Effect of Cysteamine Added to Maturation Medium on Nuclear Maturation, Glutathione and ATP Contents of Pig Oocytes

Yasuhiro Tsuzuki[1]*, Makiko Matsubara[1] and Koji Ashizawa[1]

[1]Laboratory of Animal Reproduction, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan

Abstract: The effect of cysteamine added to maturation medium on nuclear maturation, ATP content and glutathione (GSH) content of porcine oocytes was investigated. The nuclear maturation up to the metaphase stage of second meiotic division (the M-II stage) of cumulus cell-enclosed oocytes (COs) matured with various concentrations (0, 15, 45, 150, and 450 μM) of cysteamine was observed. The maturation rate of the 150 μM group was significantly higher ($P < 0.05$) than that of the control (0 μM) group. Although the ATP content of oocytes derived from COs matured with 150 μM cysteamine was the same as that of the immature group, the ATP content of COs matured without cysteamine was significantly higher ($P < 0.05$) than that of the immature group. Conversely, the GSH content of oocytes derived from COs matured with 150 μM cysteamine was significantly higher ($P < 0.05$) than that of the immature group. However, the content of the group matured without cysteamine was not significantly different from that of the immature group. These results indicate that cysteamine added to maturation medium may stimulate the nuclear maturation of porcine oocytes by increasing the GSH content without enhancing the oocyte ATP content.

Key words: Porcine oocytes, ATP, Glutathione

Introduction

Cysteamine, one of the low molecular weight thiol compounds, added to *in vitro* maturation (IVM) medium, increases the nuclear and/or cytoplasmic maturation of oocytes, and this increase stimulates subsequent embryonic development in mice [1], dogs [2], goats [3], buffalo [4], and cattle [5]. In the pig, cysteamine also facilitates oocyte maturation and the subsequent development of the embryos [6–8].

The synthesis and metabolism of GSH in living cells are linked by the γ-glutamyl cycle that includes a series of six enzyme-catalyzed reactions [9]. GSH is a tripeptide thiol compound that performs many important cellular functions such as cellular proliferation, amino acid transport, and synthesis of proteins [10]. Moreover, a major role of GSH is to maintain the redox state in cells to protect them from oxidative stress [11].

Low molecular weight thiol compounds such as cysteamine and β-mercaptoethanol increase the GSH level in oocytes by preventing the oxidation of cysteine to cystine and by promoting the conversion of cystine to cysteine [3, 8, 12, 13]. Increased GSH levels in oocytes may be an indicator of increased viability [14, 15] such as nuclear maturation, male pronuclear formation and subsequent embryonic development.

The ATP content of an oocyte could be a predictor of the developmental potential after fertilization in humans [16, 17] and cattle [18, 19]. Furthermore, the ATP content of oocytes was influenced by the addition of β-mercapotethanol, a low molecular weight thiol compound like cysteamine, during IVM in cattle [20]. However, the literature contains no reports about the relationship between thiol compounds and the ATP content of porcine oocytes during IVM. Furthermore, the literature contains no information about nuclear maturation, or ATP and GSH contents of the porcine oocytes matured in the maturation medium with or without cysteamine. Therefore, in the present study, we investigated the effects of cysteamine on the nuclear maturation, ATP content and GSH content of porcine oocytes.

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*To whom correspondence should be addressed.
e-mail: a01207u@cc.miyazaki-u.ac.jp

Materials and Methods

In vitro maturation (IVM)

