Microtubule assembly and *in vitro* development of bovine oocytes with increased intracellular glutathione level prior to vitrification and *in vitro* fertilization

H. Hara [1], I. Yamane [2], I. Noto [2], N. Kagawa [3], M. Kuwayama [4], M. Hirabayashi [5,6], S. Hochi [1,2,7]*

[1]*Interdisciplinary Graduate School of Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan*

[2]*Graduate School of Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan*

[3]*Kato Ladies Clinic, Shinjuku, Tokyo 160-0023, Japan*

[4]*Repro-Support Medical Research Centre, Shinjuku, Tokyo 160-0022, Japan*

[5]*National Institute for Physiological Sciences, Okazaki, Aichi 444-8787, Japan*

[6]*The Graduate University for Advanced Studies, Okazaki, Aichi 444-8787, Japan*

[7]*Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan*

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* Corresponding author: Tel.: +81 268 215350; fax: +81 268 215830.

E-mail address: shochi@shinshu-u.ac.jp (S. Hochi)

Summary

Although vitrification is a useful technique for preservation of bovine oocytes, yield of blastocysts derived from the vitrified oocytes is still low. We have recently reported a new type of cryoinjuries, multiple aster formation, by which pronuclear migration and development of vitrified-warmed and *in vitro*-fertilized bovine oocytes are impaired. The aim of the present study was to investigate the effect of glutathione (GSH) content of vitrified bovine oocytes on the multiple aster formation and the subsequent *in vitro* development. Treatment of bovine cumulus-oocyte complexes with β-mercaptoethanol (βME) and L-cysteine (Cys) during *in vitro* maturation resulted in 2.5-folds higher GSH content not only in fresh control but also vitrified-warmed oocytes. Proportions of normally fertilized zygotes exhibiting sperm aster(s) were as high as > 95% in all four groups (with or without βME/Cys x fresh control or vitrified). The frequency of multiple aster formation in vitrified oocytes (3-folds higher than that in fresh control oocytes) was not affected by the increased level of intracellular GSH with βME/Cys. Consequently, the migration and development of pronuclei as well as the yield of blastocysts from vitrified-warmed oocytes (17 vs 41%) were not improved. In addition, there was no effect of the increased GSH level on the yield of blastocysts in fresh control groups.

Keywords: Bovine oocytes; Intracellular glutathione; β-mercaptoethanol; Multiple asters; Vitrification

Introduction

Although successful pregnancies from cryopreserved bovine oocytes have been reported (Fuku *et al*., 1992), efficiency producing transferable blastocysts after *in vitro* fertilization (IVF) is still low even after application of vitrification protocols (Chian *et al*., 2004). Possible reasons for high sensitivity of oocytes to cryopreservation include the large cell size and low permeability of water and cryoprotective agent (Leibo, 1981), the meiotic spindle disassembly and chromosome misalignment (Shi *et al*., 2006), and the oocyte activation prior to the IVF (Larman *et al*., 2006). We have recently reported that vitrification of bovine oocytes induces frequent multiple-aster formation which leads to impaired pronuclear migration and development before the first cleavage (Hara *et al*., 2012).

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH), a major non-protein sulfydryl compound, plays an important role in protecting cells against the destructive effects of reactive oxygen species and regulating syntheses of DNA and proteins (Meister, 1983). The GSH level increases during oocyte maturation in the ovary and reaches a peak at the metaphase-II stage (Perreault *et al*., 1988). However, the GSH levels of oocytes matured under *in vitro* conditions are lower when compared with those of ovulated oocytes, as reported in some species (Ge *et al*., 2008; Brad *et al*., 2003; Rodríguez-González *et al*., 2003; Kim *et al*., 2007). The GSH synthesis of oocytes during *in vitro* maturation (IVM) may be disturbed by a low availability of cysteine (Meister, 1983; Furnus & de Matos, 1999). Low molecular weight thiol compounds, such as β-mercaptoethanol (βME) and cysteamine, can promote cysteine (cystine) uptake through formation of a mixed disulfide compound (e.g., βME-cysteine) (Ishii *et*

al., 1981; Ohmori & Yamamoto, 1983), and such thiol compounds supplemented into IVM medium can increase intracellular GSH level and developmental potential of the oocytes in domestic species including pig (Abeydeera *et al*., 1998) and cattle (de Matos *et al*., 1996).

