The interactions between cysteamine, cystine and cumulus cells increase the intracellular glutathione level and developmental capacity of goat cumulus-denuded oocytes

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Abstract

To improve *in vitro* maturation (IVM) of denuded oocytes (DOs), we observed the interactive effects of cysteamine, cystine and cumulus cells on the glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) level and developmental capacity of goat IVM oocytes. Cysteamine supplementation increased the GSH level and blastocyst rates of both cumulus–oocyte complexes (COCs) and DOs, while the addition of cystine increased the GSH level and blastulation only in the presence of cumulus cells (COCs or DOs co-cultured on a cumulus cell monolayer). Simultaneous supplementation of cysteamine and cystine increased the GSH content and blastulation of co-cultured DOs to a level similar to that of COCs matured without thiol supplementation. Co-culture without thiol supplementation improved DOs' GSH synthesis but not blastulation. The results suggest that DOs cannot utilize cystine for GSH synthesis unless exogenous cysteamine is supplied by either cumulus cells or supplementation. Thus, while the addition of cystine alone is enough to improve IVM of COCs, improvement of DOs requires supplementation of both cystine and cysteamine. Synergic actions between cysteamine, cystine and cumulus cells restore the GSH level and developmental capacity of goat DOs.

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Introduction

Removal of cumulus cells before *in vitro* maturation (IVM) was detrimental to oocyte maturation, and co-culture with cumulus–oocyte complexes (COCs) or cumulus cells restored partially the developmental potential of cumulus-denuded oocytes (DOs). Therefore, cumulus cells are considered to play an important role in oocyte maturation by regulating meiotic progression and by supporting cytoplasmic maturation (Tanghe *et al.* 2002). However, the removal of cumulus cells from oocytes or zygotes at various stages of development is inevitable for some embryo manipulation techniques. Therefore, an efficient IVM system for DOs will provide a technical approach to such procedures as germinal vesicle (GV) transfer, somatic cell haploidization and oocyte cryopreservation at the GV stage. In addition, the mechanisms by which cumulus cells improve oocyte maturation are poorly understood.

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is well known to play an important role in protecting cells against the destructive effects of reactive oxygen species, and to regulate protein and DNA synthesis by altering redox status (Meister 1983). In reproduction, GSH participates in sperm decondensation and male

pronucleus formation in hamsters (Perreault *et al.* 1988), pigs (Yoshida *et al.* 1993) and cows (Sutovsky & Schatten 1997). Moreover, cytoplasmic GSH is also defined as one of the indices of cytoplasmic maturation (Funahashi *et al.* 1994, de Matos *et al.* 1997, Abeydeera *et al.* 1998, Furnus *et al.* 1998, de Matos & Furnus 2000). Synthesis of GSH is highly dependent on the availability of cysteine in the medium (Meister & Tate 1976, Chance *et al.* 1979). It is thought that GSH synthesis under *in vitro* conditions may be impaired because of a deficiency of cysteine in the culture medium (0.6 μM in TCM-199) and its high instability and easy auto-oxidation to cystine (Bannai 1984, Sagara *et al.* 1993). In somatic cells, cysteamine can reduce cystine to cysteine, promoting cysteine uptake and hence enhancing GSH synthesis (Issels *et al.* 1988). The addition of cysteamine to the culture medium improved oocyte maturation and development by increasing the GSH content of COCs (de Matos *et al.* 1995, 2002, 2003, Abeydeera *et al.* 1998, Gasparrini *et al.* 2000, Rodriguez *et al.* 2003, Kobayashi *et al.* 2006). However, whether the cystine contained in TCM-199 (83.2 μM) is sufficient for oocyte IVM of different species is unknown. While enrichment of maturation medium with cystine increased both GSH contents and embryonic development in the cow (de Matos *et al.* 1997, de Matos & Furnus 2000)

and buffalo (Gasparrini *et al.* 2006), it increased only GSH synthesis of goat oocytes (Rodriguez *et al.* 2003).

