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Original Article

Presence of Antioxidant in *in vitro* Maturation Medium and its Effects on Glutathione Level, Spindle Area and Rate of *in vitro Fertilization*

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Abstract

Background: Effect of different doses of cysteamine on rate of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and glutathione (GSH) level was studied. Metaphase II (MII) spindle area was analyzed for quantification of shape and size of oocytes.

Methods: Female mice were primed with 5 IU of pregnant mare's stimulating gonadotrophin. Germinal vesicle (GV) oocytes were retrieved 48 hrs later. IVM medium was supplemented with 0, 50, 100, 200 and 500 mM of cysteamine. For IVM and IVF assessment in each group, 150 GV oocytes were used. Experiments also included a group of ovulated oocytes (matured *in vivo*) after priming with pregnant mare's stimulating gonadotrophin and human chorionic gonadotropin. GSH level was measured by 5,5-Dithio-bis (2nitrobenzoic acid) DTNB-GR recycling protocol in GV and MII oocytes. For IVF, MII oocytes were inseminated with mature mouse sperm and rate of two-cell embryo was measured. For immunocytochemistry of microtubule and chromosomes, MII oocytes were fixed by methanol and immunostained with α- and β-microtubule antibody and Hoechst. The spindle area was then analyzed.

Results: A dose-dependent improvement was observed in IVM and IVF rate. MII development and two-cell embryo formation were increased significantly in group which received 200 µm cysteamine compare to the control group. GSH level was increased in presence of cysteamine in group which received 200 µm cysteamine. Spindle area was increased in all groups *in vitro* except for the group which received 500 µm cysteamine. The difference between spindle area in 200 µm cysteamine and *in vivo* group was not significant ($P>0.05$).

Conclusion: Administration of cysteamine improves IVM and IVF rate in a dose-dependant manner. Also cysteamine induces glutathione synthesis in MII oocyte and improves microtubule when administered at a dose of 200 µm. Therefore, addition of cysteamine as an antioxidant can improve IVM and IVF rate by increasing of oocytes quality.
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Keywords ● Cysteamine ● GSH ● microtubule ● mouse

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Introduction

Culture conditions in which oocytes develop has critical role in rate and quality of gametes and zygotes production during in vitro maturation (IVM) and in vitro fertilization (IVF).[1]

Without doubt under culture conditions reactive oxygen species (ROS) production were increased and these molecules have negative effects on oocyte and embryo development.

Reactive oxygen species might have damage effects on cellular structures such as mitochondria and microtubules and could disturbance normal cell function.

Naturally there are several antioxidant systems in most mammalian cells, such as catalase and superoxide dismutase and thiols that act as oxidative stress scavenger.[2]

Glutathione (GSH) is a tripeptide thiol compound that has many important functions in intracellular physiology and metabolism. One of the most important roles of GSH is to maintain the redox state in cells, protecting them against harmful effects caused by oxidative injuries. Also glutathione has another function in amino acid transport, synthesis of DNA and protein and reduction of disulfides.[3] In the mouse GSH could help to progress embryo development with suppression of species- specific "block".[4]

It has been proved that during fertilization GSH has important role in sperm decondensation and transformation of the fertilizing sperm head into the male pronucleus.[3] Low molecular weight thiol compounds, such as cysteamine and b-mercaptoethanol, when added to IVM medium, improve embryo development and quality, by increasing GSH synthesis.[2] The importance of high levels of GSH following IVM is also due to the protection it affords against oxidative damages.[5] In other hand, developmental competence of oocytes in cytoplasmic level depends on intracytoplasmic synthesis of GSH.[4] Although all the components in culture media affect embryo development to some extent.[6] Several reports showed beneficial effects from antioxidants, growth factors,[7,8] and macromolecules on oocyte maturation. Thiols compounds, like cysteamine, when added to culture medium could have different effects depending on the concentration used, the species and type of oocyte in study.[9,10,11] Several reports have addressed the role of cumulus cell in GSH synthesis, a few studies also have proved the ability of oocyte to produce GSH.[2,5,10,12] but there is no report about measurement of GSH level in mouse oocyte in the presence of cysteamine and the present study seems to be among the first ones. Also there isn't any report about optimum dose of cysteamine on IVM in mouse oocytes. De Matos has an experiment with different doses of (50, 100, 200, µm cysteamine) on IVF with different type of medium and oocytes.[4] Because of that, the objective of this study was to study the effects of different doses of cysteamine during IVM of ICR mouse oocytes and detection of optimal dose.

