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

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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 μM 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 μM 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 μM 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

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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 with10% fetal bovine serum, 75 mIU follicular stimulating hormone, 10 ng/ml epidermal growth factor and 50, 100, 200 or 500 μM cysteamine. Control group was cultured without cysteamine. GV (n = 15-20) oocytes were transferred to a 50-μl droplet, covered with mineral oil and cultured in 5% CO_2 at 37ºC.

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 μM 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 miņ . 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

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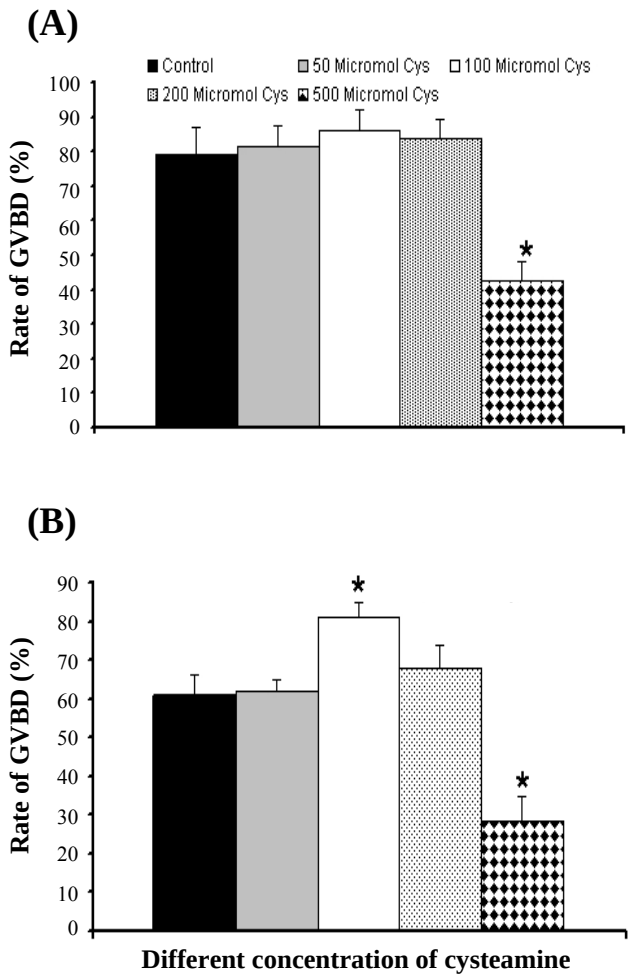

Fig. 1. (A) Rate of GVBD and **(B)** MII in different concentration of cysteamine, **P*<0.05; cys, cysteamine.

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 ± 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

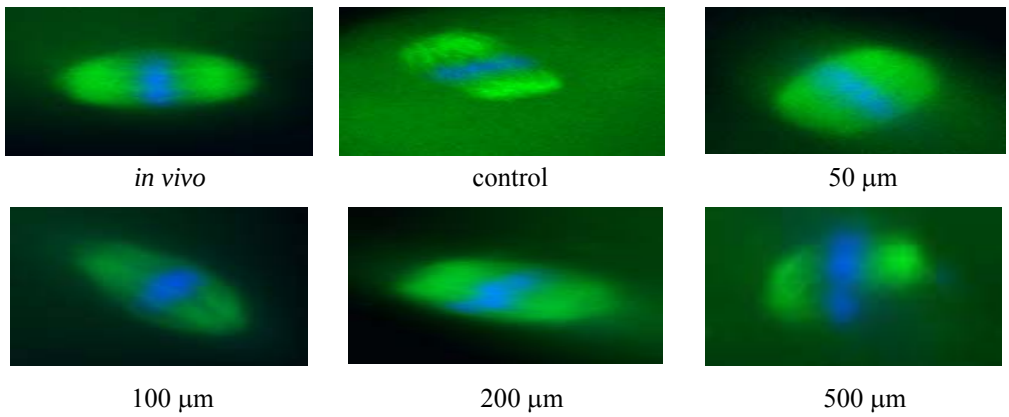

Fig. 2. MII oocyte spindle in different concentration of cysteamine. Spindle size in 500 µM cysteamine is smaller than *in vivo* and its spindle in not normal. With 100 µM cysteamine spindle shape and size in MII oocyte was similar to *in vivo*.

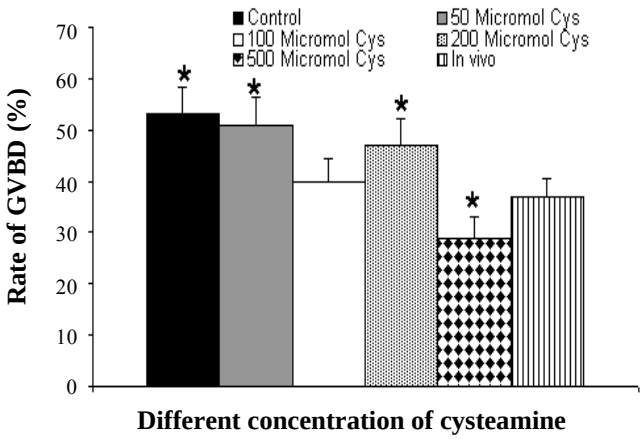

Fig 3. Mean of spindle area in different concentration of cysteamine. **P*<0.05; cys , cysteamine.

area in all *in vitro* cultured oocytes except group treated with 500 µM cysteamine increased when compared to *in vivo* group. Shape and size of spindle in group with 100 µM 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 µM cysteamine, rate of *in vitro* maturation of mouse oocyte increased significantly. Several studies also reported similar results [25-27].

In 500 µM 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 µM 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 µM 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 µM 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.

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