The GSH content of an oocyte is highly correlated with the presence of cumulus cells (Sawai *et al.* 1997). Reports indicate a lower GSH content of DOs after IVM than of COCs (de Matos *et al.* 1997, Luciano *et al.* 2005, Maedomari *et al.* 2007). However, while co-culture with intact COCs induced a significant increase in the GSH content of bovine DOs (Luciano *et al.* 2005), co-culture with a monolayer of cumulus cells had no beneficial effect on the GSH synthesis of porcine (Maedomari *et al.* 2007) and mouse (data to be published) DOs. Besides, the addition of cysteamine to culture medium increased the GSH content and formation of male pronuclei but had no effect on the nuclear maturation of porcine DOs (Yamauchi & Nagai 1999). The addition of cysteamine to culture medium also increased intracellular GSH of bovine DOs (de Matos *et al.* 1997). In addition, de Matos *et al.* (1997) found that supplementation of TCM-199 with cystine had no effect on the GSH synthesis of bovine DOs cultured alone but increased GSH levels after IVM of bovine DOs in the presence of cumulus cell monolayer. However, the effect of addition of cysteamine or cystine to maturation medium on embryonic development of DOs has not been reported.

In summary, the above review indicates that while cysteamine increases the GSH content of oocytes matured as DOs, its effect on the developmental competence of these oocytes is uncertain. Results on the effect of cystine supplementation on IVM of COCs are in conflict, and its effect on DOs needs investigation. The exact role of gap junctional communication between the oocyte and cumulus cells in GSH transport or synthesis is unclear. In addition, since the effect of GSH on oocyte developmental competence was usually evaluated after *in vitro* fertilization, whether the beneficial effect was due to an increased male pronuclear formation or an improved subsequent development is unknown, although de Matos *et al.* (1995) observed that while a higher GSH level in mature

bovine oocytes was correlated with increased blastocyst formation, it had no effect on cleavage rates. The best way to answer this question is to observe development after parthenogenetic activation. In this paper, we have studied the interactive effects of cysteamine, cystine and cumulus cells on the GSH synthesis and cytoplasmic maturation of goat oocytes, and have reported that IVM of DOs could be improved by manipulating the interactions between these factors.

Results

The optimal concentrations of cysteamine and cystine for the improvement of GSH synthesis and developmental competence of goat oocytes

When goat COCs were cultured in TCM-199 supplemented with different concentrations of cysteamine or cystine, the content of intracellular GSH increased with increasing cysteamine or cystine concentrations (Table 1). However, the highest rate of blastocyst formation was achieved only at 100- μ M cysteamine or 200- μ M cystine, with blastocyst rates decreasing at higher concentrations. A similar pattern of changes was observed in the average cell number per blastocyst among cysteamine or cystine concentrations, although differences between treatments were not always statistically significant. Rates of oocyte maturation did not differ between concentrations of cysteamine or cystine.

Effects of cysteamine and cystine alone or in combination on GSH synthesis and developmental competence of goat COCs

The addition of either 100- μ M cysteamine or 200- μ M cystine to TCM-199 improved both GSH synthesis and blastulation of goat COCs. When both cysteamine and cystine were added, the rate of blastocyst formation was further increased (Table 2). The same tendency of changes was observed in the average cell number per

Table 1 Maturation, blastocysts formation, and oocyte intracellular glutathione (GSH) concentration after culture of cumulus–oocyte complexes (COCs) in TCM-199 supplemented with different concentrations of cysteamine or cystine.