It has been reported that GSH of bovine IVM-IVF oocytes can stimulate sperm aster formation (Sutovsky & Schatten, 1997) and that GSH level of porcine oocytes is adversely affected by vitrification (Somfai *et al*., 2007). Therefore we hypothesize that decreased level of the GSH in vitrified-warmed bovine oocytes may be responsible for abnormal aster formation and poor developmental potential. In the present study, the effect of GSH content of vitrified bovine oocytes on the multiple aster formation and the subsequent *in vitro* development was investigated.

Materials and methods

In vitro maturation

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Abattoir-derived bovine ovaries were transported to the laboratory in saline (maintained at 10 to 12 ºC) within 24 h after slaughter. The contents of 2-8 mm follicles were aspirated with an 18-G needle connected to a 10-mL syringe. Oocytes surrounded with at least two layers of compact cumulus cells were cultured in Hepes-buffered Tissue Culture Medium (TCM)-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.2 mM sodium pyruvate, 0.02

AU/mL FSH (Kyoritsu Seiyaku Co., Tokyo, Japan), 1 μg/mL 17ß-estradiol, and 50 μg/mL gentamycin sulfate for 22 h at 38.5 ºC under 5% CO_2 in air. To increase the intracellular GSH level, 50 μM βME (Takahashi *et al*., 1993) and 1 mM L-cysteine (Choe *et al*., 2010) (βME/Cys) were added to the maturation medium. After the maturation culture, cumulus cells were removed by a brief vortex-mixing in the Hepes-buffered TCM-199 supplemented with 3 mg/mL bovine serum albumin (BSA), 0.2 mM sodium pyruvate, 1000 IU/mL hyaluronidase, and 50 μg/mL gentamycin sulfate. Oocytes with an extruded first polar body were defined as matured and were used for experiments.

Vitrification and warming

Matured oocytes were subjected to vitrification according to the method described previously by Hara et al. (Hara *et al*., 2012). Briefly, oocytes were equilibrated with 7.5% ethylene glycol (EG; Wako Pure Chemical Industries Co., Osaka, Japan) and 7.5% dimethylsulfoxide (DMSO; Wako) in Hepes-buffered TCM-199/20% FBS base medium for 3 min at room temperature, and then transferred into a vitrification solution consisting of 15% EG, 15% DMSO and 0.5 M sucrose in the base medium for approximately 60 s at room temperature. Within this 60 s, up to eight oocytes were loaded onto the top of the polypropylene strip of a Cryotop (Kitazato BioPharma Co., Shizuoka, Japan) with a minimal amount of the vitrification solution, and then quickly immersed into liquid nitrogen (LN_2).

After storage for 1 to 10 wk in LN_2 tank, oocytes were warmed by immersing the polypropylene strip of a Cryotop into 3 mL of the base medium containing 1 M sucrose

at 38.5 ºC, and kept for 1 min. The oocytes were transferred to the base medium at room temperature in a stepwise manner (0.5, 0.25, and 0 M sucrose for 3, 5, and 5 min, respectively). They were cultured in Hepes-buffered TCM-199 supplemented with 5% FBS, 0.2 mM sodium pyruvate and 50 μg/mL gentamycin sulfate (TCM-199/5% FBS) for 1 to 2 h at 38.5 ºC under 5% CO_2 in air before subjecting to GSH measurement or IVF.

