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—Full Paper—

Effects of Cysteine During *In Vitro* Maturation of Porcine Oocytes Under Low Oxygen Tension on Their Subsequent *In Vitro* Fertilization and Development

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Abstract. In this study, we evaluated the effect of different concentrations of cysteine in *in vitro* maturation (IVM) medium during IVM under low oxygen tension (5% O_2) of porcine oocytes on the intracellular content of glutathione (GSH) and subsequent *in vitro* fertilization (IVF) and development. Cumulus oocyte complexes (COCs) were collected from ovaries obtained at a local slaughterhouse, cultured in IVM medium supplemented with 0 (control), 0.05, 0.1, 0.2 or 0.6 mM cysteine for 44–46 h, fertilized *in vitro* and subsequently cultured for 6 days in total. The GSH content of the IVM oocytes exposed to 0, 0.05, 0.1, 0.2 or 0.6 mM cysteine increased significantly ($P<0.05$) as the concentration of cysteine increased (12.2, 14.0, 15.1, 16.4 and 16.4 pmol/oocyte, respectively). However, the rates of oocyte maturation, sperm penetration, male pronuclear formation, monospermy and even cleavage on Day 2 (the day of IVF was defined as Day 0) and blastocyst formation on Day 6 did not differ among the groups. Moreover, the cell numbers of blastomeres in blastocysts were uniform among the groups. These results indicate that supplementation with 0.05–0.6 mM cysteine during IVM under 5% O_2 tension significantly increased the intracellular GSH contents of IVM oocytes; however, it had no promoting effects on nuclear maturation, fertilization, male pronucleus formation and subsequent embryonic development to the blastocyst stage.

Key words: *In vitro* maturation, *In vitro* fertilization, Porcine

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In vitro production (IVP) of porcine embryos by *in vitro* maturation, fertilization and culture (IVM-IVF-IVC) would help in understanding early embryonic development in pigs and thus make it easier to enhance recently advancing porcine animal reproductive technologies [1]. Cellular and molecular factors that affect IVM and IVF of porcine oocytes have been investigated to contribute necessary information for establishing an optimal IVP system needed to harvest large numbers of high quality porcine embryos, which are useful for both scientific and commercial purposes. As a result, piglets have been born from IVP embryos after their transfer to recipients [2, 3]. Besides the improvements in the IVF and IVC systems by these reports, many successful experiments have been carried out to improve the IVM system to support the fertilization and developmental competences of *in vitro* matured porcine oocytes. The improvements have been achieved by elevating the male pronuclear formation rate by addition of porcine follicular fluid (pFF) [4], ascorbic acid [5, 6], epidermal growth factor [7,8] or cysteine [9–11] to the IVM medium and co-culture with extroverted follicles [12, 13]. Of these procedures, the most simple and effective method to harvest competent porcine oocytes seems to be the IVM system using IVM medium supplemented with cysteine. Supplementation of IVM medium with cysteine [9] or cysteamine [14, 15] or use of a cysteine-rich medium (e.g., Waymouth MB 75211) [16] promotes male pronucleus formation of IVM oocytes after IVF in pigs. Cysteine is a critical component amino acid of glutathione (GSH), a thiol tripeptide synthesized by the gamma-glutamyl cycle. GSH is reported to play an important role in providing cells with a reducing environment to protect against the toxic effect of oxidative damage [17], especially when cells are cultured under high oxygen tension. Consequently, the concentration of cysteine added to IVM medium decisively affects the concentration of GSH in porcine oocytes and the male pronucleus formation rate after IVF [9]. In cattle, Sutovsky and Schatten [18] suggested that the depletion of GSH during IVM blocks formation of the male pronucleus without disassembling the sperm tail connecting piece and pronuclear apposition during fertilization. Furthermore, De Matos *et al.* [19] suggested that GSH is the major non-protein sulphydryl compound in mammalian cells. Therefore, the promoting effect of GSH in IVM oocytes on MPN formation is speculated to act synergistically in the following ways: GSH promotes breaking of disulfide bonds (S-S) of protamine in the sperm head by shifting the oocyte cytoplasm into a redox state, and/or it serves as a substrate of glutathione peroxidase and acts as a scavenger of free radicals in oocytes, enhancing their competence as a whole [20]. The impact of high GSH content during porcine oocyte maturation was proven by the fact that Yoshida *et al*. [11] produced piglets