Cysteamine (μ M)	Cystine (μ M)	Oocytes cultured	% MII oocytes	Embryos cultured	% Blastocysts	Cell no./blastocyst	GSH (pmol/oocyte)
0		138	83.3 $\pm$ 2.3 ^a	103	10.7 $\pm$ 1.0 ^a	80.7 $\pm$ 3.6 ^a	2.26 $\pm$ 0.21 ^a
50		136	83.4 $\pm$ 1.9 ^a	103	18.4 $\pm$ 1.0 ^b	110.7 $\pm$ 14.2 ^{a,b}	3.51 $\pm$ 0.06 ^b
100		156	83.6 $\pm$ 2.0 ^a	104	25.9 $\pm$ 1.7 ^c	128.1 $\pm$ 17.1 ^b	4.38 $\pm$ 0.27 ^c
200		172	85.0 $\pm$ 2.2 ^a	107	21.0 $\pm$ 1.3 ^b	104.1 $\pm$ 11.3 ^{a,b}	5.08 $\pm$ 0.33 ^d
500		181	82.0 $\pm$ 3.7 ^a	114	10.9 $\pm$ 1.4 ^a	79.2 $\pm$ 3.6 ^a	5.98 $\pm$ 0.24 ^e
	0	159	81.2 $\pm$ 5.7 ^a	99	14.1 $\pm$ 2.1 ^a	93.4 $\pm$ 11.7 ^a	3.04 $\pm$ 0.23 ^a
	100	131	80.7 $\pm$ 2.5 ^a	98	10.2 $\pm$ 0.5 ^a	93.1 $\pm$ 12.5 ^a	3.67 $\pm$ 0.26 ^b
	200	156	81.7 $\pm$ 4.2 ^a	101	31.4 $\pm$ 1.8 ^c	136.7 $\pm$ 16.3 ^b	4.24 $\pm$ 0.05 ^c
	500	152	79.6 $\pm$ 1.7 ^a	100	19.7 $\pm$ 2.5 ^b	111.3 $\pm$ 15.4 ^{a,b}	4.74 $\pm$ 0.13 ^d

^{a–e}Values without a common letter in their superscripts in the same column differ ($P < 0.05$) within the cysteamine or cystine experiment. Each treatment was repeated six times and 20–30 and 15–20 oocytes were observed for maturation and embryo development, respectively, per replicate per treatment. Each sample for assay of the intracellular GSH level consisted of 30 oocytes and three samples from different experimental days were assayed for each treatment.

Table 2 Maturation, blastocyst formation, and oocyte intracellular glutathione (GSH) concentration after culture of cumulus–oocyte complexes (COCs) in TCM-199 supplemented with cysteamine, cystine, or both.

Cysteamine (100 μ M)	Cystine (200 μ M)	Oocytes cultured	% MII oocytes	Embryos cultured	% Blastocysts	Cell no./blastocyst	GSH (pmol/oocyte)
–	–	124	84.7 $\pm$ 2.3 ^a	106	13.0 $\pm$ 0.9 ^a	86.3 $\pm$ 8.4 ^a	3.16 $\pm$ 0.28 ^a
+	–	153	84.5 $\pm$ 1.6 ^a	104	24.2 $\pm$ 2.0 ^b	117.6 $\pm$ 16.0 ^{a,b}	4.61 $\pm$ 0.07 ^b
–	+	156	82.5 $\pm$ 1.5 ^a	97	27.1 $\pm$ 1.6 ^b	125.2 $\pm$ 15.6 ^{a,b}	4.25 $\pm$ 0.05 ^b
+	+	139	84.0 $\pm$ 1.1 ^a	113	34.2 $\pm$ 2.8 ^c	143.2 $\pm$ 16.8 ^b	4.67 $\pm$ 0.14 ^b

^{a,b}Values without a common letter in their superscripts in the same column differ ($P < 0.05$). Each treatment was repeated six times and 20–30 and 15–20 oocytes were observed for maturation and embryo development, respectively, per replicate per treatment. Each sample for assay of the intracellular GSH level consisted of 30 oocytes and three samples from different experimental days were assayed for each treatment.

blastocyst, although differences were not always statistically significant between treatments.

Effects of cysteamine and cystine alone or in combination on GSH synthesis and developmental competence of goat cumulus-DOs

The addition of 100- μ M cysteamine to TCM-199 improved GSH synthesis and blastulation as well as nuclear maturation of DOs (Table 3). The addition of 200- μ M cystine, however, did not have any beneficial effect on the DOs. When cysteamine and cystine were added together, GSH content and rate of blastocysts further increased ($P < 0.05$) relative to those obtained when cysteamine was added alone. The average cell number of blastocysts derived from DOs matured in the presence of cysteamine alone was lower than that of blastocysts from DOs matured in the presence of both cysteamine and cystine, although the difference was not statistically significant.

