM cysteamine. Rate of MII oocyte in the presence of 100 µM cysteamine was higher than control significantly ($P<0.05$).

Variations in meiotic spindle shape and size. For assessment of shape and size of spindle in MII oocytes cultured in different conditions, immunocytochemistry of microtubule was performed. Figure 2 indicates the morphology of MII spindle in different groups. Our analysis showed that spindle

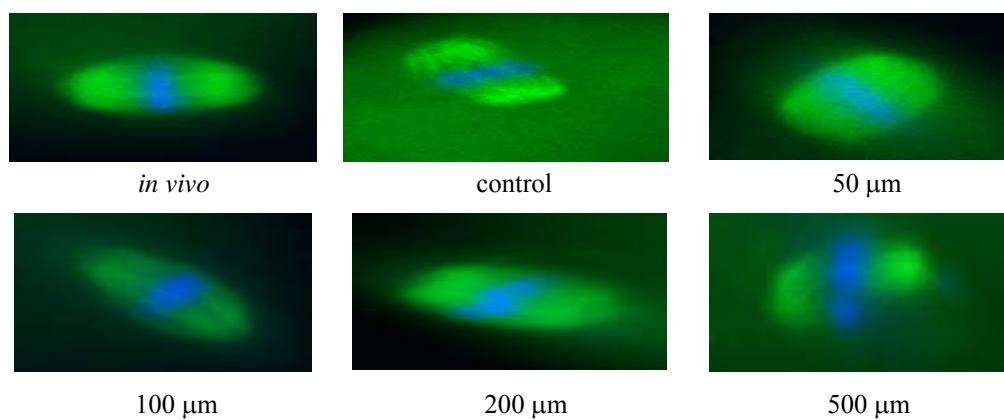


Fig. 2. MII oocyte spindle in different concentration of cysteamine. Spindle size in 500 µM cysteamine is smaller than *in vivo* and its spindle is not normal. With 100 µM cysteamine spindle shape and size in MII oocyte was similar to *in vivo*.

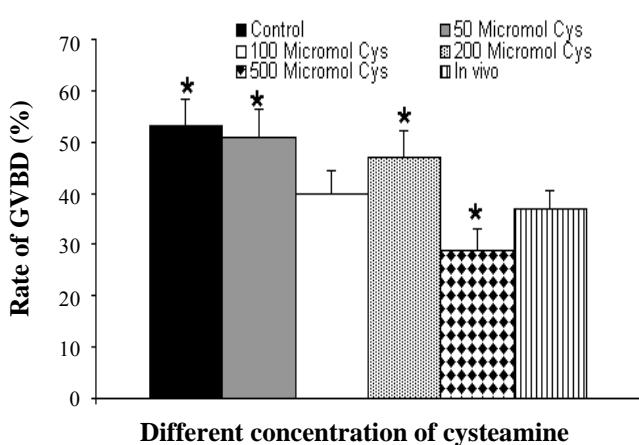


Fig 3. Mean of spindle area in different concentration of cysteamine. * $P<0.05$; cys, cysteamine.

area in all *in vitro* cultured oocytes except group treated with 500 μ M cysteamine increased when compared to *in vivo* group. Shape and size of spindle in group with 100 μ M cysteamine was close to *in vivo* group and difference between them was not significant (Fig. 3). Overall, the results indicate variations in meiotic spindle shape and size.

DISCUSSION

In this study, effect of different concentrations of cysteamine on IVM rate and cytoplasmic maturation of oocytes were investigated. GVBD and MII development increased in the presence of cysteamine in a dose dependant manner. In oocytes treated with 100 μ M cysteamine, rate of *in vitro* maturation of mouse oocyte increased significantly. Several studies also reported similar results [25-27].

In 500 μ M cysteamine, IVM rate decreased significantly when compared to control group. This means that the dose had toxic effects on oocytes development. Degeneration of oocytes in this group was higher than other groups (data not shown).

