STUDIES ON MALE PRONUCLEAR FORMATION AFTER *IN VITRO* FERTILIZATION OF PIG OOCYTES

March 1997

Ken SAWAI

The Graduate School of
Natural Science and Technology
(Doctor Course)
OKAYAMA UNIVERSITY

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PREFACE

The experiments described in this dissertation were carried out at the Graduate school of Natural Science and Technology (Doctor Course), Okayama University, Japan, from April 1994 to October 1996, under the supervision of Professor K. Niwa. These studies are original works by the author and any assistance and collaboration from others are specifically acknowledged.

This dissertation has not been submitted previously whole or in part to council, a University or any other professional institution for degree, diploma or other professional qualification.

Ken Sawai
March, 1997

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ABSTRACT

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The objective of the present study was to examine the effects of various factors responsible for male pronuclear (MPN) formation associated with glutathione (GSH) synthesis of pig oocytes. In the first series of experiments, when cumulus-enclosed oocytes were cultured for 24 h in maturation medium supplemented with 0.57 mM cysteine, GSH concentration of oocytes was increased. However, the activation of oocytes after sperm penetration *in vitro* was inhibited and thereby MPN formation of the oocytes was not promoted. The MPN formation in activated oocytes after sperm penetration at any stages of maturation was promoted when cysteine was added to maturation medium. The results indicate that an increased concentration of GSH and intimate synchronization with oocyte activation are essential for MPN formation of pig oocytes. In the next series of experiments, it was shown that, when cysteine was removed from maturation medium at 36 h of culture, both the incidence of MPN formation after *in vitro* fertilization and GSH concentration of matured oocytes were lower than when cysteine was present during whole period of culture for 48 h. In contrast, the presence of cysteine in maturation medium only between 42 and 48 h of culture, when oocytes reached to the late metaphase-I to metaphase-II stage, could promote oocyte GSH synthesis and thereby MPN formation after *in vitro* fertilization. The results indicate that the presence of cysteine in maturation medium for 6 h of the last phase of maturation is essential for oocyte GSH synthesis enough to induce MPN formation with the same efficiency as in oocytes matured in the

presence of cysteine from the start of culture. The effects of cystine, an oxidized form of cysteine, added to maturation medium at various times after culture on the MPN formation and GSH synthesis of cumulus-enclosed and cumulus-free oocytes were examined in the third series of experiments. When maturation medium was supplemented with 0.57 mM cystine, MPN formation and GSH synthesis of cumulus-enclosed oocytes were accelerated, but not in cumulus-denuded oocytes. On the other hand, cysteine (0.57 mM) could promote MPN formation and GSH synthesis in both cumulus-enclosed and cumulus-denuded oocytes. The results indicate that cystine is associated with increased GSH synthesis and MPN formation of pig oocytes and that the presence of cumulus cells is essential for the utilization of the cystine by oocytes. In contrast, cysteine is utilized directly by oocytes without cumulus cells for GSH synthesis and MPN formation. Taken together, it is concluded from these results that synchronization of oocyte activation and sperm penetration, and the addition of exogenous compounds such as cysteine or cystine to maturation medium are important factors for GSH synthesis of matured oocytes and MPN formation of oocytes following fertilization. The presence of cumulus cells associated with utilization of cystine by oocytes is also an important factor.

INTRODUCTION

The new biotechnologies in mammals such as gene transfer and cloning, in particular using embryonic stem cells, involve the manipulation of oocytes and/or embryos *in vitro*. Thus, there is an urgent requirement for reliable *in vitro* maturation and *in vitro* fertilization procedures. In pigs, the first successful *in vitro* fertilization was reported by Iritani *et al.* (1978) who used epididymal and ejaculated spermatozoa incubated in the isolated female genital tracts. However, penetration rates were very low. Then, Nagai *et al.* (1984) tried to fertilize oocytes *in vitro* by epididymal spermatozoa preincubated at very high sperm concentration and obtained high penetration rates. However, when epididymal spermatozoa was used for *in vitro* fertilization, repeated use of spermatozoa with the same characteristics is not possible. Wang *et al.* (1991) have reported successful penetration *in vitro* of oocytes matured *in vitro* by frozen-thawed ejaculated spermatozoa. This makes it possible to analyse correctly various factors which affect on *in vitro* maturation and penetration of oocytes.