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derived from IVM-IVF oocytes by using a culture medium (TALP) comprised of relatively simple components supplemented with cysteine for IVM. Thus, in practice, elevating the GSH levels in oocytes is one of the easiest ways to expand the chances of successful embryonic development in pigs. However, this promoting effect of cysteine to synthesize GSH has been reported mostly in events from sperm penetration until MPN formation. The information available about the effect of elevated GSH in porcine oocytes exposed to cysteine during IVM on subsequent embryo development after IVF and IVC is limited, and only one report is available [21]. Although low oxygen tension has been reported to be beneficial in protecting cells [17, 22] and mammalian gametes [23] from oxidative stress, Abeydeera *et al*. [21] matured oocytes under high oxygen tension (20% O_2) and reported the benefit of cysteine (0.825–3.3 mM) and 25 μM β-mercaptoethanol supplementation in IVM medium on oocyte GSH content and subsequent *in vitro* development to the blastocysts after IVF; however, no significant differences in GSH content and developmental ability among the oocytes cultured in the medium with cysteine at different concentrations were observed. Furthermore, when porcine oocytes were matured in IVM medium supplemented with 0.6 mM cysteine and 50 μM β-mercaptoethanol under low oxygen tension (5%), the resultant IVM/IVF/IVC embryos could be carried to term after transfer to recipients [3]. However, this previous report did not investigate the concentration of cysteine in IVM medium that is effective for *in vitro* production of porcine embryos. In the present study, we investigated the maturation and subsequent fertilization and developmental competences of porcine oocytes matured under low oxygen tension (5% O_2) in IVM medium supplemented with different concentrations of cysteine and 50 μM β-mercaptoethanol.

Materials and Methods

Oocyte collection and in vitro maturation (IVM)

Collection, *in vitro* maturation and fertilization and subsequent culture of oocytes were carried out according to Kikuchi *et al*. [3]. Briefly, ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory at 35 C. Cumulus-oocyte complexes (COCs) from follicles 3–6 mm in diameter were collected in TCM-199 with Hanks' salt (Sigma Chemical, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 20 mM Hepes (Dojindo Laboratories, Kumamoto, Japan), 100 IU/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate. Only COCs with compact cumulus cell layers and evenly granulated cytoplasm were used. About 40–50 COCs were cultured for 20–22 h, as the first step IVM, in 500 μl of maturation medium in a four-well dish (Nunclon Multidishes; Nalge Nunc International, Roskilde, Denmark). The maturation medium consisted of a modified North Carolina State University (NCSU)-37 solution supplemented with 10% (v/v) porcine follicular fluid, which was collected in advance and cryopreserved until the time of usage, 50 μM β-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan), 10 IU/ml PMSG (Peamex, Sankyo, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Puberogen, 500 U; Sankyo) and 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma). The maturation media were further supplemented with 0 (control), 0.05, 0.1, 0.2 or 0.6 mM cysteine (Sigma). As the second step IVM, they were subsequently cultured in the same maturation medium but without dbcAMP and hormones for 24 h. IVM was carried out at 38.5 C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2.

In vitro fertilization (IVF) and embryo culture

Frozen spermatozoa from the Landrace breed were thawed and preincubated for 15 min at 38.5 C in TCM-199 with Earle's salts (Invitrogen) with the pH adjusted to 7.8 as reported by Kikuchi *et al*. [24]. A portion (10 μl) of the preincubated spermatozoa was introduced into 90 μl of fertilization medium containing 10–15 COCs covered with paraffin oil (Paraffin Liquid; Nakarai Tesque, Kyoto, Japan). The fertilization medium was pig-FM [25] supplemented with 0.5% (w/v) bovine serum albumin (BSA; Fraction V, Sigma) and 2 mM caffeine (Sigma). The final sperm concentration was 1×10^5/ml. Three hours after co-incubation with spermatozoa, cumulus cells surrounding putative zygotes were removed by pipetting with a small-bore pipette and cultured in IVC medium. The basic medium for IVC was modified NCSU-37 medium containing 4 mg/ml BSA and 50 μM β-mercaptoethanol. IVC was carried out at 38.5 C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in basic IVC medium supplemented with 0.17 mM sodium pyruvate (Sigma Chemical) and 2.73 mM sodium lactate (Sigma Chemical) for the first two days and then in basic IVC medium supplemented with 5.55 mM D-glucose (Sigma) for the following four days.