In mammals, oxidative stress interfere severely gamete viability and embryo development. A series of anti-oxidant enzymes and non-enzymatic processes protect gametes and embryos against ROS damage during oocyte maturation and early stage of development [28]. In ovine, GSH synthesis stimulated by cysteamine during IVM may improves embryo development by reducing intracellular peroxide levels [29]. In our previous study, in the presence of 100 μ M cysteamine on IVM medium, GSH level in MII oocytes was increased. We also showed that highest level of GSH is produced in MII oocytes cultured with 100 μ M cysteamine [30].

The GSH synthesis during oocyte maturation is reported to be a pre-requisite for sperm chromatin decondensation and hence for male pronucleus formation after sperm penetration of mouse and hamster oocytes [31].

Cytoplasmic GSH, defined as one of the indices of cytoplasmic maturation, plays an important role in protecting not only somatic cells but also mammalian gametes from oxidative stress [7]. The cumulus cells surrounding the oocytes are structurally and metabolically linked with the oocytes by gap junctional communications, and are reported to regulate GSH synthesis and accumulation in the ooplasm of pig and cow [32]. These reports suggested that cumulus cells are responsible for maintenance of cytoplasmic GSH concentrations through their gap junctional communications with the oocytes [32].

Immuno-cytochemistry of spindle in MII oocytes also showed that spindle area of MII oocytes in different culture condition was higher than *in vivo* group except group with 500 μ M cysteamine. Sanfins *et al.* [20] showed that *in vivo*-derived oocytes have larger reductions in both spindle length and area than those of IVM oocytes. This finding is concurrent with the result obtained in our study. It has been proved that ROS play an important role on microtubule organization. The higher production of ROS by defective mitochondria may decrease the intracellular reduced GSH/oxidized GSH (GSSG, oxidative form of GSH) ratio. Each of these parameters acting separately or together may affect the dynamic equilibrium of microtubules (assembling/disassembling of tubulins) and/or microfilaments (assembling/disassembling of actin) [22, 23].

The *in vitro* culture environment differs from *in vivo* conditions in many aspects: the oxygen concentration is higher *in vitro* and in such conditions; there is higher ROS concentration in simple culture media. This could be attributed to the lack of suitable culture conditions that can dramatically impair oocyte quality. Tarian *et al.* [33] have demonstrated that a modification in standard culture medium composition significantly improves permission rate and the number of oocytes with normal spindle organization.

Therefore, cysteamine as an anti-oxidant acts as a scavenger of free radical in culture medium and improved microtubule organization in MII oocytes in a dose dependant manner and subsequently increase the rate of GVBD and MII during *in vitro* maturation of oocytes.

REFERENCES

1. Han, D., Zhao, B.T., Liu, Y., Li, J.J., Wu, Y.G., Lan, G.C. and Tan, J.H. (2007) Interactive effects of low temperature and roscovitine (ROS) on meiotic resumption and developmental potential of goat oocytes. *Mol. Reprod. Dev.* 75: 838-846.

2. Min, K.K., Mohammad, S.H., Hyun, J.O., Heru, Y.F., Goo, J., Hye, J.K., So, G.H., Jung Eun, E.P., Sung, K.K., and Byeong Chun, C.L. (2007) Glutathione content of *in vivo* and *in vitro* matured canine oocytes collected from different reproductive stages. *J. Vet. Med. Sci.* 69: 627-632.

3. Brad, A.M., Bormann, C.L., Swain, J.E., Durkin, R.E., Johnson, A.E., Clifford, A.L., and Krisher, R.L. (2003) Glutathione and adenosine triphosphate content of *in vivo* and *in vitro* matured porcine oocytes. *Mol. Reprod. Dev.* 64: 492-498.

4. Tatimoto, H., Ootaki, K., Shigeta, K. and Muto, N. (2001) Enhancement of developmental competence after *in vitro* fertilization of porcine oocytes by treatment with ascorbic acid 2-O-alpha-glucoside during *in vitro* maturation. *Biol. Reprod.* 65: 1800-1806.

5. Agarwal A., Gupta, S., and Sharma, R.K. (2005) Role of oxidative stress in female reproduction. *Reprod. Biol. Endocrinol.* 3: 28 (Review).