Interactive effects of cysteamine, cystine and cumulus cells on GSH synthesis and developmental competence of goat DOs

In the presence of neither cysteamine nor cystine, co-culture with cumulus monolayer increased nuclear maturation and GSH synthesis, but not blastulation of DOs (Table 4). Co-cultured with cumulus cell monolayer, the addition of either cysteamine or cystine improved both GSH synthesis and blastulation of DOs.

When cysteamine and cystine were added together, both the GSH level and the rate of blastocysts of the co-cultured DOs increased further to the same level as that of COCs cultured with no thiol supplementation (Table 2). The average cell number per blastocyst was significantly higher when both cysteamine and cystine were added together than when either one was added alone.

Discussion

The present results suggest that goat DOs cannot utilize cystine for GSH synthesis, unless in the presence of either supplemented cysteamine or cumulus cells. In the cow, an increase in GSH level stimulated by cystine was observed only in the presence of cumulus cells, either in COCs or in DOs matured on a co-culture monolayer (de Matos *et al.* 1997). However, embryonic development was not observed in that study. Takahashi *et al.* (1995) showed that bovine embryos can hardly utilize cystine for GSH synthesis, but in the presence of a cumulus–granulosa cell co-culture, they can utilize components derived from cystine metabolism secreted to the culture medium by the cell monolayer. Cysteine is an external substrate required for GSH synthesis in maturing bovine oocytes (de Matos *et al.* 1996). However, the concentration of cysteine in TCM-199 is very low (0.6 μ M), and outside the cell, essentially no cysteine will be present in TCM-199 due to autoxidation to cystine (Bannai 1984, Sagara *et al.* 1993). Therefore, it is possible that this cystine is converted into cysteine by

Table 3 Maturation, blastocyst formation, and oocyte intracellular glutathione (GSH) concentration after culture of denuded oocytes (DOs) in TCM-199 supplemented with cysteamine, cystine, or both.

Cysteamine (100 μ M)	Cystine (200 μ M)	Oocytes cultured	% MII oocytes	Embryos cultured	% Blastocysts	Cell no./blastocyst	GSH (pmol/oocyte)
–	–	158	59.8 $\pm$ 1.8 ^a	92	0.0 $\pm$ 0.0 ^a	–	1.43 $\pm$ 0.21 ^a
+	–	150	80.4 $\pm$ 1.9 ^b	104	5.0 $\pm$ 1.8 ^b	71.3 $\pm$ 1.9 ^a	2.50 $\pm$ 0.25 ^b
–	+	142	62.6 $\pm$ 1.8 ^a	103	0.0 $\pm$ 0.0 ^a	–	1.60 $\pm$ 0.10 ^a
+	+	147	80.8 $\pm$ 1.1 ^b	102	9.6 $\pm$ 1.5 ^c	103.2 $\pm$ 17.5 ^a	3.10 $\pm$ 0.16 ^c

^{a–c}Values without a common letter in their superscripts in the same column differ ($P < 0.05$). Each treatment was repeated six times and 20–30 and 15–20 oocytes were observed for maturation and embryo development, respectively, per replicate per treatment. Each sample for assay of the intracellular GSH level consisted of 30 oocytes and three samples from different experimental days were assayed for each treatment.

Table 4 Maturation, blastocyst formation, and oocyte intracellular glutathione (GSH) concentration after culture of denuded oocytes (DOs) in the presence of cumulus cell monolayer in TCM-199 supplemented with cysteamine, cystine, or both.

Cysteamine (100 μ M)	Cystine (200 μ M)	Oocytes cultured	% MII oocytes	Embryos cultured	% Blastocysts	Cell no./blastocyst	GSH (pmol/oocyte)
—	—	158 ^a	59.8 $\pm$ 1.8 ^a	105	0.0 $\pm$ 0.0 ^a	—	1.52 $\pm$ 0.17 ^a
—	—	155	83.0 $\pm$ 1.2 ^b	103	1.0 $\pm$ 1.0 ^a	—	2.65 $\pm$ 0.12 ^b
+	—	159	84.8 $\pm$ 1.5 ^b	112	8.4 $\pm$ 1.4 ^b	76.6 $\pm$ 10.6 ^a	3.27 $\pm$ 0.31 ^c
—	+	153	81.6 $\pm$ 1.4 ^b	94	8.7 $\pm$ 2.1 ^b	72.1 $\pm$ 7.3 ^a	2.53 $\pm$ 0.08 ^b
+	+	163	84.7 $\pm$ 1.2 ^b	104	14.3 $\pm$ 0.5 ^c	115.1 $\pm$ 15.9 ^b	3.36 $\pm$ 0.22 ^c