On the other hand, although some piglets have also been obtained after transfer of embryos resulted from oocytes matured and fertilized *in vitro*, the success is still limited in a few laboratories (Yoshida, 1987; Mattioli *et al.*, 1989; Yoshida *et al.*, 1993a; Funahashi and Day, 1996; Funahashi *et al.*, 1997). This might be due to high incidence of polyspermy and reduced incidence of male pronuclear (MPN) formation which are characteristically observed in pig oocytes matured and fertilized *in vitro* (see Niwa, 1993; Nagai, 1994; Mattioli, 1994; Day and Funahashi, 1996 for reviews). Under *in vivo* conditions, fertilization of pig oocytes occurs soon after ovulation and is monospermic in over 95% of cases (Hunter, 1967; 1972). However, a high rate of polyspermy was observed *in vivo* when the number of spermatozoa at the fertilization site was artificially increased (Hunter, 1976). These

reports suggest that conditions for fertilization might be more important to achieve a high percentage of normal fertilization than the conditions for oocyte maturation. In fact, it has been reported that the rate of polyspermy can be reduced by controlling the conditions of the medium used for treatment of spermatozoa and fertilization of oocytes (Nagai and Moor, 1990; Funahashi and Day, 1993a). In contrast, although oocytes can be penetrated *in vitro* by spermatozoa under appropriate conditions, incomplete oocyte maturation may result in low rate of MPN formation (see Niwa, 1993; Nagai, 1994 for reviews). In the majority of species, after incorporation of the sperm nucleus into the oocyte cytoplasm, cytoplasmic factors react with sperm chromatin to induce molecular changes, including cleavage of disulfide bonds, partial enzymatic degradation of nuclear proteins, the release of chromatin-associated protamines and their immediate replacement by oocyte-derived histones (Zirkin *et al.*, 1989). The oocyte cytoplasmic activity that controls MPN formation has been defined as the MPN growth factor (Thibault and Gerard, 1973; Thibault *et al.*, 1975) or the sperm pronucleus development factor (Yanagimachi, 1981).

The ability of pig oocytes to form a male pronucleus is affected by exogenous hormonal levels (Mattioli *et al.*, 1988a; Funahashi and Day, 1993b; Funahashi *et al.*, 1994a; Wang and Niwa, 1995), follicular secretions (Mattioli *et al.*, 1988b; Naito *et al.*, 1988; Yoshida *et al.*, 1992a; Funahashi and Day, 1993c; Nagai *et al.*, 1993), and intercellular ionic strength (Funahashi *et al.*, 1994a, b). In addition, it is clearly indicated that the incidence of MPN formation is positively associated with oocyte glutathione (GSH) concentration at the end of maturation (Yoshida, 1993; Yoshida *et al.*, 1993b).

Yoshida *et al.* (1992b) found at first that the rate of MPN formation was significantly higher in pig oocytes matured in Waymouth MB 752/1 medium than in those matured in mTCM-199 or mTLP-PVA medium. Waymouth medium contains a higher concentration of GSH and cysteine than in mTCM-199 or mTLP-PVA. Since permeability of plasma membranes to GSH is low (De Felici *et al.*, 1987), cysteine

in the medium might affect MPN formation of pig oocytes. It has been suggested that the synthesis of GSH during maturation is a prerequisite for MPN formation in mouse (Calvin *et al.*, 1986) and hamster (Perreault *et al.*, 1988; Perreault, 1990) oocytes. The GSH is a tripeptide that has intracellular functions in the reduction of the disulfide linkages of proteins and other molecules, amino acids transport, and synthesis of proteins and DNA (Kosower and Kosower, 1978; Meister and Anderson, 1989). The GSH seems to be associated with the reduction of disulfide bond cross-linking of sperm protamine (Perreault, 1990), and the intracellular concentration of GSH depends on the availability of cysteine (Meister, 1983). Then, Yoshida *et al.* (1993b) have found that pig oocyte GSH concentration increased during maturation when cysteine was added to maturation medium (mTLP-PVA) but decreased without added cysteine, and indicated that GSH is an important cytoplasmic factor for MPN formation in pig oocytes.

As described above, synthesis of sufficient GSH during *in vitro* maturation of pig oocytes may be required for MPN formation and cysteine has an important role for synthesis of GSH in the oocyte. However, detailed mechanism of GSH synthesis during maturation and the relation to MPN formation in pig oocytes after *in vitro* fertilization are unknown with only a few hypotheses (Yoshida, 1993; Yoshida *et al.*, 1993b; Day and Funahashi, 1996). It is suggested in another species that several factors such as oocyte activation (Borsuk and Manaka, 1988; Yanagimachi, 1994), nuclear stage of oocyte (Perreault, 1990), and reducing environment of oocyte culture (Takahasi *et al.*, 1993) affected GSH concentration and MPN formation of oocytes. To clarify the factors affecting MPN formation of pig oocytes is especially important since, with increased interest in producing transgenic pigs, there is an increased demand for production of normal pig embryos *in vitro* (Niwa, 1993).