6. Bedaiwy, M.A., Falcone, T., Mohamed, M.S., Aleem, A.A., Sharma, R.K., Worley, S.E., Thornton, J., and Agarwal, A. (2004) Differential growth of human embryos *in vitro*: role of reactive oxygen species. *Fertil. Steril.* 82: 593-600.

7. Guérin, P., Mouatassim, E.S. and Ménézo, Y. (2001) Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum. Reprod. Update* 7: 175-189 (Review).

8. Kim, I.H., Van Langendonck, A., Van Soom, A., Vanroose, M.G., Casi, A.L., Hendriksen, P.J. and Bevers, M.M. (1999) Effect of exogenous glutathione on the *in vitro* fertilization of bovine oocytes. *Theriogenology* 52: 537-547.

9. Kitagawa, Y., Suzuki, K., Yoneda, A., and Watanabe, T. (2004) 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* 62: 1186-1197.

10. Mohammad, S.H., Min, K., Goo, J., Hynt, J.O., Okjae, K., Jeong, J.K., Sung, K.K., Byeong, C.L. and Woo, S.H. (2007) Effects of thiol compounds on *in vitro* maturation of canine oocytes collected from different reproductive stages. *Mol. Reprod. Develop.* 74: 1213-1220.

11. Ping, Z., Yan-Guang, W., Qing, L., Guo-Cheng, L., Gang, W., Da, G. and Jing-He, T. (2008) The interactions between cysteamine, cystine and cumulus cells increase the intracellular glutathione level and developmental capacity of goat cumulus-denuded oocytes. *Reproduction* 135: 605-611.

12. Gasparini, B., Boccia, L., Marchandise, J., Palo, R.D., Georgr, F., Donay, I., and Zicarelli, L. (2006) Enrichment of *in vitro* maturation medium for buffalo (*Bubalus bubalis*) oocytes with thiol compounds: Effects of cystine on glutathione synthesis and embryo development. *Theriogenology* 65: 275-287.

13. Furnus, C.C., de Matos, D.G. and Moses, D.F. (1998) Cumulus expansions during *in vitro* maturation of bovine oocytes: relationship level and its role on subsequent embryo development. *Mol. Reprod. Develop.* 51: 76-83.

14. Gasparini, B., Neglia, G., Palo, R.D., Campanile, G. and Zicarelli, L. (2000) Effect of cysteamine during *in vitro* maturation on buffalo embryo development. *Theriogenology* 54: 1537-1542.

15. Kobayashi, M., Lee, E.S., and Fukui, Y. (2006) Cysteamine or beta-mercaptoethanol added to a defined maturation medium improves blastocyst formation of porcine oocytes after intracytoplasmic sperm injection. *Theriogenology* 65: 1191-1199.

16. Balasubramanian, S. and Rho, G.J. (2007) Effect of cysteamine supplementation of *in vitro* matured bovine oocytes on chilling sensitivity and development of embryos. *Anim. Reprod. Sci.* 98: 282-292.

17. Song, K. and Lee, E. (2007) Modification of maturation condition improves oocyte maturation and *in vitro* development of somatic cell nuclear transfer pig embryos. *J. Vet. Sci.* 8: 81-87.

18. De Matos, D.G., Gasparini, B., Pasqualini, S.R. and Thompson, J.G. (2002) Effect of glutathione synthesis stimulation during *in vitro* maturation of ovine oocytes on embryo development and intracellular peroxide content. *Theriogenology* 5: 1443-1451.

19. Rossi, G., Macchiarelli, G., Palmerini, M.G., Canipari, R. and Cecconi, S. (2006) Meiotic spindle configuration is differentially influenced by FSH and epidermal growth factor during *in vitro* maturation of mouse oocytes. *Hum. Reprod.* 21: 1765-1770.

20. Sanfins, A., Lee, G.Y., Plancha, C.E., Overstrom, E.W. and Albertini, D.F. (2003) Distinctions in meiotic spindle structure and assembly during *in vitro* and *in vivo* maturation of mouse oocytes. *Biol. Reprod.* 69: 2059-2067.