Porcine ovaries were obtained from a local slaughterhouse and immersed in physiological saline (0.9% NaCl; 27–32°C) supplemented with 400 U/ml penicillin G potassium salts (No. 26239-42, Nacalai Tesque, Kyoto, Japan) and 500 μg/ml streptomycin sulfate (No. 32237-72, Nacalai Tesque). The oocytes were aspirated from superficial follicles (from 2 to 6 mm in diameter) by using an 18-gauge needle (No. NN-1838R, Terumo, Tokyo, Japan) attached to a 5-ml syringe (No. NN-05sZ, Terumo). This syringe contained a small amount of TCM-199 (Hank's salt, No. M-0393, Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 2% (v/v) heat inactivated calf serum (CS; 56°C; 30 min; No. 16170-086; Invitrogen Co., Carlsbad, CA, USA). The oocytes, which were enclosed by more than one layer of cumulus cells (COs), were selected and washed four times with TCM-199 without CS. These COs were matured in BSA-free NCSU37 medium [21] for 42 to 46 hours under 5% CO_2, 95% air, and 100% humidity at 39°C. The NCSU37 medium [21] used for IVM in this study was modified with 51.5 mM taurine (No. T-7146; Sigma-Aldrich Co.), 1.2 mM cysteine (No. 10309-12; Nacalai Tesque), 2% MEM essential amino acid (No. 11130-051; Invitrogen Co.), 1% non-essential amino acid (No. 11140-050; Invitrogen Co.), 15 ng/ml epidermal growth factor (No. E-4127, Sigma-Aldrich Co.), 5 μg/ml insulin (No. I-6634, Sigma-Aldrich Co.), 0.23 μg/ml LH (No. L-9773, Sigma-Aldrich Co.), 0.63 μg/ml FSH (No. F-2293, Sigma-Aldrich Co.), 5% (v/v) fetal calf serum (FBS, 56°C, 30 min heat-inactivated, No. 16140-063; Invitrogen Co.), and antibiotics (100 U/ml penicillin G potassium, No. P-4687, Sigma-Aldrich Co; 100 μg/ml streptomycin sulfate, No. S-1277, Sigma-Aldrich Co; and 100 μg/ml dibekacin sulfate, No. DBK, Meiji Seika Co., Tokyo Japan) [22].

Nuclear maturation test

A few immature oocytes, derived from COs aspirated from the ovaries and denuded of their cumulus cells by a vortex mixture, were fixed with acetic-alcohol (alcohol: acetic acid = 3:1, v/v) for few days at room temperature and stained with 1% orcein (No. 7100, Merck, Darmstädt, Germany) [22].

The remaining COs were divided into 5 groups, which were matured with the various concentrations of cysteamine (No. M-6500, Sigma-Aldrich, 0, 15, 45, 150 or 450 μM), and their nuclear maturation rates up to the M-II stage were assessed. The culture volume for IVM was 4.2 to 5.0 μl/CO for all groups. After IVM, the COs in each group were denuded of their cumulus cells, fixed and stained by the same method used for the immature oocytes.

ATP assay

COs were matured with or without 150 μM cysteamine in the same culture volume used (4.2–5.0 μl/CO) in the nuclear maturation test. They were randomly divided into two groups without further selection. One group (10 to 20 COs/assay) was used for the measurement of the ATP content of the oocytes. The other group (10 to 20 COs/assay) was used for the measurement of the GSH content, as described below. The ATP content of the oocytes was measured by a method described in a previous report [22]. Briefly, the oocytes derived from the matured COs by the removal of cumulus cells were washed with physiological saline supplemented with 2% CS (2% CS-PS), transferred to an 1.5-ml Eppendorf tube (No. A-150, Assist Co., Tokyo, Japan) with 50 μl 2% CS-PS, and boiled at 100°C for 4 min after the addition of 100 μl pure water, then stored at –20°C. The 100-μl sample and the ATP stock solution were loaded into a lumicounter (No. A-237, Advantec Co., Tokyo, Japan), and pure luciferase (No. 60311, Kikkoman, Noda, Chiba, Japan) was added to measure the ATP content. The ATP content of immature oocytes was also measured by the same method.

GSH assay

For the measurement of the GSH content, the oocytes derived from COs by removal of cumulus cells were loaded into a 1.5-ml Eppendorf tube with 5 μl 2% CS-PS, and stored at –20°C until measurement. For the assay, 1.25 M H_3PO_4 (No. 27618-55, Nacalai Tesque) was added to thawed samples, as well as 700 μl buffer (pH 7.2) supplemented with 0.33 mg/ml NADPH (No. 309-50471, Oriental Yeast Co., Osaka), 100 μl of 5,5'-dithibis (2-nitrobenzoic acid) (DTNB, No. 047-16401, Wako Pure Chemical Industries, Osaka), 190 μl of water and 10 μl GSSG-reductase (No. G-3664, 250 U/ml, Sigma-Aldrich). The formation of TNB in samples was measured at 412 nm on a spectrophotometer (UV-150-02, Shimazu Co., Tokyo, Japan) every 30 sec for three minutes at room temperature. The GSH content of the immature oocytes was also assessed. Standards were prepared for each assay and the GSH content per sample was determined from a standard curve [23].