Oocyte, zygote and embryo evaluation with orcein staining

For evaluation of the meiotic stage of oocytes, fertilization status of putative zygotes and developmental ability of embryos after IVF and subsequent IVC, oocytes after IVM for 44–46 h, putative zygotes after 10 h of insemination and embryos after IVC for 2 days and 6 days from different groups were fixed, respectively, for at least 3 days with a mixture of acetic acid and ethanol (1:3, v/v). They were then stained with aceto–orcein for 30 seconds and mounted with aceto-glycerol solution (acetic acid:glycerol:distilled water=1:1:3, v/v). Subsequently, all of them were examined under a phase-contrast microscope (Olympus, Tokyo, Japan) at ×40 and ×100 magnification. Oocytes were evaluated for maturation to metaphase of the second meiotic division (M-II), and putative zygotes were evaluated for fertilization parameters, such as rates of sperm penetration, monospermic fertilization and MPN formation, while embryos on Day 2 and Day 6 (the day of fertilization was defined as Day 0) were evaluated for cleavage rate and blastocyst formation rate and cell number, respectively.

Measurement of intracellular GSH content

Intracellular GSH content was measured as described previously [26–28]. For each replicate, we placed pools of 10–15 *in vitro* matured oocytes in 5 μl of 0.2 M sodium phosphate buffer containing 10 mM Na_2-EDTA (pH 7.2) and 5 μl of 1.25 M phosphoric acid in microtubes, and then all the oocytes were frozen at –80 C. The concentrations of GSH in the oocytes were determined by dithionitrobenzoic acid - glutathione disulphide (DTNB-GSSG) reductase recycling assay [29]. Briefly, the samples were thawed, and then 175 μl sodium phosphate buffer containing 0.33 mg/ml nicotina-

Table 1. *In vitro* maturation (IVM) of porcine oocytes* with different concentrations of cysteine

Cysteine concentration (mM)	Total No. of oocytes examined	No. (%) of oocyte matured to M-II stage
0	110	75 (68.2 ± 3.2)
0.05	119	84 (70.6 ± 7.7)
0.1	109	76 (69.7 ± 15.9)
0.2	112	85 (75.9 ± 7.7)
0.6	170	117 (68.8 ± 8.0)

Means ± SEM are presented. M-II: Metaphase of the second meiotic division.
* The first half IVM was carried out at 38.5C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in a modified NCSU-37 medium supplemented with 10% (v/v) porcine follicular fluid, 50 μM β-mercaptoethanol, 10 IU/ml PMSG, 10 IU/ml human chorionic gonadotropin and 1 mM dibutyryl cyclic AMP for 20–22 h. The second IVM was carried out for 24 h under the same conditions in the same medium but without dbcAMP and hormones.

Table 2. Glutathione concentration in porcine oocytes after *in vitro* maturation (IVM) with different concentrations of cysteine

Cysteine concentration (mM)	Glutathione concentration* (pmol/oocyte)
0	12.2 ± 0.6^{a}
0.05	14.0 ± 0.8^{ab}
0.1	15.1 ± 0.5^{bc}
0.2	16.4 ± 0.4^{c}
0.6	16.4 ± 0.5^{c}

Means ± SEM are presented. *The glutathione concentrations of IVM oocytes were measured after 44–46 h of IVM. $^{a-c}$ Values with different superscripts are significantly different ($P<0.05$).

Table 3. *In vitro* fertilization of porcine oocytes after *in vitro* maturation (IVM) with different concentrations of cysteine*

Cystein concentration (mM)	Total No. of oocytes examined	No. (%) of oocytes penetrated by sperm	No. (%) of oocytes with MPN	No. (%) of monospermic oocytes
0	106	73 (68.9 ± 21.6)	70 (95.9 ± 2.4)	38 (35.9 ± 9.8)
0.05	88	55 (62.5 ± 11.3)	55 (100.0 ± 0.0)	30 (34.1 ± 4.9)
0.1	107	69 (64.5 ± 11.55)	64 (92.8 ± 4.6)	41 (38.3 ± 7.7)
0.2	124	67 (54.0 ± 8.9)	63 (94.0 ± 4.1)	35 (28.2 ± 6.1)
0.6	106	66 (62.3 ± 6.0)	61 (92.4 ± 2.7)	36 (34.0 ± 4.6)

Means ± SEM are presented. MPN: male pronuclear formation. * IVM oocytes were inseminated with spermatozoa for 10 h and fixed to evaluate fertilization parameters.

mide adenine dinucleotide phosphate (NADPH; Sigma), 25 μl of 6 mM DTNB (Wako) and 40 μl of water were added to each sample tube. The samples were warmed at room temperature for 15 min, and then the assay was initiated with the addition of 5 μl of 125 IU glutathione disulphide reductase (Wako). Absorbance was measured five times by spectrophotometer (DU7500; Beckman Coulter, Fullerton, CA, USA) at 30-sec intervals at a wavelength of 412 nm. A GSH standard and sample blank lacking GSH were also assayed. Standards were prepared for each assay, and the total GSH content per sample was determined from a standard curve of GSH. The GSH concentration per oocyte was calculated by dividing the total concentration per sample by the number of oocytes present in the sample.