^{a–c}Values without a common letter in their superscripts in the same column differ ($P < 0.05$). Each treatment was repeated six times and 20–30 and 15–20 oocytes were observed for maturation and embryo development, respectively, per replicate per treatment. Each sample for the assay of the intracellular GSH level consisted of 30 oocytes and three samples from different experimental days were assayed for each treatment.

^aDOs were cultured without cumulus cell monolayer.

cumulus cells and then incorporated into GSH synthesis during IVM (Yoshida *et al.* 1993, Takahashi *et al.* 1995). Under physiological conditions, only 10–20% of the total free cystine is present as the reduced form (Meier & Issels 1995).

In this study, cysteamine improved GSH synthesis and blastulation of DOs when added alone but increased GSH synthesis and the blastocyst formation further in the presence of either cumulus cell monolayer or cystine supplementation. The addition of cysteamine alone to IVM medium also increased GSH levels of bovine (de Matos *et al.* 1997) and porcine DOs (Yamauchi & Nagai 1999) in the absence of cumulus cells, but the effect on embryo development was not reported. To our knowledge, for the first time we have reported the beneficial effect of simultaneous addition of cystine and cysteamine on the GSH synthesis and embryo development of DOs. In somatic cells, cysteamine can reduce cystine to cysteine, promoting cysteine uptake and hence enhancing GSH synthesis (Issels *et al.* 1988, Meier & Issels 1995). While this helps to explain the mechanism by which cysteamine promotes the utilization of cystine, the synergic effect of the supplemented cystine and cysteamine on the non-co-cultured DOs in this study would suggest a deficiency of cystine in TCM-199. Then, how can one explain the synergic effect between cysteamine and the cumulus cells under the same concentration of cystine? According to the Michaelis–Menten equation (Michaelis & Menten 1913), at a constant enzyme concentration, the turnover velocity (v) of an enzyme increases as a function of the substrate concentration. Therefore, our explanation is that when either cysteamine or cumulus cells are involved, more substrates (cystine) are needed to produce enough cysteine for GSH synthesis and for the procurement of the ability to form blastocysts. When both cysteamine and cumulus cells are involved, however, more cysteine would be reduced from less cystine. The fact that when cysteamine and cystine were added together, both the rate of blastocysts and the GSH content of the co-cultured DOs increased to the same level as that of COCs cultured with no thiol supplementation further supports our hypothesis.

The removal of cumulus cells before IVM impaired both the developmental capacity (Schroeder & Eppig 1984, Chian *et al.* 1994, Wongsrikeao *et al.* 2005) and the GSH synthesis (de Matos *et al.* 1997, Luciano *et al.* 2005, Maedomari *et al.* 2007) of oocytes. Many efforts have been made to improve the developmental potential of oocytes after cumulus denudation. However, co-culture with COCs or cumulus cells could only partially restore the developmental potential of DOs (Zhang *et al.* 1995, Hashimoto *et al.* 1998, Luciano *et al.* 2005, Ge *et al.* 2007), and whether co-culture would improve GSH synthesis of DOs is inconclusive (Luciano *et al.* 2005, Maedomari *et al.* 2007). The irreparability for the detrimental effect of cumulus removal has been attributed to the disruption of the gap junctions between cumulus cells and the oocyte, because co-culture with the cumulus cell monolayer completely restored the competence of corona-enclosed DOs (Hashimoto *et al.* 1998, Ge *et al.* 2008). However, this study demonstrated that in the presence of a cumulus cell monolayer, simultaneous addition of cysteamine and cystine to the culture medium increased the GSH content and the capacity to blastulate in DOs to the same level as in COCs matured under routine culture conditions. This suggests that it is possible to restore completely the developmental potential of DOs by regulating the IVM conditions, and will contribute to our understanding of the mechanisms by which cumulus cells promote oocyte maturation and to the establishment of an efficient DO IVM system.