Measurement of intracellular GSH

Intracellular GSH content was measured by a 5,5'-dithio-bis(2-nitrobenzoic acid)-GSH reductase recycling assay with a total glutathione quantification kit (Dojin Molecular Technologies Inc., Kumamoto, Japan). Oocytes were washed three times with Ca^{2+}/Mg^{2+}-free PBS containing 1 mg/mL polyvinylpyrrolidone (PVP). According to the instructions, pools of 35 to 40 oocytes from each treatment were transferred to 12 μl of 10 mM HCl in a 0.6-mL microfuge tube. Then the oocytes were frozen in LN_2 and were thawed at room temperature. This freeze-thaw procedure was repeated twice and the oocytes were stored at -80 ºC until assay. After final thawing, 3 μL of 5% 5-sulfosalicylic acid was added to the samples and the tubes were centrifuged for 10 min at 8000 × *g*. Ten μL of the supernatant was diluted with 40 μL pure water, and then 20 μL of the sample solution was transferred to each well of 96-well microplate preloaded with 20 μL co-enzyme working solution, 120 μL buffer solution and 20 μL enzyme working solution. After incubation for 10 min at 37.0 ºC, 20 μL of substrate working solution was added to the each well. The absorbance at 405 nm was determined by a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA) following

incubation for 30 min at room temperature. The total GSH content (pmol/oocyte) was calculated by reference to a standard curve prepared with authentic GSH.

In vitro fertilization and culture

Commercially available frozen semen from a Japanese Black bull was used. After thawing in a water bath at 37 ºC for 30 s, the contents of a 0.5-mL straw was layered on the top of Percoll density gradient consisting of 2 mL of 45% Percoll above 2 mL of 90% Percoll in a 15-mL conical tube, and centrifuged for 20 min at 700 × *g*. The pellet was re-suspended in 4 mL of modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan) supplemented with 5 mM theophylline, washed twice (5 min at 300 × *g* each) and then re-suspended in the mBO medium supplemented with 5 mg/mL BSA and 10 µg/mL heparin (IVF medium) to yield a concentration of 1.5×10^7 sperm cells/mL. Ten to 12 matured oocytes in the IVF medium were co-incubated with the above sperm suspension at a final concentration of 3×10^6 sperm cells/mL for 6 h in 100-µL microdrops under mineral oil at 38.5 ºC under 5% CO_2 in air.

Up to 30 presumptive zygotes were cultured in a 250-µL microdrop of modified synthetic oviduct fluid (mSOF) (Holm *et al.*, 1999), supplemented with 30 µL/mL essential amino acids solution (× 50, Gibco-11130), 10 µL/mL non-essential amino acids solution (× 100, Gibco-11140) and 5% FBS at 39.0 ºC under 5% CO_2, 5% O_2 and 90% N_2 for up to 8 d. Cleavage rate was determined on Day-2 (Day-0 was defined as the day of IVF), and number of blastocysts were recorded on Day-7 and -8.

Immunostaining of pronuclear zygotes

To assess the aster formation of pronuclear zygotes, inseminated oocytes were cultured for an additional 4 h in TCM-199/5% FBS at 38.5 ºC under 5% CO_2 in air, and then immunostained according to the method described previously (Hara *et al*., 2011). The zygotes were extracted for 15 min by buffer M (25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$ 0.1 mM EDTA, 1 mM EGTA and 50 mM imidazole hydrochloride, pH 6.8) containing 5% (v/v) methanol and 1% (v/v) Triton X-100, after zonae pellucidae had been removed with 0.75% protease in M2 medium. The zygotes were then fixed with cold methanol for 10 min and permeabilized overnight in PBS containing 0.1% (v/v) Triton X-100. Microtubules were labeled with a monoclonal antibody against α-tubulin (T5168; diluted 1:1000). The primary antibodies were detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (F1010; diluted 1:200). Nuclear DNA was visualized by counterstaining with 2.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI). Preparations were mounted with coverslips in antifade agent, and digital images were collected at 2 μm distance using a confocal laser scanning microscope (FV1000-D; Olympus, Tokyo, Japan). The digital images were stacked and assessed with Image-J software (National Institutes of Health, Bethesda, ML, USA; accessed on-line). Zygotes with 2-PN were defined as those fertilized normally, and the larger pronucleus was defined as male pronucleus.

