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Effect of Cysteamine on *In Vitro* Maturation, Fertilization and Culture of Porcine Oocytes*

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ABSTRACT

This study was carried out to investigate the effect of cysteamine addition during *in vitro* maturation, fertilization and culture of porcine oocytes. Oocytes were matured for the first 22 h in mTCM-199 media supplemented with or without 150 μM cysteamine. They then were matured for an additional 22 h in mTCM-199 media without hormones supplemented with or without 150 μM cysteamine. When cumulus-oocyte complexes (COCs) were matured in the mTCM-199 media supplemented with cysteamine, the rates of GVBD and maturation (metaphase Ⅱ) were enhanced as compared to the media without the addition of cysteamine. Also, when COCs were matured in the mTCM-199 media supplemented with cysteamine, the rates of sperm penetration, male pronucleus formation, cleavage and blastocyst formation after *in vitro* fertilization were enhanced as compared to the media without the addition of cysteamine. In conclusion, it was suggested that oocytes matured for the first 22 h in mTCM-199 media supplemented with 150 μM cysteamine increased the rates of metaphase Ⅱ, sperm penetration, male pronucleus and blastocyst formation were higher as compared to the media without addition of cysteamine.

(Key words : Maturation, Cysteamine, Male pronucleus, Blastocyst)

Ⅰ. INTRODUCTION

Cysteamine addition to an *in vitro* maturation medium including a hormonal condition has been reported to promote male pronucleus formation after *in vitro* maturation and fertilization of pig oocytes (Yoshida et al., 1992; Yoshida, 1993a; Grupen et al., 1995; Nagai, 1996).

Yamauchi and Nagai (1999) reported that cysteamine, a thiol with reducing function, increased the content of glutathione (GSH) and promoted male pronucleus formation even in cumulus-free porcine oocyte. Probably in this case, cysteamine converted cystine to cysteine in TCM-199, a cysteine-rich medium.

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Grupen et al. (1995) demonstrated that the addition of cysteamine to TCM-199 culture medium increased male pronuclear formation in porcine cumulus-oocyte complexes matured and fertilized *in vitro*. In addition, cysteamine increased intracellular GSH in mouse lymphoma cells (Ishii et al., 1981; Zmuda and Friedenson, 1983) and CHO cells (Isseles et al., 1988). Synthesis of GSH during oocyte maturation occured in mice (Calvin et al., 1986), hamsters (Perreault et al., 1988), pigs (Yoshida et al., 1993b), and cattle (Miyamura et al., 1995; de Matos et al., 1996). After penetration, GSH participated in sperm decondensation in parallel with oocyte activation, as well as in the transformation of the penetrated sperm head into the male pronucleus (Perreault et al., 1984; Calvin et al., 1986; Perreault et al., 1988; Naito et al., 1992; Yoshida et al., 1992; Yoshida, 1993a; Funahashi et al., 1996). Further, oocyte GSH content affected not only male pronuclear development but also histone H1 kinase (H1K) activitiy (Funahashi et al., 1996), which has also been reported to affect male pronuclear formation after sperm penetration and subsequent early embryonic development (Naito et al., 1992).

The present study was conducted to examine the effect of cysteamine on *in vitro* maturation, fertilization and culture of immatured porcine oocytes.

Ⅱ. MATERIALS AND METHODS

1. Oocyte Collection and *In Vitro* Maturation

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in 0.9% NaCl solution containing 75 ㎍/ml potassium penicillin G, 50 ㎍/ml streptomycin sulfate and 0.1% BSA at 30~35℃. Cumulus oocyte complexes (COCs) were aspirated from antral follicles (3 to 6 mm diameter) using a 18 gauge needle fixed to a 10 ml disposable syringe. COCs were washed three times in mTLP-PVA and were washed two times with a maturation medium. 30~40 COCs were transferred to 500 ㎕ of the same medium and that had been covered with mineral oil in a 4-well multidish (Nunc, Rosklide, Denmark) and equilibrated at 38.5℃, 5% CO_2 in air. The medium used for oocyte maturation was modified tissue culture medium (mTCM)-199 supplemented with 26.19 mM sodium bicarbonate, 0.9 mM sodium pyruvate, 10 ㎍/ml insulin, 2 ㎍/ml vitamin B_{12}, 25 mM HEPES, 10 ㎍/ml bovine apotransferrin, 150 μM cysteamine, 10 IU/ml PMSG, 10 IU/ml hCG, 10 ng/ml EGF, 0.4% BSA, 75 ㎍/ml sodium penicillin G, 50 ㎍/ml streptomycin sulfate and 10% pFF. After about 22 h of culture, oocytes were cultured without hormones for an additional 22 h at 38.5℃, 5% CO_2 in air.

