

## Intracytoplasmic glutathione level in MII oocyte during in vitro maturation of germinal vesicle: Effect of cysteamine

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### ABSTRACT

In the present study the kinetics of glutathione (GSH) concentration during in vitro maturation in the presence of Cysteamine in culture medium was examined. Also the effects of different doses of Cysteamine on Germinal Vesicle Breakdown (GVBD) and MetaphaseII (MII) development were investigated. Germinal vesicle (GV) oocytes was obtained from ICR mouse and cultured in Tissue culture medium (TCM199) supplemented with 0, 50, 100, 200 and 500  $\mu$ M cysteamine. Number of GVBD and MII oocytes were recorded at 4 and 24 hours after culture respectively. For GSH assay 5, 5'-dithio-bis (2-nitrobenzoic acid)-glutathione disulfide (DTNB-GSSG) reductase recycling assay was employed. Our results showed that 100  $\mu$ m cysteamine can improve GVBD and MII development significantly higher than control group ( $P < 0.05$ ). Also all Cysteamine groups increased GVBD and MII development compared to control group except 500  $\mu$ m cysteamine groups. Developmental competence in 500  $\mu$ m group was significantly lower than control group ( $P < 0.05$ ). In vivo GSH assay indicated that glutathione concentration in MII oocyte is significantly higher than GV stage and in vitro maturation MII oocytes. Also our results showed that 100  $\mu$ m cysteamine in culture medium increased GSH level in MII oocyte significantly compared to control ( $P < 0.05$ ). GSH level in 500  $\mu$ m cysteamine was lower than control group but it was not significant. Presence of cysteamine in culture medium affects oocyte development competence in vitro dose dependently. Cysteamine as a thiole is able to improve development of GVBD and MII via synthesis of glutathione as a major antioxidant in the mammalian cells.

**Keywords:** In vitro maturation of oocyte, Cysteamine, Glutathione, Germinal Vesicle.

### INTRODUCTION

It is well known that in vitro mammalian oocyte development is negatively affected by the increased oxidative stress occurring under culture conditions. The oxidative damage of cell components via reactive oxygen species interferes with proper cell function. Most mammalian cells possess efficient antioxidant systems such as catalase and superoxide dismutase, as well as thiol compounds that act as metabolic buffers which scavenge reactive oxygen species (1).

Thiol compounds such as cysteamine (Cys) or b-mercaptoethanol (b-ME) have been reported to stimulate glutathione (GSH) synthesis (2, 3) and to improve embryonic development (4, 5).

Thiols compounds like Cysteamine when added to culture mediums depending on the concentration,

the species and the types of oocytes and medium used could have different effects. Since mouse genome is similar to human and due its easy accessibility and low cost, it was used as a model in the present study. Also there isn't any report about optimum dose of cysteamine on IVM on mouse oocytes (6). For these reasons, the objective of this study was to investigate the effect of cysteamine on rate of GVBD and MII during IVM of germinal vesicle oocytes in TCM199 and to detect optimal dose of cysteamine during IVM.

Several studies have demonstrated the positive effect of high intracytoplasmic glutathione (GSH; c-glutamylcysteinylglycine) levels on embryo development by protecting cells against oxidative

stress (7). Synthesis of GSH during oocyte maturation has been reported in pig, cow, sheep and goat oocytes (8) but there is not any report indicating effects of cysteamine on GSH synthesis in MII stage oocytes resulting from IVM in mouse. Moreover, the role of cumulus cells in the synthesis of GSH in oocytes is still obscure. Therefore in the present study synthesis of GSH in oocytes without cumulus cells (Denude oocytes) was investigated.

## METHODS AND MATERIALS

### Reagents

Cysteamine, tissue culture medium (TCM) 199, fetal Bovine serum (FBS), sodium pyruvate), EDTA, 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB), GSH, glutathione reductase, NADPH, estradiol-17 $\beta$ , PBS, and penicillin G were purchased from Sigma Chemical Company. All reagents were of cell culture-tested quality.

### Animals

Germinal vesicle Oocytes were obtained from immature female ICR mice (Tohoku university animal house, Japan) 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.