The possibility to use spindle analysis as a parameter to assess oocyte quality, derived from studies conducted on oocytes of various mammalian species.[13,14,15,16] and has been recently utilized to assess effects of toxicants and drugs.[17,18,19] Reactive oxygen species can affect on microtubule organization and spindle formation. Culture conditions are able to alter spindle shape and size. GSH level and spindle parameters are two factors that detect cytoplasmic maturation during in vitro maturation of oocyte. So effect of cysteamine on spindle shape and size was investigated.

Methods and Materials

Animals

Oocytes were obtained from immature female ICR mice with age 4 weeks that were kept under controlled light and temperature conditions with free access to water and food. They had 12 hour light and 12 hour dark condition. Animal experiments were approved by the ethnical committee of Tehran medical university and performed with accordance with the university guidelines.

Collection of Germinal Vesicle oocytes

For obtaining GV stage oocytes, mice were primed with 5 IU of Pregnant Mare's Stimulating Gonadotrophin intraperitoneally (PMSG, Sigma). Forty-five to fifty hours later the animals were sacrified by cervical dislocation. And the ovaries placed in Minimum essential Medium eagle (MEME) with 10% Fetal bovine serum (FBS), 100 IU Penicilin –streptomycin. Cumulus oocytes complexes (COCs) were retrieved directly from the follicles under a stereomicroscope by two 27 gauge needles.

In Vitro Maturation (IVM)

In vitro maturation (IVM) medium consisted of MEME supplemented with 10% fetal Bovine serum, 75mIU follicular stimulating factor (FSH), 10ng/ml EGF (Epidermal growth factor, sigma) and different concentration of Cysteamine (Sigma) 0, 50, 100, 200 and 500 µM. 15-20 COC oocytes were transferred to 50µl droplet covered with Mineral oil and cultured in 37ºc and 5 % Co_2. For IVM assessment in each group 150 oocytes were cultured.

Assessment of in vitro maturation of oocytes

20 hours after IVM, germinal vesicle breakdown oocytes (GVBD) and Metaphase II oocytes were analysed. Germinal Vesicle disappears in GVBD oocytes and MII oocytes were indicated with presence of first polar body.

Collection of in vivo MII oocytes

To obtaining In vivo oocytes, 4 weeks female ICR mice were primed with 5 IU of pregnant Mare's serum gonadotrophin (PMSG, Sigma) and with 5 IU of human chorionic gonadotrophin (hCG, Sigma) 48 h later. About 14–15 h post-hCG, MII-arrested oocytes, released from the oviductal ampullae, were collected into MEME supplemented with 0.23 mM sodium pyruvate, 10% fetal bovine serum and 100 IU penicillin- stereptomysin (Collection medium). Cumulus cells were removed by brief treatment with hyaluronidase at room temperature.

In vitro fertilization

Epididymal sperm suspentions were prepared from 8-10 weeks adult male mice and preincubated for 2 hr in IVF medium to ensure capacitation. IVF medium consisted of human tubal fluid (HTF) medium supplemented with 3% bovine serum albumin (BSA) fraction V (sigma chemical co). After IVM, MII oocytes washed in IVF medium, placed in 50 µl microdrops under mineral oil and 2×10^6 spermatozoa/ ml were added. Sperm and oocytes were incubated for 4 hours after which oocytes were removed, washed, and placed in 50 µl microdroplets of in vitro fertilization medium (HTF with 4mg/ml BSA). After 24 hours numbers of two cell embryos were counted.

GSH assay

For GSH assay, oocytes(Germinal vesicle and MII stage) were washed three times in the stock phosphate buffer (0.2 M sodium phosphate buffer containing 10 mM EDTA, pH 7.2), and groups of 10-15 oocytes in 5 µl of stock buffer were transferred to 1.5-ml microfuge tubes. Samples were stored at -80°C until assay. Briefly, 700 µl of 0.33 mg/ml NADPH in the stock buffer, 100 µl of 6 mM DTNB in the stock buffer, and 190 µl of distilled water were added and mixed in a microfuge tube.10 microliter of 250 units/ml glutathione reductase (Sigma) was added to initiate the reaction. The absorbance was monitored continuously at 412 nm using a spectrophotometer (Japan) for 2 min and its quantity determined based on its standard curve. The experiments were repeated 3 times with 10-15 oocytes for each group.

