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

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Summary

Although vitrification is a useful technique for preservation of bovine oocytes, the yield of blastocysts derived from the vitrified oocytes is still low. We have recently reported a new type of cryoinjury, 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 multiple aster formation and 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-fold higher GSH content not only in fresh control but also in vitrified–warmed oocytes. The percentage of normally fertilized zygotes exhibiting sperm aster(s) was >95% in all four groups (with or without βME/Cys × fresh control or vitrified). The frequency of multiple aster formation in vitrified oocytes (three-fold 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 versus 41%) were not improved. In addition, there was no effect of 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), the efficiency of 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 large cell size and low permeability of water and cryoprotective agent (Leibo, 1981), meiotic spindle disassembly and chromosome misalignment (Shi *et al*., 2006), and oocyte activation prior to IVF (Larman *et al*., 2006). We have recently reported that vitrification of bovine oocytes induces frequent multiple aster formation and 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 the protection of cells against the destructive effects of reactive oxygen species and regulating syntheses of DNA and proteins (Meister, 1983). 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

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of oocytes matured under *in vitro* conditions are lower when compared with those of ovulated oocytes, as reported in some species (Brad *et al*., 2003; Rodríguez-González *et al*., 2003; Kim *et al*., 2007; Ge *et al*., 2008). GSH synthesis in 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). In addition, such thiol compounds supplemented into IVM medium can increase intracellular GSH level and the developmental potential of the oocytes in several domestic species including pig (Abeydeera *et al*., 1998) and cattle (de Matos *et al*., 1996).

It has been reported that GSH in bovine IVM–IVF oocytes can stimulate sperm aster formation (Sutovsky & Schatten, 1997) and that the GSH level of porcine oocytes is adversely affected by vitrification (Somfai *et al*., 2007). Therefore, we hypothesize that the decreased level of 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 multiple aster formation and 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°C) within 24 h of slaughter. The contents of 2–8 mm follicles were aspirated with an 18-G needle connected to a 10-ml syringe. Oocytes that were 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 17β-estradiol, and 50 μg/ml gentamycin sulfate for 22 h at 38.5°C 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 brief vortex mixing in 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*. (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 that consisted 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 weeks in a liquid nitrogen tank, oocytes were warmed by immersing the polypropylene strip of a Cryotop into 3 ml of the base medium that contained 1 M sucrose at 38.5°C, and left 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). Oocytes 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 1–2 h at 38.5 °C under 5% CO_2 in air before being subjected 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 phosphate-buffered saline (PBS) that contained 1 mg/ml polyvinylpyrrolidone (PVP). According to the instructions, pools of 35–40 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 °C until being assayed. After the 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*. Next, 10 μl of supernatant was diluted with 40 μl purified 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

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and 20 μl enzyme working solution. After incubation for 10 min at 37.0°C, 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°C for 30 s, the contents of a 0.5-ml straw were layered on top of a Percoll density gradient that consisted of a 2 ml layer of 45% Percoll above 2 ml of 90% Percoll in a 15-ml conical tube, and then centrifuged for 20 min at 700 *g*. The pellet was re-suspended in 4 ml of modified Brackett & 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°C 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°C under 5% CO_2, 5% O_2 and 90% N_2 for up to 8 days. Cleavage rate was determined on day 2 (day 0 was defined as the day of IVF), and number of blastocysts were recorded on days 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°C 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) that contained 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 that contained 0.1% (v/v) Triton X-100. Microtubules were labelled 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). 2-PN zygotes were defined as those that 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 analysis of variance (ANOVA). When the ANOVA was significant, differences among means were analyzed by 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 presence of the first polar body, treatment of the cumulus–oocyte complexes with βME/Cys did not influence the maturation rate (62%, 634/1029 versus 66%, 658/1004 in the 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 at 2.5-fold 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

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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 aster-formed	No. (%) of aster-formed zygotes	
		Inseminated	Fertilized as 2-PN		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 ± standard error of the mean (SEM) of six replicates in each group.
^{a,b}Different superscripts denote significant difference within a column ($P < 0.05$).