### Collection of GV oocytes

For obtaining GV stage oocytes, female mice were primed with 10 IU of PMSG (Sigma). Forty-five to fifty hours later the animals were sacrificed by cervical dislocation and placed in TCM199 with 100IU 10% FBS, Penicillin-streptomycin. Cumulus oocytes complexes (COCs) were retrieved directly from the follicles under a stereomicroscope by means of two 27 gauge needles. COC oocytes were denuded with frequent pipette.

### In Vitro Maturation (IVM)

In vitro maturation (IVM) medium consisted of TCM199 supplemented with 10% fetal bovine serum, 75mIU FSH, 10ng/ml EGF (Epidermal growth factor, sigma) and of Cysteamine (Sigma) in concentration of 0, 50, 100, 200 and 500  $\mu$ M. Oocytes (15-20) were transferred to 50 $\mu$ l droplet covered with Mineral oil (sigma, embryo tested) and cultured at 37°C and 5% CO<sub>2</sub>.

### Assessment of in vitro maturation of oocytes

Twenty hours after germinal vesicle oocytes culture (figure 1), germinal vesicle breakdown oocytes (GVBD) (figure 2) and metaphase II (Figure 3) oocytes were analysed. Indicator for GVBD oocytes was disappearance of germinal

vesicle and for MII oocytes were the presence of first polar body.

### Collection of in vivo MII oocytes

To obtain in vivo MII oocytes, 4 weeks female ICR mice were primed with 10 IU of pregnant Mare's serum gonadotrophin (PMSG, Sigma) and 10 IU of hCG (Sigma) 48 hrs later. About 14-15 hrs post-hCG, MII-arrested 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 (1 min) with hyaluronidase (1 mg/ml) at room temperature.

### GSH assay

For this purpose, Oocytes (germinal vesicle and MII stage) were washed three times in the stock buffer (0.2 M sodium phosphate buffer containing 10 mM EDTA, pH 7.2), and groups of 10-15 oocytes in 5  $\mu$ l of stock buffer were transferred to 1.5-ml microfuge tubes. Samples were stored at -80°C until assay. The intracellular concentration of GSH in oocytes was determined using 5,5'-dithio - bis (2 - nitrobenzoic acid) - glutathione disulfide (DTNB-GSSG) reductase recycling assay by a described method (9). Briefly, 700  $\mu$ l of 0.33 mg/ml NADPH and 100  $\mu$ l of 6 mM DTNB in the stock buffer, and 190  $\mu$ l of distilled water were mixed in a microfuge tube. Then 10 microliters of 250 units/ml glutathione reductase (Sigma) was added to initiate the reaction and the absorbance was monitored continuously at 412 nm using a spectrophotometer (Japan) for 2 min and its quantity was determined from a standard curve which was constructed.

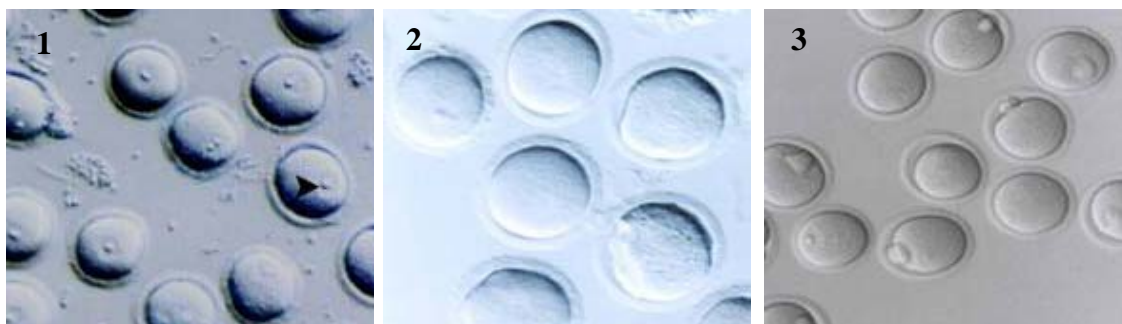
### Statistical Analysis

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  $\pm$  SD. A probability of  $P < 0.05$  was considered to be statistically significant.