Table 1. Effect of various concentrations of cysteamine added to the maturation medium on the nuclear maturation of porcine oocytes

Concentration of cysteamine (μM)	N	Nuclear maturation rate to the M-II stage (%)
0	316	58.2 ± 7.07[b]
15	302	68.6 ± 5.09[ab]
45	308	73.0 ± 6.33[a]
150	302	78.2 ± 1.38[a]
450	303	76.8 ± 1.21[a]

[a, b]: Values with different superscripts in a column are significantly different ($P < 0.05$). Values are mean ± SE of 5 replicates.

Table 2. Effect of cysteamine on the ATP content of CO-derived oocytes

Treatment	ATP contents (pmol/oocyte)
Immature	0.6 ± 0.10[b]
Matured –	1.0 ± 0.08[a]
Matured +	0.7 ± 0.12[b]

[a, b]: Values with different superscripts in a column are significantly different ($P < 0.05$). Values are mean ± SE of 4 replicates. +, presence of 150 μM cysteamine; –, absence of cysteamine (0 μM cysteamine).

Table 3. Effect of cysteamine added to the IVM medium on the GSH content of CO-derived oocytes

Treatment	GSH contents (pmol/oocyte)
Immature	1.7 ± 0.11[b]
Matured –	2.4 ± 0.48[ab]
Matured +	3.6 ± 0.54[a]

[a, b]: Values with different superscripts in a column are significantly different ($P < 0.05$). Values are mean ± SE of 4 replicates. +, presence of 150 μM cysteamine; –, absence of cysteamine (0 μM cysteamine).

Statistical analysis

All data were analyzed with ANOVA followed by Duncan's Multiple Range Test.

Results

Of the 134 immature oocytes collected in 5 replicates, 86.1 ± 3.89% were found to be at the germinal vesicle stage, 4.8 ± 1.63% oocytes were at the prometaphase I stage and the remainder were at the metaphase I stage before IVM treatment. The nuclear maturation rate up to the M-II stage of the oocytes matured with various concentrations of cysteamine are presented in Table 1. The maturation rate of the 150 μM cysteamine group was the highest, and was significantly higher ($P < 0.05$) than that of the control (0 μM) group.

The ATP content of the oocytes in each group are presented in Table 2. Although the ATP content of the oocytes derived from COs matured with 150 μM cysteamine was the same as that of the immature group, the ATP content of the oocytes matured without cysteamine was significantly higher ($P < 0.05$) than that of the immature group.

The GSH content of each group is presented in Table 3. Unlike ATP contents, the GSH content in the oocytes derived from the COs matured with 150 μM cysteamine was significantly higher ($P < 0.05$) than that of the immature group. However, the GSH content of the oocytes derived from the COs matured without cysteamine was not significantly different from that of the immature group.

Discussion

In the present study, the nuclear maturation rate up to the M-II stage of the oocytes matured with 150 μM cysteamine was the highest of all the groups, and this rate was significantly higher ($P < 0.05$) than that of the control (0 μM) group. This suggests that 150 μM cysteamine may facilitate nuclear maturation of porcine oocytes. This concentration has been used by other researchers [8, 12, 24] who matured porcine oocytes. However, Bing *et al.* [8] reported that under high oxygen tension (20%, the same as the present study), no increase in the nuclear maturation rate up to the M-II stage of the porcine COs was seen in a group supplemented with 150 μM cysteamine compared with a control group without cysteamine. In contrast, we found that cysteamine improved nuclear maturation of COs in this study. The effects of the cysteamine in IVM medium on the nuclear maturation of porcine oocytes is dependent on hormones such as estradiol-17β [25].

Although Bing *et al.* [8] added estradiol-17β to the maturation medium, we did not. The lack of estradiol-17β may be one of the reasons for the discrepancy between our results and those reported by Bing *et al.* [8].