Statistical analysis

Maturation rate of cumulus-oocyte complexes and morphological survival rate of

vitrified oocytes were compared between non-treated and βME/Cys-treated groups by Student's *t*-test. Arcsin-transformed data for cleavage rate, blastocyst yield, fertilization rate, polyspermic penetration rate and aster formation rate, as well as data for GSH content, distance between male and female pronuclei and pronuclear size were compared by one-way ANOVA. When the ANOVA was significant, differences among means were analyzed by a Tukey's test. A value of $P < 0.05$ was defined as a significant difference.

Results

Effect of βME/Cys in IVM medium on intracellular GSH content

As oocyte maturation rate was assessed with the presence of the first polar body, treatment of the cumulus-oocyte complexes with βME/Cys did not influence the maturation rate (62%, 634/1,029 vs 66%, 658/1,004 in non-treated group, $P > 0.05$). The GSH content of fresh control oocytes matured in the presence of βME/Cys was significantly higher than that of non-treated oocytes ($P < 0.05$; Fig. 1). After vitrification and warming, all the matured oocytes (n = 318 and 326 for βME/Cys and non-treated groups, respectively) appeared morphologically normal. The GSH content of the vitrified-warmed oocytes in the βME/Cys group remained to be 2.5-folds higher than that of those in the non-treated group ($P < 0.05$).

Effect of increased GSH content on aster formation

Incidences of normal fertilization (2-PN), assessed by DAPI staining, were comparable among all the four groups ($P > 0.05$; Table 1). Polyspermic penetration occurred at similar rates between non-treated and βME/Cys groups regardless of vitrification (Fresh control; 17 vs 16%, Vitrified; 24 vs 24%). Immunostaining for α-tubulin indicated that proportions of 2-PN zygotes exhibiting sperm aster(s) were high as > 95% in all groups (Table 1). However, ratios of zygotes exhibiting multiple asters were > 3-folds higher in vitrified group than those in fresh control group ($P < 0.05$).

Pronuclear migration and development of the βME/Cys-treated 2-PN zygotes, regardless of vitrification, were comparable with those of the non-treated zygotes (Table 2), as far as zygotes with a single aster were concerned. While zygotes with multiple asters exhibited an impaired migration and development of their pronuclei, neither treatment with βME/Cys nor vitrification also did not influence the extent of these parameters.

Effect of increased GSH content on embryonic development

Cleavage rates of presumptive zygotes were comparable in all four groups ($P > 0.05$; Table 3). On the other hand, developmental potential of vitrified oocytes into blastocysts until Day-8 was not improved by increasing intracellular GSH level with βME/Cys treatment ($P > 0.05$) and still lower than that of fresh control oocytes ($P < 0.05$). Within fresh control groups, increased level of intracellular GSH did not contribute to improve the blastocyst yield ($P > 0.05$).

Discussion

Effect of low molecular weight thiol compound during IVM (to increase ooplasmic GSH level) on developmental potential of cryopreserved bovine oocytes has not been investigated to date, while there is only one report attempting at such an approach, without success, in pig (Gupta *et al*., 2010). Oocyte maturation rate was similar between non-treated and βME/Cys-treated groups, but mean GSH level in βME/Cys-treated oocytes (16.2 pmol/oocyte) was significantly higher than that in non-treated oocytes (6.5 pmol/oocyte; Fig. 1). Mizushima & Fukui (2001) reported an enhanced maturation rate of bovine oocytes in the presence of βME. The composition of IVM medium, the density of oocytes during IVM and the period of ovary storage were different from those employed in the present study. Cryotop vitrification procedure did not decrease the GSH level of post-warm oocytes in both non-treated and βME/Cys-treated groups. Somfai et al. (Somfai *et al*., 2007) reported a significant decrease of GSH levels in porcine oocytes after solid-surface vitrification procedure. This may depend upon species difference and/or suitability of vitrification procedure.