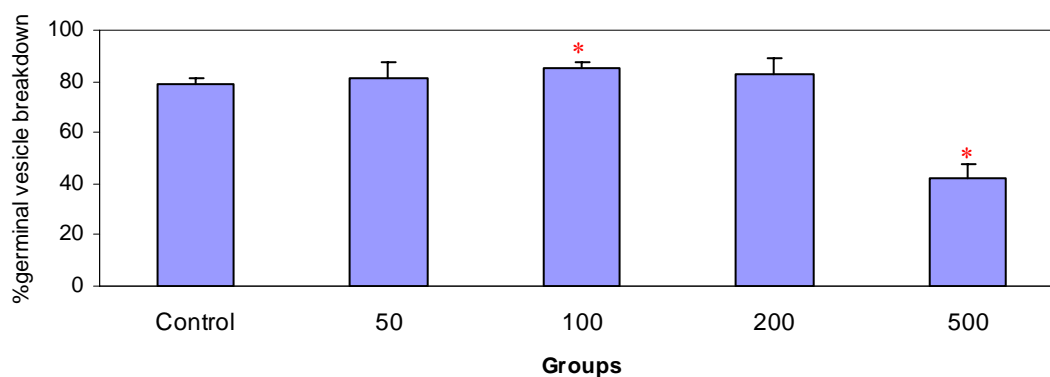
## RESULTS

### Effect of different doses of cysteamine on GVBD development

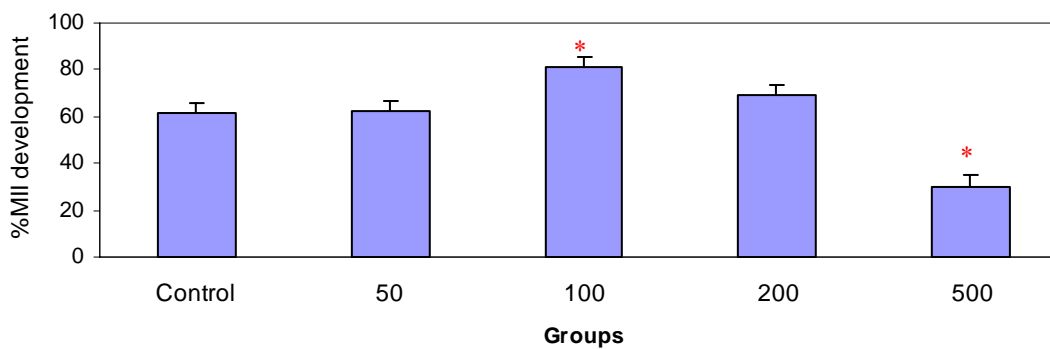
Figure 4 shows that rate of GVBD development in the presence of different doses of cysteamine in TCM199 after culturing for 4 hours. In all Cysteamine groups except of concentration of 500  $\mu$ M, the rate of GVBD improved. Rate of GVBD in 100  $\mu$ M cysteamine was significantly higher than the control group (86% versus 72%,  $p < 0.05$ ).



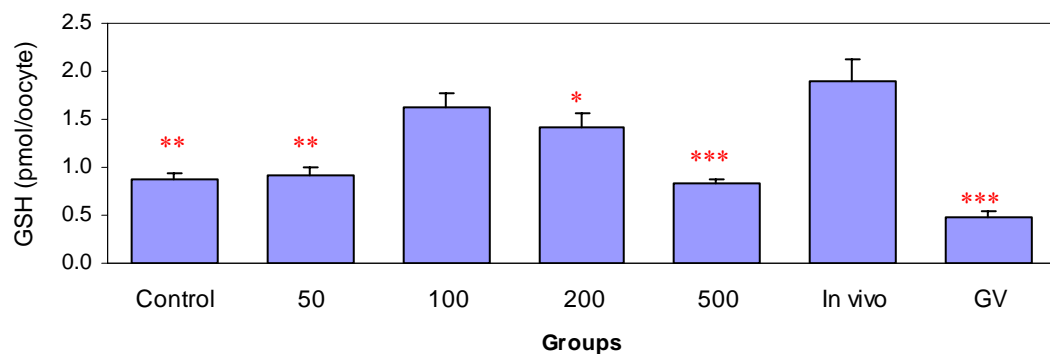
**Figure 1.** Oocytes in germinal vesicle stage. Nucleus and nucleolous are shown with arrow and arrow tip respectively  
**Figure 2.** Oocytes in GVBD. Germinal vesicle is disappeared in this stage.  
**Figure 3.** Oocytes in MII stage. First polar body is shown in periviteline line space.