The ATP content of the oocytes matured without cysteamine in this study was 1.0 ± 0.08 p mol/oocyte. This value is comparable that of a previous report [26], which suggests that our measurement method for ATP content is accurate. Furthermore, the ATP content of the oocytes matured without cysteamine was significantly higher ($P < 0.05$) than that of the immature ones. This result was consistent with that reported by Brevini *et al.* [27], who noted an increase in ATP contents of porcine oocytes after maturation. Although the ATP content of the oocytes matured with cysteamine was comparable to that of the immature oocytes, it was significantly lower ($P < 0.05$) than that in the oocytes matured without cysteamine. Tsuzuki *et al.* [20] reported that the ATP contents of bovine oocytes matured with 100 μM β-mercaptoethanol (a low-molecular weight thiol compound) was significantly lower ($P < 0.05$) than that of oocytes matured without it. They postulated that the decrease in ATP content of oocytes matured with β-mercaptoethanol might be caused by consumption of ATP to polymerize actin for cytoplasmic maturation events such as relocation of the oocytic cortical granules [28], or synthesis of cellular molecules such as GSH (described below). If such events took place in the oocytes matured with cysteamine, it would explain the decrease of the ATP content seen in the present study.

The average GSH content of the oocytes matured with or without cysteamine was 2.4 to 3.6 p mol/oocyte in this study, and these values were comparable to those of previous reports [6, 12, 13]. This indicates that our methods for the measurement of the GSH content as well as our method for the measurement of oocyte ATP content may be accurate. Although the GSH contents of the oocytes matured with cysteamine were significantly higher ($P < 0.05$) than that of the immature group, the GSH content of the oocytes matured without cysteamine was not significantly different from that of the immature group in our study. It is well known that thiol compounds can enhance GSH synthesis in oocytes [12, 13] by preventing the oxidation of cysteine to cystine and by promoting the conversion of cystine to cysteine in the medium [8]. In our study, 150 μM cysteamine added to the maturation medium may have stimulated GSH synthesis, the processes described above, by resulting in an increase in the GSH content after IVM. The GSH content of the oocytes matured with cysteamine was significantly higher ($P < 0.05$) than that of the immature oocytes. In contrast, the ATP content of the oocytes matured with cysteamine were significantly lower ($P < 0.05$) than that of the immature group. It appears that the ATP content of the oocytes might be related to the higher GSH content. Glutathione is synthesized in two consecutive ATP-dependent reactions [10, 29]. Under oxidative stress, cysteamine may increase the consumption of ATP, which in turn would accelerate glutathione synthesis within the oocytes. This would result in oocytes matured with cysteamine showing increased GSH content, and decreased ATP content, as seen in this study. Related to this, Brad *et al.* [26] reported that the ATP content of porcine oocytes matured *in vitro* may not be related to the development of embryos after fertilization; however, the GSH content may be in part responsible, for lower developmental competence. To clarify the role of GSH, further studies including examination of the embryonic development will be necessary.

GSH synthesis is dependent on the availability of cysteine, one of the constituent amino acids of GSH, in the medium [30, 31]. Although cysteine is unstable and easily oxidized to cystine in the medium [31], supplementation of low thiol compounds such as cysteamine and β-mercaptoethanol to cysteine-containing medium can promote GSH synthesis in bovine and porcine oocytes by reducing the cystine to cysteine, as described above [5, 7, 8, 12]. We used NCSU37 medium supplemented with 2% essential amino acid solution and cysteine with changing the concentration from 0.6 mM [32] to 1.2 mM for IVM. Although NCSU37 medium does not contain any cystine, 2% essential amino acid solution contains 24 mg/l (0.1 mM) cystine [21, 32, Invitrogen catalog 2006–2007]. If all the cystine in the medium used in this study were converted to cysteine, the final concentration of cysteine in the medium would be 1.4 mM, because cystine is cysteine disulfide [33]. Abeydeera *et al.* [34] reported that when porcine oocytes were matured in the NCSU23 medium, which has almost the same components as NCSU37 medium except for hypotaurine, taurine and sorbitol [21], supplemented with 0.8, 1.7 or 3.3 mM cysteine in the presence of 25 μM β-mercaptoethanol, the GSH content of the oocytes and the embryonic development rates after fertilization were not significantly different among the cysteine groups. Accordingly, we suggest that 1.2 mM cysteine added to the maturation medium used in the present

study may not have had an inhibitory effect on GSH synthesis in the porcine oocytes.

In conclusion, 150 μM cysteamine added to IVM medium enhanced nuclear maturation and the GSH content but did not increase the ATP content of porcine oocytes.

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