Statistical analysis

Data were expressed as means ± SEM. The percentage data were subjected to arc-sine transformation. All data were subjected to ANOVA followed by Tukey-Kramer test. Analyses were carried out using the GLM procedure of the Statistical Analysis System (SAS; SAS Institute, Cary, NC, USA).

Results

Maturation rate and GSH content of oocytes after IVM

As shown in Table 1, the maturation rates to the MII stage after IVM did not differ among the control (0 mM) and experimental groups (68.2 to 75.9%). As shown in Table 2, the GSH content of the IVM oocytes exposed to 0.05 mM cysteine (14.0 pmol/oocyte) did not increase compared to the control (12.2 pmol/oocytes). However, when exposed to 0.1, 0.2 or 0.6 mM cysteine, the GSH content (15.1, 16.4 and 16.4 pmol/oocyte, respectively) increased significantly ($P<0.05$) compared with the control; the GSH content plateaued when oocytes were exposed to 0.1, 0.2 and 0.6 mM cysteine.

Fertilization status after IVF

As shown in Table 3, the rates of sperm penetration, monospermic fertilization and MPN formation did not differ among the control and experimental groups (54.3 to 68.9%, 92.4 to 100% and 28.2 to 38.3%, respectively).

Table 4. *In vitro* development of oocytes matured *in vitro* with different concentrations of cysteine after fertilization *in vitro**

Cysteine concentration (mM)	Day 2**		Day 6**		
	Total no. of examined oocytes	No. (%) of cleaved oocytes	Total no. of examined oocytes	No. (%) of blastocysts	Cell no. / blastocyst
0	116	35 (30.2 ± 13.8)	264	39 (14.8 ± 5.3)	38.7 ± 3.5
0.05	120	40 (33.3 ± 14.7)	264	48 (18.2 ± 5.2)	40.2 ± 3.1
0.1	129	53 (41.1 ± 9.3)	280	68 (24.3 ± 8.6)	37.5 ± 3.0
0.2	133	69 (51.9 ± 7.7)	268	52 (19.4 ± 3.7)	36.2 ± 3.3
0.6	128	62 (48.4 ± 5.0)	222	47 (21.2 ± 6.8)	43.8 ± 4.0

Means ± SEM are presented. * *In vitro* culture (IVC) was carried out at 38.5 C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in basic IVC medium supplemented with 0.17 mM sodium pyruvate and 2.73 mM sodium lactate for the first two days of IVC and then in basic IVC medium supplemented with 5.55 mM D-glucose for the following four days (6 days of IVC in total). **Cleavage and development of IVM/IVF oocytes to the blastocyst stage were observed on Day 2 (the day of IVF was defined as Day 0) and Day 6, respectively.

In vitro development of IVP embryos after IVC

As shown in Table 4, the rates of cleavage and blastocyst formation after 2 and 6 days, respectively, of IVC did not differ among the control and experimental groups (30.2 to 51.9% and 14.8 to 24.3%, respectively). Moreover, the cell numbers of blastomeres in blastocysts did not differ among the groups (37.5 to 43.8 cells).