Immunofluorescence analysis

In vivo and IVM oocytes matured under the different experimental conditions (control, 50, 100, 200 and 500 µm cysteamine) were labelled for α and β tubulin. Oocytes were fixed 10 min at room temperature (RT) with methanol. Oocytes were stored at 4ºC in 0.01% pbs-Triton X100 blocking solution. Then MII oocytes were incubated in the presence of a mouse monoclonal anti- alpha and beta tubulin (1:100, Sigma) followed by donkey anti-mouse IgG (1:40) for 40 min at room tempratura (RT). Finally, the oocytes chromosomes were labelled with Hoechst 33342 (1 ng/ml) in blocking solution for 10 min at room temperature and mounted on slides. 2 poles of spindle, width and length of spindle were measured in all groups according to Hu et al.[13] Spindle areas was calculated according to sanfins et al formula.[20] in order to assess spindle area in each group 40 oocytes were analyzed.

Statistical Analysis

Statistical analyses of data from five replicate trials were carried out by INSTAT view. Comparisons between groups of oocytes were performed using Chi-square and ANOVA with Tukey-Kramer Multiple Comparison Test as a post test. All values are expressed as Mean ± SD. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Effects of different doses of cysteamine on GVBD and MII development

Effects of cysteamine supplementation on IVM rate were examined in present study. In all Cysteamine groups, except 500 µM (40%), rate of GVBD improved compare to control group. Rate of GVBD in group with 200µM cysteamine was significantly higher than control group (86% versus 62.3%, $p<0.5$). 500µM cysteamine showed detrimental effects on oocytes and degeneration of oocytes increased. Also rate of GVBD development in this group was significantly lower than control group. Rate of MII development in all cysteamine groups was increased except in group with 500µM cysteamine. Rate of MII development was highest in group with 200µM cysteamine (figure1 (a) and (b)).

Effects of different doses of cysteamine on rate of two cell embryo

Percent of two cell embryo development in different groups was 40% in control, 38% in 50 µm, 42% in 100 µm, 51% in 200 µm and 19% in 500 µm. Rate of two cell embryo development was higher in presence of cysteamine

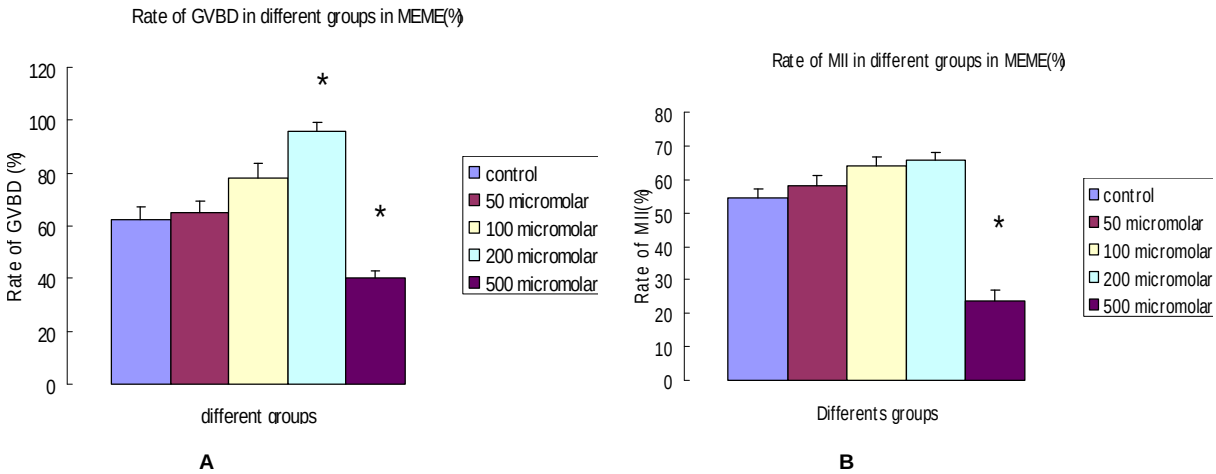

Figure1: (A): shows rate of GVBD in different doses of cysteamine. (B): showes rate of MII in different doses of cysteamine.

compare to control except in 500 µm cysteamine. Rate of two cell embryo in 200 µm was higher than contol group significantly (P<0.05).

Intracytoplasmic level of Glutathione in different groups

In this experiment, for comparison of GSH level between different groups of IVM and in vivo the intracytoplasmic GSH levels in samples of oocytes at oocyte collection time (0 h) and after IVM (24 hours culture) was analysed. The purpose of this study was to assess relationship between synthesis of intracytoplasmic GSH level and different doses of cysteamine in mouse Oocytes. Level of glutathione in in vivo oocytes was significantly higher than other groups. Our results indicate that level of glutathione in MII oocyte was significantly higher than GV stage oocytes (P<0.001). Also level of Glutathione in 200µm cysteamine was significantly higher than control group (P<0.05) (figure2).