Our results indicate that both cysteamine and cystine improve the GSH synthesis and developmental competence of goat COCs in a concentration-dependent manner. However, although the content of intracellular GSH increased with increasing concentrations of cysteamine or cystine up to 500- μ M, the highest rate of blastocyst formation was achieved only at 100- μ M cysteamine or 200- μ M cystine, and blastocyst rates decreased at higher concentrations of the thiol compounds. Yamauchi & Nagai (1999) also noticed that the addition of cysteamine in the culture medium at a concentration of 500 μ M resulted in the degeneration of porcine DOs, and none of them matured to

metaphase II. However, they did not measure the GSH content of the DOs cultured under the same condition. To explain the decreased developmental capacity of oocytes matured under a high concentration of cysteamine or cystine, we speculate that too high a level of intracellular GSH would upset the redox homeostasis that is essential for normal functions such as gene expression of the cell (Arrigo 1999). Besides, in this study, co-culture without thiol supplementation increased only the GSH level but did not improve blastocyst formation of DOs. Furthermore, the mouse DOs showed impaired development while having a similar level of GSH as COCs after IVM (Ge *et al.* 2008). Therefore, it is suggested that the competence for embryonic development is not necessarily correlated with the intracellular GSH level of oocytes following IVM.

In conclusion, we have studied the interactive effects between cysteamine, cystine, and cumulus cells on the GSH synthesis and developmental competence of goat oocytes. The results suggest that DOs cannot utilize cystine for GSH synthesis unless exogenous cysteamine is supplied by either cumulus cells or supplementation. Therefore, while addition of cystine alone is enough to improve the IVM of COCs, improvement of DOs requires the supplementation of both cystine and cysteamine. Synergic actions between cysteamine, cystine, and cumulus cells restore the GSH level and developmental capacity of goat DOs. The data will contribute to our understanding of the mechanisms by which cumulus cells promote oocyte maturation and to the establishment of an efficient DO IVM system.

Materials and Methods

Unless otherwise indicated, all chemicals and drugs were purchased from Sigma Chemical Co.

Recovery of oocytes

Caprine ovaries were obtained from a local abattoir, and transported within 3 h to the laboratory in sterilized saline containing 100 IU/ml penicillin and 0.05 mg/ml streptomycin maintained at 30–35 °C. Collection and culture of oocytes were as described previously (Lan *et al.* 2006). Oocyte aspiration and selection were performed in Dulbecco's PBS (D-PBS), supplemented with 0.1% of polyvinyl alcohol. The COCs were aspirated with a syringe from antral follicles 2–5 mm in diameter and were examined under a stereomicroscope. Only COCs with more than three complete layers of cumulus cells and a finely granulated homogeneous ooplasm were used. The DOs were obtained by mechanically removing cells from COCs with a small-bore pipette. Before culture, DOs and COCs were washed with D-PBS and finally with culture medium.

Maturation culture

The maturation medium was TCM-199 (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco), 1 µg/ml 17β-estradiol, 24.2 mg/l sodium pyruvate, 0.05 IU/ml follicle-stimulating hormone, 0.05 IU/ml luteinizing hormone and 10 ng/ml epidermal growth factor. Depending on the experiment, different concentrations of cysteamine or cystine were added to the maturation medium. Cysteamine and cystine stocks were prepared in advance at concentrations of 20 mM and 100 mM respectively and diluted to the desired concentrations before use. Groups of 20–30 oocytes were transferred into a 100-µl culture medium under mineral oil. The COCs and DOs were cultured in IVM medium at 38.5 °C in 5% CO₂ in humidified air for 24 h.