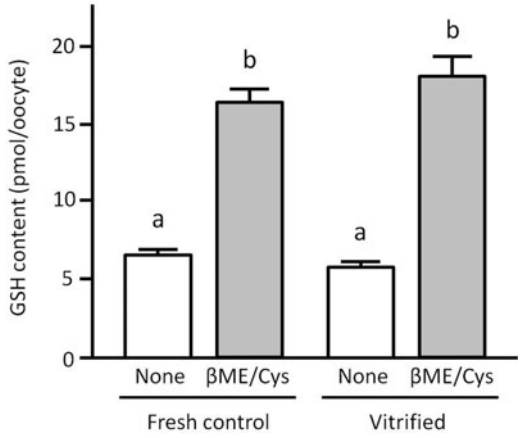

Figure 1 Glutathione (GSH) content of bovine oocytes treated with β-mercaptoethanol and L-cysteine (βME/Cys) during *in vitro* maturation. Half the total of denuded mature oocytes were subjected to vitrification before GSH measurement. Mean ± standard error of the mean (SEM). ^{a,b}Different letters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

occurred at similar rates between non-treated and βME/Cys groups regardless of vitrification (fresh control; 17 versus 16%, vitrified; 24 versus 24%). Immunostaining for α-tubulin indicated that the percentage of 2-PN zygotes that exhibited sperm aster(s) was high as >95% in all groups (Table 1). However, ratios of zygotes exhibiting multiple asters were more than three-fold higher in the vitrified group than those in the 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

The effect of addition of low-molecular-weight thiol compound during IVM (to increase ooplasmic GSH level) on the developmental potential of cryopreserved bovine oocytes has not been investigated to date; while there is only one report on this methodology, attempted 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*. (2007) reported a significant decrease in GSH levels in porcine oocytes after a solid-surface vitrification procedure. This decrease may depend upon species difference and/or suitability of the vitrification procedure.

After vitrification and IVF, bovine oocytes with increased GSH level exhibited similar incidence of

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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}	$259 \pm 15^{a,b,c}$	$130 \pm 10^{a,b}$
		Multiple	50 ± 6^{b}	159 ± 22^{d}	$83 \pm 6^{c,d}$
	βME/Cys	Single	29 ± 2^{a}	$264 \pm 9^{a,b}$	142 ± 9^{a}
		Multiple	47 ± 8^{b}	139 ± 42^{d}	$77 \pm 11^{c,d}$
Vitrified	None	Single	24 ± 2^{a}	288 ± 11^{ab}	$113 \pm 6^{a,b,c,d}$
		Multiple	46 ± 3^{b}	$177 \pm 16^{c,d}$	78 ± 7^{d}
	βME/Cys	Single	29 ± 3^{a}	312 ± 25^{a}	120 ± 13^{abc}
		Multiple	42 ± 3^{b}	$228 \pm 14^{b,c,d}$	$94 \pm 9^{b,c,d}$

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	Developed to blastocysts on days 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 ± standard error of the mean (SEM) of four replicates in each group.
[a,b]Different superscripts denote significant difference within a column ($P < 0.05$).

multiple aster formation compared with the oocytes without increased GSH level (Table 1). Sutovsky & Schatten (1997) reported that sperm aster formation in bovine IVF oocytes was disturbed when the oocytes were treated with buthionine sulfoximine, a specific inhibitor of γ-glutamyl-cysteine synthetase. Yoshida *et al*. (1993) also reported that intracellular GSH plays an important role in male pronuclear development of porcine IVF oocytes. To make the sperm centrosome act as a microtubule-organizing centre (MTOC), reducing activity for disulfide bonds is required (Schatten, 1994). However, our data suggest that a GSH level of <6 μmol/oocyte is enough to support the function of the sperm centrosome as an 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 components other than the GSH. 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 the pericentriolar material.

The extent of delayed or arrested pronuclear development (Table 2) and impaired development into the blastocyst stage (Table 3) in vitrified–warmed bovine oocytes following IVF was consistent with our previous study (Hara *et al*., 2012). There are several reports that deal with improved yields of bovine blastocysts after treatment of oocytes with thiol compound during IVM (de Matos *et al*., 1995, 1996, 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, 1996, 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 a result of 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, the vitrification procedure did not decrease the intracellular GSH level of bovine oocytes stimulated by treatment with βME/Cys. However, high content of GSH in matured oocytes did not result in suppression of the high incidence of

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

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