Therefor, the present study was carried out to examine some factors responsible for MPN formation of pig oocytes after *in vitro* fertilization.

CHAPTER 1

GENERAL METHODOLOGY

1. Medium

The basic medium used for the manipulation of oocytes and spermatozoa was tissue culture medium (TCM) 199 (with Earle's salts; Gibco, Grand Island, NY, USA) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 75 μg /ml potassium penicillin G, 50 μg/ml streptomycin sulphate and 1 mg/ml PVA. This medium was essentially the same as TCM-199B used by Wang *et al*. (1991) except that calcium lactate was deleted and PVA was substituted for fetal calf serum (FCS) and therefore designated modified TCM-199B (mTCM-199B).

2. Collection and culture of oocytes

Ovaries were collected from about 6.5 mo old maturing gilts (Landrace × Yorkshire × Duroc; about 110 kg body weight) at a local slaughterhouse and transported to the laboratory within 1-1.5 h in 0.9% (w/v) NaCl solution containing 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate at 37-39°C. Immature oocytes were aspirated from antral follicles (2-5 mm in diameter) with an 18-gauge needle fixed to a 10-ml disposable syringe. Oocytes were washed four times with maturation medium, mTCM-199B (pH 7.4) supplemented with 10 IU/ml eCG and 10 IU/ml hCG. Ten oocytes with compact cumulus were transferred to 100 μl of the same medium, which had been previously covered with warm paraffin oil in a polystyrene culture dish (35 × 10 mm; No. 1008, Becton and Dickinson, NJ, USA) and equilibrated in an atmosphere of 5% CO_2 in air for about 3 h. Oocytes were cultured for various periods according to different experiments at 39°C in the same atmospheric condition. After culture, the oocytes were used for *in vitro*

fertilization, examination of nuclear maturation or assay of GSH.

3. Cryopreservation of spermatozoa

Sperm-rich portion of the ejaculated semen collected from boars by the glove method and filtered through double gauze was kept at 15°C for 2-3 h. Semen samples were then diluted 1:2 with Hülsenberger VIII diluent (Richter *et al.*, 1975) at 15°C and centrifuged for 15 min at 300 **g**. The supernatant was discarded and spermatozoa were resuspended in Beltsvill F5 (BF-5) extender (Pursel and Johnson, 1975) to give 4×10^7 cells/ml. After cooling to 5°C over 1 h, the sperm suspensions were diluted with the same volume of BF-5 solution containing 2% (v/v) glycerol. The sperm suspensions were frozen in a 200-μl pellet on solid CO_2 and stored in liquid nitrogen (-196°C) until required for *in vitro* fertilization.

4. Penetration of oocytes and spermatozoa for *in vitro* fertilization

After maturation culture, oocytes were washed twice with fertilization medium, mTCM-199B (pH 7.8) containing 10 mM caffeine-sodium benzoate (Sigma Chemical Co., St Louis, MO, USA), 2.92 mM calcium lactate, and 10% (v/v) FCS instead of PVA, which had been previously covered with paraffin oil in a culture dish. Ten oocytes were placed into 50 μl of the same medium and kept in a CO_2 incubator (5% CO_2 in air at 39°C) for about 30 min until spermatozoa were added for fertilization.

Frozen ejaculated spermatozoa (three pellets with a 200-μl volume and containing about 4×10^6 spermatozoa each) were thawed in 2 ml fertilization medium without caffeine at 37°C for 1 min for each insemination. The medium had been previously equilibrated in an atmosphere of 5% CO_2 in air for about 3 h. After thawing, 6-8 ml of the same medium was added and then spermatozoa were washed three times by centrifugation, each time at 550 **g** for 5 min. After washing, the sperm pellet was resuspended to give a concentration of 1×10^7 spermatozoa/ml. A 50-μl

aliquot of the sperm suspension was introduced into 50 μl of fertilization medium containing the oocytes, thus producing a mixture with the final concentrations of 5 × 10^6 spermatozoa/ml and 5 mM caffeine. After insemination, the dishes were put into an incubator (5% CO_2 in air at 39°C) until examination.