**Figure 4.** Rate of GVBD development in different groups.



**Figure5.** Rate of MII development of oocytes.



**Figure 6.** Glutathione level in different groups in TCM199

Cysteamine at concentration of 500 $\mu$ M showed detrimental effects on oocytes and degeneration of oocytes increased in this group. Also the rate of GVBD development in this group was significantly lower than the control group (72% versus 45.2%,  $p < 0.5$ ).

*Effects of different doses of cysteamine on MII development:*

Figure 5 indicates the rate of MII development in the presence of cysteamine. Rate of MII formation was higher in the presence of cysteamine compared to control except when the concentration of cysteamine was 500  $\mu$ M. Rate of MII oocyte in the presence of 100  $\mu$ M cysteamine was higher than another groups and in comparison with the control was significant ( $P < 0.05$ ).

*Determination of intracytoplasmic level of Glutathione in different groups:*

In this experiment, the intracytoplasmic GSH levels in samples of oocytes obtained from mouse at oocyte collection time (0 h) was analysed after IVM (24 hours culture). Also the level of Glutathione in In vivo MII oocytes was measured. From the figure 6 it appears that level of glutathione in In vivo oocytes was significantly higher than other groups. Our results indicate that the level of glutathione in MII oocyte was significantly higher in GV stage oocytes ( $P < 0.001$ ). Also the level of Glutathione in 100 $\mu$ M cysteamine was significantly higher than the control group ( $P < 0.05$ ).

### DISCUSSION

This study is the first in mouse to show the role of cysteamine in the synthesis of glutathione during in vitro maturation of oocytes. Similar to other species such as equine, goat, cow and pig, intra-oocyte GSH concentration increases during both in vivo and IVM (5, 8).

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

The biosynthesis of GSH is strictly dependent on the availability of cysteine in extracellular

compartment. However, cysteine is rapidly oxidized in cystine, thus the presence of other thiols, as cysteamine, may maintain cysteine in its reduced form and promotes its cellular availability (2). In this study, 100  $\mu$ M of cysteamine was the effective concentration on the rate of maturation in TCM199. There are several studies that verify these results (2, 3, 4, 10).

In the present study, addition of cysteamine affects not only GSH content in MII oocytes but also maturation rate of mouse germinal vesicle oocytes.

In mammals, oxidative stress interferes severely gamete viability and embryo development. A series of antioxidant enzymes and nonenzymatic processes protects gametes and embryos against ROS damage during oocyte maturation and early stage of development (1). In bovine, it was demonstrated that 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 the rate and quality of embryo development (11). In ovine, it has been shown (5) that cysteamine and BME increased intracellular GSH levels of the oocyte and only addition of cysteamine had a positive influence on subsequent embryo development rates.

Our results showed that in vivo level of glutathione in MII oocytes was significantly higher than that of in vitro condition. In a report using hamster oocytes it was shown that the significant increase in GSH occurred during the transition from a nuclear or fibrillar GV to the time that chromosomes was condensed and spindle began to form (12). Therefore this finding verifies our results about comparison of glutathione level in GV and MII oocytes.

In ovine, GSH synthesis stimulated by cysteamine during IVM may improves embryo development by reducing intracellular peroxide levels (8). In porcine it has been observed that concentrations of 50 and 500  $\mu$ M of cysteamine in the maturation medium positively affected the MPN formation rate, and only at higher concentration improved blastocyst yield (13). It has been concluded that increase in levels of thiol compounds within the oocyte cytoplasm, accumulated during maturation with cysteamine, might persist as the embryo develops (13).

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