After vitrification and IVF, bovine oocytes with increased GSH level exhibited similar incidence of multiple aster formation compared to the oocytes without increased GSH level (Table 1). Sutovsky & Schatten (1997) reported that sperm aster formation of bovine IVF oocytes was disturbed when the oocytes were treated with buthionine sulfoximine, a specific inhibitor of γ-glutamyl-cysteine synthetase. Yoshida et al. (Yoshida *et al*., 1993) also reported that intracellular GSH plays an important role in male pronuclear development of porcine IVF oocytes. To make sperm centrosome active as microtubule-organizing center (MTOC), reducing activity for disulfide bonds is required (Schatten, 1994). However, our data suggest that GSH level as < 6

μmol/oocyte is enough to support the function of sperm centrosome as MTOC and to form both pronuclei in our IVF system. The higher incidence of multiple aster formation observed in vitrified-warmed bovine oocytes may be triggered by change of other ooplasmic component other than the GSH. Shimizu et al. (Shimizu *et al*., 2009) reported that knockdown of cyclin G-associated kinase by siRNA in HeLa S3 cells caused multiple aster formation, which was due to abnormal fragmentation of pericentriolar material.

Extent of the delayed or arrested pronuclear development (Table 2) and the impaired development into blastocyst stage (Table 3) in vitrified-warmed bovine oocytes following IVF was co-insistent with our previous study (Hara *et al*., 2012). There are several reports dealing with improved yields of bovine blastocysts after treatment of oocytes with thiol compound during IVM (de Matos *et al*., 1995; de Matos *et al*., 1996; de Matos *et al*., 2002; Balasubramanian & Rho, 2007), but our study failed to improve the blastocyst yield by increasing the GSH level even in fresh control oocytes (both 41% on Day 8, Table 3). In those reports (de Matos *et al*., 1995; de Matos *et al*., 1996; de Matos *et al*., 2002; Balasubramanian & Rho, 2007), the blastocyst yields of fresh oocytes without thiol treatment were all less than 20% of the cultured oocytes. It is still unclear how much the developmental loss of cryopreserved bovine oocytes is involved from the abnormal microtubule assembly. Further study is required to elucidate the mechanism responsible for multiple aster formation and poor developmental potential of vitrified-warmed bovine oocytes.

In conclusion, vitrification procedure did not decrease intracellular GSH level of bovine oocytes stimulated by treatment with βME/Cys. However, the high content of the GSH in the matured oocytes did not result in suppression of the high incidence of

multiple aster formation and improvement of the poor developmental potential into blastocyst stage.

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Table 1

Aster formation in pronuclear-stage bovine zygotes matured in the presence of βME/Cys, vitrified-warmed, and fertilized *in vitro*.

Groups	Chemical treatment	No. (%) of oocytes		No. (%) of 2-PN zygotes	No. (%) of aster-formed zygotes	
		Inseminated	Fertilized as 2-PN	Aster-formed	With single aster	With multiple asters
Fresh control	None	84	58 (69 ± 3)	57 (98 ± 2)	47 (83 ± 4)[a]	10 (17 ± 4)[a]
	βME/Cys	87	63 (73 ± 3)	60 (95 ± 2)	50 (83 ± 5)[a]	10 (17 ± 5)[a]
Vitrified	None	87	57 (67 ± 4)	57 (100 ± 0)	27 (46 ± 5)[b]	30 (54 ± 5)[b]
	βME/Cys	86	61 (69 ± 7)	59 (97 ± 3)	22 (38 ± 2)[b]	37 (62 ± 2)[b]

Percentages were expressed as mean ± SEM of six replicates in each group.

[a,b] Different superscripts denote significant difference within a column ($P < 0.05$).

Table 2

Migration and development of pronuclei in bovine zygotes with a single aster or multiple asters.