5. Assessment for sperm penetration and oocyte nuclear maturation

At various time after maturation or fourteen hours after insemination, oocytes were mounted, fixed for 72-96 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined under a phase-contrast microscope at magnifications of 200× and 400×. Oocytes were considered penetrated when they had one or more morphologically changed sperm nuclei and the corresponding sperm tails. When polyspermic oocytes contained sperm nuclei at different stages of transformation, they were classified according to the most prevalent stage.

The stages were classified as germinal vesicle (GV), prometaphase-I (Pro M-I), metaphase-I (M-I), anaphase-I (A-I), telophase-I (T-I) and metaphase-II (M-II).

6. Assay of glutathione

At various times after maturation, concentration of GSH in oocytes was assayed. Oocytes were freed from cumulus and corona radiata cells by agitation using a narrow bore glass pipette in PBS with 1 mg/ml PVA (PBS-PVA) and washed 3 times with the same medium. Subsequently, a group of 30 oocytes in 10 μl of PBS-PVA were transferred to a 1.5-ml polypropylene microfuge tubes using a micropipette and frozen (-20°C). The frozen samples were thawed at room temperature, and 5 μl of 1.25 M H_3PO_4 was added. The samples were refrozen for storage at -20°C until used for the assay of GSH. Blanks containing 5 μl of PBS-PVA without oocytes were similarly treated.

Glutathione was determined by the enzymatic cycling assay of Tietze (1969).

The samples were thawed, diluted with 1.2 ml of H_2O, and transferred to glass tubes. The following solutions were subsequently added to the tubes: 5 μl of 1.25 M H_3PO_4, 1.2 ml of 0.2 M potassium phosphate in 10 mM EDTA buffer (pH 7.2), 100 μl of 10 μM 5,5-dithiobis-(2-nitrobenzonic acid) (DTNB; Sigma), 50 μl of 20 units/ml glutathione reductase (Type III; Sigma), and 50 μl of 4 μM NADPH (Kohjin Co., Tokyo, Japan). After the addition of NADPH, the absorbance was immediately monitored at 412 nm with a spectrophotometer (Hitachi, Tokyo, Japan) and was recorded at 0.5 and 5 min after the addition of NADPH. Both the reagent blank and GSH (Sigma) standards (0.1-1.0 nmol) were also assayed under the same conditions. The total amount of GSH measured was divided by the number of oocytes in the sample to obtain the total GSH content/oocyte. To calculate the mean cell volume, a total of 100 oocytes was photographed using by microscope (Nikon, Tokyo, Japan) and the diameter of the ooplasm was measured. The mean diameter of the ooplasm in pig oocyte was 118.6±0.7 μm, and it was calculated by the equation $V = 4\pi r^3/3$ that the mean cell volume of pig oocyte was 873.3 pL. Then, the GSH concentration/oocyte was estimated by determining total glutathione content/oocytes and mean cell volume as described by Calvin *et al.* (1986).

Glutathione measured in the present study contains both reduced (GSH) and oxidized (GSSG) forms. However, since the high ratio (about 100:1) of GSH to GSSG is found intracellularly (Meister, 1983) and GSSG is suggested to be reduced immediately or rapidly in most cellular systems (Meister and Anderson, 1989) and mammalian oocytes (Perreault, 1990), glutathione we concerned in the present study is mostly reduced form.

7. Statistical analysis

The percentages of experimental data were subjected to an arc-sine transformation, and the transformed values were analyzed using for one-way or two-way analysis of variance (ANOVA). Since the raw GSH concentrations were

normally distributed, the data were analyzed using ANOVA without transformation. When ANOVA revealed a significant treatment effect, the treatments were compared by Duncan's multiple range test.

CHAPTER 2

EFFECTS OF CYSTEINE IN SERUM-FREE MATURATION MEDIUM ON MALE PRONUCLEAR FORMATION OF MATURING PIG OOCYTES PENETRATED *IN VITRO*

As described in the Introduction, faulty cytoplasmic maturation of oocytes *in vitro*, affecting MPN formation after sperm penetration, has been one of major problems for *in vitro* production of pig embryos. However, the problem has recently been partly broken through by culturing pig oocytes in maturation medium supplemented with cysteine. Cysteine could increase the synthesis of GSH in pig oocytes. The increased GSH level is believed to be prerequisite for sperm nuclear decondensation, and consequently to induce MPN formation in mouse (Calvin *et al.*, 1986), hamster (Perreault *et al.*, 1988) and pig (Yoshida, 1993) oocytes.