21. Ueno, S., Kurome, M., Ueda, H., Tomii, R., Hiruma, K. and Nagashima, H. (2005) Effects of maturation conditions on spindle morphology in porcine MII oocytes. *J. Reprod. Dev.* 51: 405-410.

22. Won-Jun, C., M.D., Jashoman, B., Tommaso, F., James, B., Ashok, A. and Rakesh, K.S. (2007) Oxidative stress and tumor necrosis factor-a-induced alterations in metaphase II mouse oocyte spindle structure. *Fertil. Steril.* 88: 1220-1231.

23. Juan, J., Tari, N.L., Francisco, J.V., Jorge, T. and Antonio, C. (1998) Antioxidant therapy counteracts

the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes. *Mol. Hum. Reprod.* 4: 281-288.

24. Hu, Y., Betzendahl, I., Cortvrindt, R., Smitz, J. and Eichenlaub-Ritter, U. (2002) Effects of low O₂ and aging on spindles and chromosomes in mouse oocytes from pre-antral follicle culture. *Hum. Reprod.* 16: 737-748.

25. Gasparini, B., Boccia, L., Marchandise, J., Palo, R.D., Georgeb, F., Donnay, I. and Zicarelli, L. (2006) Enrichment of *in vitro* maturation medium for buffalo (Bubalus bubalis) oocytes with thiol compounds: Effects of cystine on glutathione synthesis and embryo development. *Theriogenology* 65: 275-287.

26. Whitaker, B.D. and Knight, J.W. (2004) Exogenous gamma-glutamyl cycle compounds supplemented to *in vitro* maturation medium influence *in vitro* fertilization, culture, and viability parameters of porcine oocytes and embryos. *Theriogenology* 62: 311-322.

27. De matos, D.G., Nogueira, D.R., Cortvrinda, C., Herrera, T., Adriaenssens, R.S., Pasqualini, J., and Smitz, J. (2003) Capacity of Adult and Prepubertal Mouse Oocytes to Undergo Embryo Development in the Presence of Cysteamine. *Mol. Reprod. Dev.* 64: 214-218.

28. Luberda, Z. (2005) The role of glutathione in mammalian gametes. *Reprod. Biol.* 5: 5-17.

29. Mohammadi Roushandeh, A., Noori Mooghahi, M.H., Pasbakhsh, P., Abdolvahhab, M., Akbari, M., Shokrgozar, M.A., Sobhani, A., Amidi, F., Abbasi Habibi, M. and Habibi Roudkenar, M. (2007) Intracytoplasmic glutathione level in MII oocyte during *in vitro* maturation of germinal vesicle: Effect of cysteamine. *DARU* 15: 12-16.

30. Zuelke, K.A., Jones, D.P. and Perreault, S.D. (1997) Glutathione oxidation is associated with altered microtubule function and disrupted fertilization in mature hamster oocytes. *Biol. Reprod.* 57: 1413-1419.

31. Mohammadi Roushandeh, A., Pasbakhsh, P., Alizadeh, Z. and Habibi Roudkenar, M. (2007) *In vitro* maturation media, cysteamine concentration and glutathione level affect blstocysts development in mouse. *Iran. J. Reprod. Med.* 5: 159-163.

32. Maedomari, N., Kikuchi, K., Ozawa, M., Noguchi, J., Kaneko, H., Ohnuma, K., Nakai, M., Shino, M., Nagai, T. and Kashiwazaki, N. (2007) Cytoplasmic glutathione regulated by cumulus cells during porcine oocyte maturation affects fertilization and embryonic development *in vitro*. *Theriogenology* 67: 983-993.

33. Tarian, J.J., Vendrell, F.J. and Ten, J. (1996) The oxidizing agent butyl hydroperoxide induces disturbances in spindle organization, c-meiosis, and aneuploidy in mouse oocytes. *Mol. Hum. Reprod.* 2: 895-901.