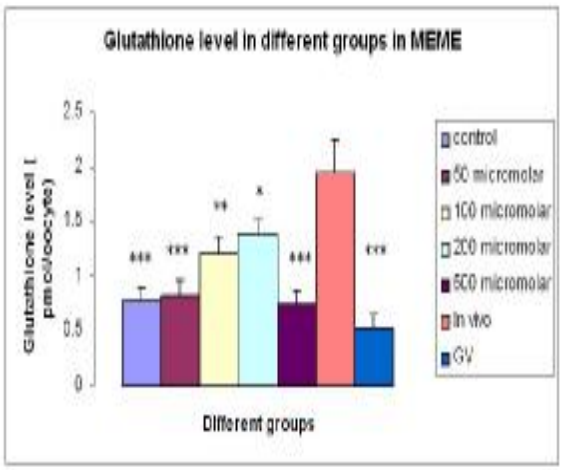

Figure 2: Indicates glutathione level in different groups.

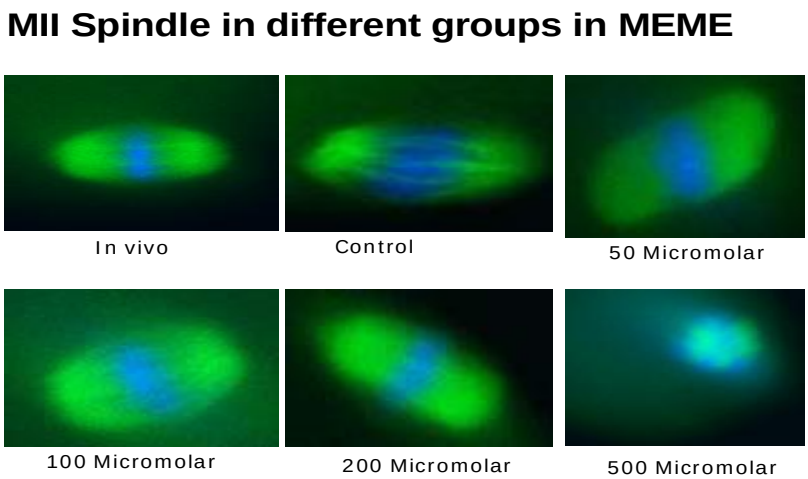

Figure3: Immunocytochemistry of MII oocytes spindle labled with alpha and beta tubulin in in vivo, control, 50 µM cysteamine, 100 µM cysteamine , 200 µM cysteamine , 500 µM cysteamine.

Variations in Meiotic Spindle Shape and Size (spindle area)

Spindle organization was analysed in IVO and IVM oocytes cultured as reported above. Spindle area in all IVM groups except 500µm increased compare to In vivo group (figure3). According to figure 4, control, 50 and 100µm cysteamine groups had higher spindle area compared to in vivo (P<0.05). Spindle area in 200µm cysteamine was close to in vivo and difference between them was not significant (P>0.05). In 500µm cysteamine spindle area was significantly lower than control groups (P <0.05).

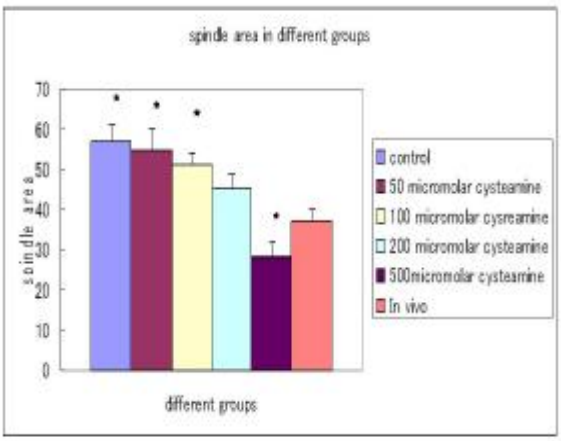

Figure 4: indicates the spindle area in different groups.