Co-culture of DOs with cumulus cell monolayer

The COCs obtained from ovaries were pipetted in maturation medium with a narrow-bore pipette to release cumulus cells. After the oocytes were removed, cumulus cells were collected and vigorously pipetted to allow separation of the cells. Then, the cells were counted in a hemocytometer chamber, and aliquots of the cell suspension (100 µl, 3 × 10⁵ cells/ml in maturation medium) were placed in the wells of a 96-well culture plate under mineral oil and cultured at 38.5 °C in a humidified atmosphere of 5% CO₂ in air. The medium was renewed every 48 h until >80% confluence, which was normally attained within 4–5 days. A RIA of progesterone confirmed that the cumulus cells were not luteinized after 5 days of culture in the oocyte maturation medium. When cumulus cells grew to 80% of confluence, the spent medium in the wells was replaced with 100 µl fresh maturation medium. After a 3 h of equilibration in a CO₂ incubator, DOs were placed in wells (20–30 DOs per well) and cultured for 24 h at 38.5 °C in a humidified atmosphere of 5% CO₂ in humidified air.

Chemical activation

At 24 h of maturation culture, COCs were stripped of their cumulus cells by pipetting with a thin pipette in D-PBS containing 0.1% hyaluronidase, and those with an intact first polar body were selected for chemical activation. The DOs with a first polar body were also selected. Oocytes selected were first exposed to 5 µM ionomycin for 2 min at room temperature, and then incubated at 38.5 °C under 5% CO₂ in humidified air for 3 h in CR1aa containing 2 mM 6-dimethylaminopurine (6-DMAP) followed by culture for 3 h in CR1aa without 6-DMAP. The ionomycin and 6-DMAP stocks were prepared in dimethyl sulfoxide at concentrations of 500 µM and 400 mM respectively and diluted to the desired concentrations in CR1aa supplemented with 5% FCS before use.

Embryo culture

Cumulus cells were recovered from *in vitro* matured goat COCs and cultured in DMEM/F12 supplemented with 10% FCS in the wells of a 96-well culture plate. The DMEM/F12 in wells with growing monolayer were replaced with CR1aa containing

3 mg/ml BSA and 5% FCS and equilibrated for 12 h prior to embryo culture. After activation treatments, oocytes were placed in the wells (15–20 oocytes per well) with CR1aa and cumulus cell monolayer and cultured for 9 days at 38.5 °C under 5% CO₂ in humidified air. At the end of culture, embryo development was examined under a phase contrast microscope. Some of the blastocysts were mounted on a slide, stained with Hoechst 33342 and observed for cell counts under a fluorescent microscope.

Assay of GSH

Intracellular content of GSH was measured as described by Funahashi *et al.* (1994). Each sample consisted of 30 oocytes and three samples were assayed for each treatment. Oocytes were denuded of cumulus cells (if any) and washed three times in Ca²⁺-, Mg²⁺-free PBS. Five microliters of distilled water containing 30 oocytes was transferred to a 1.5 ml microfuge tube, and then 5 µl of 1.25 M phosphoric acid was added to the tube. Samples were frozen at –70 °C and thawed at room temperature. This procedure was repeated three times. Then the samples were stored at –20 °C until analyzed. Concentrations of GSH in the oocyte were determined by the 5,5'-dithio-bis (2-nitrobenzoic acid)-glutathione disulfide (DTNB-GSSG) reductase-recycling assay. Briefly, 700 µl of 0.33 mg/ml NADPH in 0.2 M sodium phosphate buffer containing 10 mM EDTA (stock buffer, pH 7.2), 100 µl of 6 mM DTNB in the stock buffer, and 190 µl distilled water were added and mixed in a microfuge tube. Ten microliters of 250 IU/ml GSH reductase were added with mixing to initiate the reaction. The absorbance was monitored continuously at 412 nm with a spectrophotometer for 3 min, with reading recorded every 0.5 min. Standards (0.01, 0.02, 0.1, 0.2, and 1.0 mM) of GSH and a sample blank lacking GSH were also assayed. The amount of GSH in each sample was divided by the number of oocytes to get the intracellular GSH concentration per oocyte.

Data analysis

To observe oocyte maturation and embryo development, each treatment was repeated six times and 20–30 and 15–20 oocytes were observed for maturation and embryo development, respectively, per replicate per treatment. For an assay of intracellular GSH level, each sample contained 30 oocytes and three samples were assayed for each treatment. Percentage data were arc sine transformed and analyzed with ANOVA; a Duncan multiple comparison test was used to locate differences. The software used was Statistics Package for Social Science (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± s.e.m. and *P* < 0.05 was considered significant.

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