Groups	Chemical treatment	Aster formation	Distance between pronuclei (μm)	Pronuclear size (μm^2)	
				Male	Female
Fresh control	None	Single	27 ± 2^{a}	259 ± 15^{abc}	130 ± 10^{ab}
		Multiple	50 ± 6^{b}	159 ± 22^{d}	83 ± 6^{cd}
	βME/Cys	Single	29 ± 2^{a}	264 ± 9^{ab}	142 ± 9^{a}
		Multiple	47 ± 8^{b}	139 ± 42^{d}	77 ± 11^{cd}
Vitrified	None	Single	24 ± 2^{a}	288 ± 11^{ab}	113 ± 6^{abcd}
		Multiple	46 ± 3^{b}	177 ± 16^{cd}	78 ± 7^{d}
	βME/Cys	Single	29 ± 3^{a}	312 ± 25^{a}	120 ± 13^{abc}
		Multiple	42 ± 3^{b}	228 ± 14^{bcd}	94 ± 9^{bcd}

Numbers of 2PN-zygotes analyzed correspond to those in Table 1.

$^{a-d}$ Different superscripts within a column denote significant difference ($P < 0.05$).

Table 3

In vitro development of bovine oocytes with an increased level of GSH after vitrification and *in vitro* fertilization.

Groups	Chemical treatment	No. (%) of oocytes			
		Inseminated	Cleaved	Developed to blastocysts on	
				Day 7	Day 7 + 8
Fresh control	None	104	68 (65 ± 4)	37 (36 ± 6)[a]	43 (41 ± 5)[a]
	βME/Cys	98	61 (62 ± 6)	38 (39 ± 5)[a]	40 (41 ± 5)[a]
Vitrified	None	100	65 (65 ± 3)	9 (9 ± 3)[b]	16 (16 ± 4)[b]
	βME/Cys	94	56 (60 ± 8)	10 (10 ± 3)[b]	16 (17 ± 3)[b]

Percentages were expressed as mean ± SEM of four replicates in each group.

[a,b] Different superscripts denote significant difference within a column ($P < 0.05$).

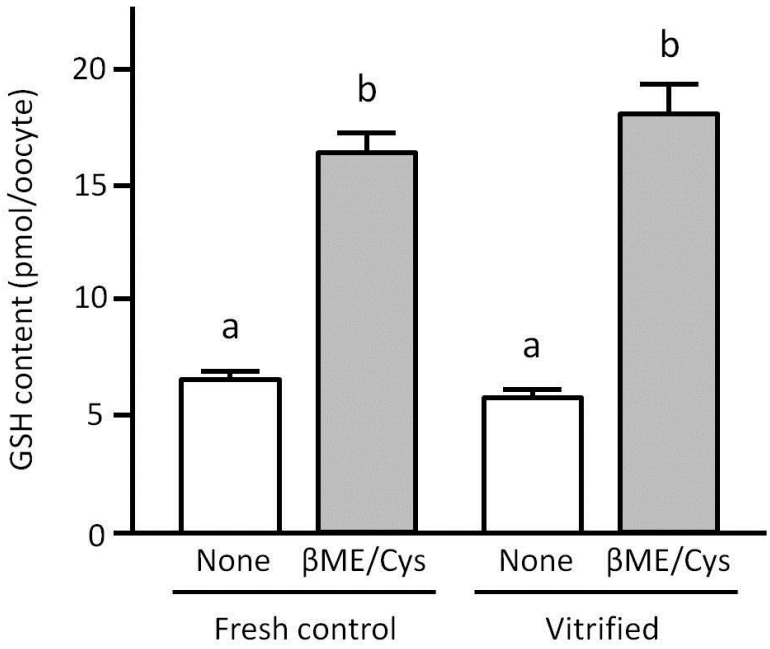

Figure 1. Glutathione (GSH) content of bovine oocytes treated with β-mercaptoethanol and L-cysteine (βME/Cys) during *in vitro* maturation. Half of denuded mature oocytes were subjected to vitrification before the GSH measurement. Mean ± SEM. Different letters on SEM bars denote significant difference ($P < 0.05$).