Discussion

The present results indicate that the rates of oocyte maturation, sperm penetration, MPN formation and monospermy did not differ among the control (0 mM) and cysteine-supplemented (0.05–0.6 mM) groups. However, this observation is slightly different from those reported by Yoshida *et al*. [9], who matured porcine oocytes in mTLP supplemented with various concentrations of cysteine, but without β-mercaptoethanol, a reducing reagent, under high (20%) oxygen tension. They found that when cysteine at a concentration of 0.04 mM or higher was added to the medium, the MPN formation rate increased significantly compared with oocytes matured in medium without (0 mM) or with a low concentration (0.02 mM) of cysteine. The reason for this discrepancy in rate of MPN formation among these studies is not clear. However, considering that Yoshida *et al*. [9] did not add β-mercaptoethanol into the IVM media and that the oocytes and putative zygotes were cultured under 20% oxygen tension, the different redox states in the IVM systems might have caused the different MPN formation rates. The reducing status, fundamentally obtained by low oxygen tension and addition of 50 μM β-mercaptoethanol to IVM media, in this study might maintain cytoplasmic GSH above the threshold level for MPN formation, resulting in high MPN formation rates, because the GSH level of the oocytes in the control group of the present study (12.2 pmol/oocyte) seems to have been higher than those of the control and low cysteine addition groups (without cysteine, 4.0 pmol/oocytes; 0.02–0.08 mM cysteine supplemented, 5.3–8.2 pmol/oocyte) in the report of Yoshida *et al*. [9]; the MPN formation rate of the oocytes in the 0.14 mM cysteine addition group, which had a GSH concentration of 12.8 pmol/oocyte, showed a similarly high MPN formation rate (87%) to that of the control group (95.9%) in the present study and that of oocytes matured without cysteine (5.5 pmol/oocyte) reported by Abeydeera *et al*. [21]. The GSH level seems to be the same as those of oocytes matured with cysteine supplementation (12.8–15 pmol/oocyte) in the report by Yoshida *et al*. [9] or of oocytes matured in the medium supplemented with higher concentration of cysteine (0.825–3.3 mM cysteine supplemented: 13–15 pmol/oocyte) [21].

On the other hand, in the present study and the reports by Yoshida *et al*. [9] and Abeydeera *et al*. [21], cysteine supplementation elevated GSH to significantly higher levels (14.0–16.4, 5.3–15 and 13–15 pmol/oocyte, respectively) compared with the control groups (12.2, 4.0 and 5.6 pmol/oocyte, respectively). As mentioned above, this difference in GSH level did not contribute to the incidences of MPN formation after IVF and subsequent development to the blastocyst stage in the present study but was shown to reflect embryo developmental ability [9]. Furthermore, the presence of cysteine in IVM medium significantly increases the blastocyst formation rates [21]. In contrast, under low oxygen tension and in the presence of 50 μM β-mercaptoethanol in the present study, no significant difference was found in either the cleavage or blastocyst rates between the control (0 mM) and cysteine-supplemented groups (0.05–0.6 mM; Table 4). Moreover, the cell numbers of blastomeres in blastocysts were uniform among the groups (Table 4). Thus, although GSH in matured oocytes is considered to enhance their developmental competence after IVF, in other words, cytoplasmic maturation [20], a slight difference in GSH level may affect the developmental ability of resultant embryos after IVF. In the present study, such a difference in GSH content was also achieved between oocytes cultured in the media supplemented with different concentrations of cysteine. However, IVC of IVP embryos was carried out in a medium supplemented with β-mercaptoethanol under low oxygen tension, which might have lowered the oxidative stress on the embryos during IVC, resulting in the high developmental rate to the blastocyst stage even

in the control group (oocytes with relatively low GSH level).

In the report of Abeydeera *et al*. [21], higher concentrations (0.825–3.3 mM, higher than those in the present study: 0.05–0.6 mM) of cysteine in the IVM medium did not further promote either the GSH level or embryo development. From this and our studies, it could be inferred that a given redox state, in the form of ooplasm GSH content, is sufficient for the development of porcine IVP embryos, and excess GSH does not further improve embryo development. In the present study, the optimal redox state might be created by low oxygen tension during IVM of oocytes and IVC of IVP embryos and by β-mercaptoethanol in the IVM and IVC media. These might have kept the IVM oocytes and IVP embryos in a safer redox state than required for subsequent in vitro development to the blastocyst stage. For those reasons, the higher and excess GSH level in the cysteine-supplemented groups could not promote embryonic development.

The results of the present study contribute to the hypothesis that there is a limited redox state of ooplasm necessary for MPN formation and development of IVF porcine embryos, which could be achieved by one of the two following conditions: 1) presence of cysteine, which is crucial in GSH synthesis, under high oxygen tension, or 2) low oxygen tension, which could naturally maintain a deoxidized state of ooplasm even without cysteine supplementation during IVM and IVC periods. This necessary state could also be additionally maintained in IVC medium by supplementation with a reducing thiol compound, β-mercaptoethanol.

In conclusion, supplementation of 0.05–0.6 mM cysteine during IVM of porcine oocytes under 5% oxygen tension significantly increased intracellular GSH synthesis in a concentration-dependent manner but had no promoting effects on nuclear maturation, fertilization and male pronucleus formation or subsequent embryonic development to the blastocyst stage (with probable assistance of β-mercaptoethanol supplementation in the IVC medium). Thus, oxygen tension during IVM of oocytes or in a further extension, conditions that help to maintain the ooplasm redox state, is suggested to be important for *in vitro* production of porcine blastocysts.

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