2. *In Vitro* Fertilization and Culture of Oocytes

After the completion of culture of oocytes for IVM, cumulus cells were removed with 0.1% hyaluronidase in mTLP-PVA and washed two times with mTBM. Thereafter, 30~40 oocytes were transferred into each well of a 4-well multidish containing 500 ㎕ mTBM fertilization medium that had been covered with mineral oil and equilibrated at 38.5℃, 5% CO_2 in air. The dishes were kept in a CO_2 incubator until spermatozoa were added for insemination.

For IVF, one frozen 5 ml straw was thawed at 52℃ in 40 sec and was diluted with 20 ml Beltsville thawing solution (BTS) at room temperature. Two ml of the diluted sperm was then added to 8 ml of mTLP-PVA and centrifuged two times for 5 min at 800×g. Washed sperm was resuspended with mTBM, and oocytes were inseminated with 2×10^7/ml sperm concentration. At 6 h after IVF, oocytes were transferred into 500 ㎕

HEPES buffered (25 mM) NCSU-23 culture media, respectively, containing 0.4% BSA for further culture.

3. Examination of Oocytes

At 44 h after maturation and at 12 h and 48 h after insemination, oocytes were fixed for 48 h in 25% acetic acid (v:v) in ethanol at room temperature, and stained with 1% (w:v) orcein in 45% (v:v) acetic acid to examine metaphase Ⅱ, sperm penetration, polyspermic oocytes, male pro-nucleus and cleaved oocytes under a phase-contrast microscope at ×400 magnification. Blastocysts on day 6 were stained with Hoechst 33342 and were counted nucleus number under fluorescent microscope (Olympus, Japan).

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MD, USA).

4. Statistical Analysis

Analyses of variance (ANOVA) were carried out using the SAS package (SAS, 1996) in a completely randomized design. Duncan's multiple range test was used to compare mean values of individual treatment, when the F-value was significant ($P<0.05$).

Ⅲ. RESULTS

1. Effects of Cysteamine Addition during *In Vitro* Maturation in mTCM-199

As shown in Table 1, COCs were matured in the mTCM-199 media supplemented with cysteamine, the rates of GVBD and maturation (MⅡ) were significantly enhanced as compared to the media without the addition of cysteamine. Also, the addition of cysteamine during the first culture for 22 h was important for the rates of GVBD and maturation as compared to with and without the addition of cysteamine during the second culture for 22 h.

2. Effects of Cysteamine Addition in Maturation Media on *In Vitro* Fertilization of Pig Oocytes

Table 1. Effects of cysteamine addition (150 μM) during *in vitro* maturation in mTCM-199 media

Cysteamine addition		No. of oocytes	No. of oocytes			GVBD[2] (%)	MⅡ[2] (%)
22 h*	22 h**		GV[1]	GVBD - MⅠ[1]	MⅡ[1]		
–	–	150	18	7	125	88.1±1.6[b]	83.4±2.7[b]
+	–	153	8	2	143	94.9±1.1[a]	93.6±1.5[a]
–	+	153	13	4	136	91.7±1.9[ab]	89.1±2.3[ab]
+	+	155	9	3	143	94.3±1.1[a]	92.3±1.3[a]

* First maturation time with 10 IU/ml PMSG and 10 IU/ml hCG in mTCM-199 maturation medium.

** Second additional maturation time without 10 IU/ml PMSG and 10 IU/ml hCG in mTCM-199 maturation medium.

[1] GV : germinal vesicle, GVBD : germinal vesicle breakdown, MⅠ: metaphase Ⅰ and MⅡ: metaphase Ⅱ.

[2] Mean±SE. Experiments were repeated five times.

[ab] Values in the same column with different superscripts differ significantly ($P<0.05$).

As shown in Table 2, when COCs were matured in the mTCM-199 media supplemented with cysteamine, the rates of sperm penetration and male pronucleus formation after *in vitro* fertilization were significantly enhanced as compared to with those without cysteamine.