Discussion

This study revealed that 200µm cysteamine in MEME improved IVM of GV oocyte. Rate of GVBD and MII was higher in 200µm cysteamine compared to control. De matos et al (2003) showed that Cysteamine failed to improve oocytes development (P>0, 05) and 2-cell embryo rate with 50, 100, 200 µm cysteamine.[4] They used different medium (MEM-α), oocytes with cumulus and different strain of mouse.[4] Gasparini et al (2006) reported positive effects of cysteamine in buffalo.[2] Addition of 100 µm cysteamine in culture medium of buffalo oocytes improved rate of IVM and IVF and increased significantly intrcytoplasmic level of GSH.[2]

Synthesis of GSH during oocyte maturation has been reported in the hamster.[3,11,21] our study is the first in the mouse to measure of cytoplasmic level of GSH after presence of Cysteamine during in vitro maturation of oocytes.

Moreover, our study demonstrates that culture conditions stimulate a significant increase in oocyte GSH content during meiotic progression, from GV stage through MII in oocytes. The augmentation has beneficial effect on Rate of GVBD and MII developmental competencies of mouse oocytes.

In the present study, the addition of cysteamine not only improved the GSH content in MII oocytes but also had positive effect on the maturation rate of mouse Germinal vesicle oocytes.

In mammals, oxidative stress interferes severely gamete viability and embryo Development.[22] A series of antioxidant enzymes and nonenzymatic processes protects gametes and embryos against ROS damage during oocyte maturation and early stage of development.[23] In bovine, it was demonstrated that the addition of cysteamine, BME, cysteine, and cystine to the IVM medium increased the intracellular GSH content of oocytes after in vitro maturation, and also improved embryo development rates and quality.[2] In ovine, De Matos showed that cysteamine and BME increased intracellular GSH levels of the oocyte, but only the addition of cysteamine had a positive influence on subsequent embryo development rates.[11] The synthesis of GSH during oocyte maturation is reported to be a prerequisite for sperm chromatin decondensation and hence for male pronucleus formation after sperm penetration of mouse and hamster oocytes.[21,24] the unpacking of sperm DNA during fertilization requires a reversal of at least some of the changes undergone by sperm nuclei during spermiogenesis and epididymal maturation: protamine disulfide bonds must be reduced and protamine must be removed. Reduction of sperm nuclear disulfide bonds is the first step in the induction of sperm nuclear decondensation.[12] Intracellular reducing powers are generally maintained by GSH, and GSH could directly induce sperm nuclear decondensation in vitro.

Our results revealed that level of glutathione in In vivo MII oocytes was significantly higher than IVM. Zuelke et al. (2003) using with hamster oocytes showed that the significant increase in GSH occurred during the transition from a nuclear or fibrillar GV to when the chromosomes have condensed and the spindle is beginning to form.[5] This finding is in consistence with our results about comparison of glutathione level in GV and MII oocytes.

In ovine, GSH synthesis stimulated by cysteamine during IVM may improve embryo development by reducing intracellular peroxide levels.[11] In porcine, Grupen et al. (1995) observed that concentrations of 50 and 500 mm of cysteamine in the maturation medium positively affected the male pronuclear (MPN) formation rate, but only the higher concentration improved blastocyst yield.[9] Grupen et al. (1995) concluded that increased levels of thiol compounds within the oocyte cytoplasm, accumulated during maturation with cysteamine, might persist as the embryo develops.[9] In our study,

200 µm cysteamine had high level of GSH compare to control group and rate of GVBD and MII was the highest in this concentration.

Given the apparent differences in developmental potential exhibited by IVO and IVM oocytes in various mammals,[25,26] and the role of spindle positioning in the establishment of egg polarity.[27] We asked whether conditions of meiotic maturation influence the spindle area in mouse oocytes. There was difference between the oocytes matured in the presence of different concentrations of cysteamine with those in vivo. According to our results all IVM oocytes had higher spindle area compared to in vivo. IVM groups except 200µm cysteamine had significant difference in spindle area compared to in vivo, more probably due to the highest level of glutathione synthesized in this concentration. Not only GSH acts as scavenger of free radicals but also it participates in microtubule assembly. Sanfins has reported in mice that in vivo derived oocytes have a larger reduction in both spindle length and area than those of IVM oocytes (sanfins et al 2003). Maturation of mammalian oocytes has two aspects: nuclear and cytoplasmic maturation.[28] Medium composition such as follicular fluid, growth factors, cystine, cysteamine, gonadotrophin and hyaloronic acid has been reported that influence cytoplasmic maturation.[29] GSH level and spindle parameters are two factors that detect cytoplasmic maturation during in vitro maturation of oocyte and our data revealed that cysteamine in dose dependence manner can improve spindle shape and size and increase GSH synthesis.

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