3. Effects of Cysteamine Addition in Maturation Media for Pig Oocytes on Embryo Development during *In Vitro* Culture

As shown in Table 3, when COCs were matured in the mTCM-199 media supplemented with cysteamine, the rates of cleavage and blastocyst formation were significantly enhanced as compared to with those without cysteamine.

Ⅳ. DISCUSSION

Grupen et al. (1995) reported that the addition of cysteamine to the maturation medium with 10 μg /ml FSH, 5 μg/ml LH and 1 μg/ml E_2 did not significantly affect the rate of oocyte maturation, and Yamauchi and Nagai (1999) demonstrated that the addition of 150 μM cysteamine had no effect on meiotic maturation of porcine oocytes cultured in the medium with 1 μg/ml E_2, 10 IU/ml eCG and 10 IU/ml hCG. Also, he reported that the addition of cysteamine to culture medium increased oocyte GSH content and promoted male pronuclear formation after sperm penetration of porcine cumulus -denuded oocytes but had no effect on their maturation rates or kinase activities. Bing et al. (2001) reported that cysteamine significantly enhanced male pronucleus formation, a result inconsistent with many studies (Yoshida, 1993a; Grupen et al., 1995; Nagai, 1996).

Although the mechanism for the effect of cysteamine and E_2 on oocyte maturation is not clear, it is of interest that the addition of cysteamine to the maturation medium promotes the synthesis of glutathione (GSH) in the oocytes resulting in a high male pronucleus formation after *in vitro* fertilization (Yoshida, 1993a; Nagai, 1996). Furthermore, considering the fact that GSH enhances *in vitro* development of IVM-IVF bovine oocytes by reacting as a substrate of glutathione peroxidase and

Table 2. Effects of cysteamine addition (150 μM) in maturation media for pig oocytes on sperm penetration and male pronucleus formation after *in vitro* fertilization

Cysteamine addition		No. of oocytes inseminated	% of oocytes penetrated[1]	% of polyspermic oocytes[1]	% of oocytes with male pronucleus[1]
22 h*	22 h**				
–	–	148	47.0±2.6[b]	2.3±0.8	37.3±3.5[b]
+	–	154	55.7±2.1[a]	4.6±1.4	46.1±2.7[a]
–	+	155	52.9±2.6[a]	2.5±0.7	43.3±1.7[a]
+	+	155	54.1±2.1[a]	3.2±0.9	45.7±2.5[a]

* First maturation time with 10 IU/ml PMSG and 10 IU/ml hCG in mTCM-199 maturation medium.

** Second additional maturation time without 10 IU/ml PMSG and 10 IU/ml hCG in mTCM-199 maturation medium.

[1] Mean±SE. Experiments were repeated five times.

[ab] Values in the same column with different superscripts differ significantly ($P<0.05$).

Table 3. Effects of cysteamine addition (150 μM) in maturation media for pig oocytes on embryo development during *in vitro* culture[1]

Cysteamine addition		No. of embryos cultured[2]	% of cleaved oocytes[3]	% of blastocyst from cleaved oocytes[3]
22 h*	22 h**			
–	–	96	62.6±2.8[b]	5.5±1.8[b]
+	–	99	73.9±3.4[a]	15.1±2.7[a]
-	+	101	71.1±3.1[a]	11.2±2.1[a]
+	+	100	72.7±3.3[a]	12.6±2.8[a]

* First maturation time with 10 IU/ml PMSG and 10 IU/ml hCG in mTCM-199 maturation medium.

** Second additional maturation time without 10 IU/ml PMSG and 10 IU/ml hCG in mTCM-199 maturation medium.

[1] Mean±SE. Experiments were repeated four times.

[2] Cultured oocytes were selected after *in vitro* fertilization (IVF).

[3] Rates of cleavage and blastocyst were examined at 48 and 144 h after IVF, respectively.

[ab] Values in the same column with different superscripts differ significantly ($P<0.05$).

acting as a scavenger of free radicals in the fertilized oocytes (Takahashi et al., 1993), GSH might be involved in this enhancement of oocyte maturation by scavenging free radicals in oocytes.

In conclusion, we found out that the addition of 150 μM cysteamine to maturation medium enhanced the rates of metaphase Ⅱ, male pronuclear formation and blastocyst formation as compared to without addition of cysteamine.

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