In contrast to oocytes cultured without cysteine, GSH concentration in pig oocytes continued to increase until 36 h of culture in medium with cysteine (Yoshida *et al.*, 1993b). According to Wang *et al.* (1994), immature pig oocytes culturing in medium without cysteine are penetrable by spermatozoa *in vitro* and have high ability to support sperm nuclear decondensation. In this case, however, the ability of penetrated oocytes to support MPN formation was very poor. It remains to be determined whether high level of GSH in immature pig oocytes could directly promote MPN formation from decondensed sperm nuclei.

The present study was designed to examine this possibility in immature pig oocytes cultured in medium with cysteine. It has been reported that the presence of FCS in maturation medium is detrimental to MPN formation of pig oocytes (Naito *et al.*, 1998; Funahashi and Day, 1993c) and that pig oocytes have been successfully cultured in serum-free media (Funahashi and Day, 1993c; Wang and Niwa, 1995).

Therefore, the present experiments were conducted using a serum-free medium which was supplemented with polyvinyl alcohol (PVA) rather than FCS.

Materials and Methods

The medium used for the oocyte maturation was mTCM-199B with or without 0.57 mM cysteine (Sigma). After culture for various periods, oocytes were inseminated in fertilization medium in the presence or absence of 0.57 mM cysteine. Some oocytes were assayed for GSH.

The morphological changes of sperm nuclei were classified into decondensed chromatin, recondensed chromatin, metaphase chromosomes and MPN according to the description by Abeydeera and Niwa (1992) for immature cattle oocytes penetrated *in vitro*. When polyspermic oocytes contained sperm nuclei at different stages of transformation, they were classified according to the most prevalent stage.

In Experiment 1, immature pig oocytes were cultured in maturation medium with or without cysteine. At 12, 24, 36 and 48 h after culture, nuclear maturation and GSH concentration of oocytes were examined. Some oocytes cultured for 24, 36 and 48 h were fertilized in fertilization medium without cysteine and examined after 14 h of insemination for sperm penetration, activation and MPN formation of oocytes.

In Experiment 2, effects of the presence of cysteine during *in vitro* fertilization on activation and MPN formation of penetrated oocytes were examined. Oocytes cultured for 24 h in maturation medium with cysteine were further cultured for 14 h with spermatozoa in fertilization medium with or without cysteine.

In Experiment 3, to examine whether activation and MPN formation of oocytes are promoted by prolonged incubation of penetrated oocytes, those cultured for 24 h in maturation medium with cysteine and for 14 h with spermatozoa in fertilization medium with or without cysteine were further cultured for 24 h in hormones-free maturation medium without cysteine.

Results

Experiment 1

Oocytes were difficult to mature to M-II within 12 and 24 h of culture (Table 1). The proportions of M-II oocytes were increased with prolonged culture periods and reached 88-90% at 48 h of culture. No differences were detected in the proportions of M-II oocytes at each time of culture between the presence and absence of cysteine. When oocytes were inseminated *in vitro*, high proportions (74-93%) of the oocytes were penetrated irrespective of the culture period and the presence of cysteine (Table 2). Although higher ($P < 0.05$) proportions of oocytes were activated forming female pronucleus after penetration when they were inseminated 36 and 48 h than 24 h after maturation culture both in the presence and absence of cysteine, no differences were observed in MPN formation among the three different insemination times. The proportions of activated oocytes with MPN were higher ($P < 0.01$) in the presence (71-85%) than absence (22-36%) of cysteine at any times of insemination.

The concentration of GSH in oocytes before culture was 6.7 ± 0.4 mM (Fig. 1). This value was not different with values ($5.3 \pm 0.8 \sim 5.9 \pm 0.5$ mM) obtained in oocytes cultured for 12, 24 or 36 h in the absence of cysteine. At each time of culture except 12 h, the concentrations of GSH were increased ($P < 0.05$) in the presence ($10.4 \pm 0.9 \sim 11.9 \pm 0.7$ mM) than in the absence of cysteine. However, there was no difference in GSH levels in oocytes cultured in the presence of cysteine among different culture periods.

Experiment 2

High proportions (80-85%) of oocytes were penetrated regardless of the presence of cysteine during *in vitro* fertilization (Table 3). However, the proportion of oocytes activated forming female pronucleus after penetration was very low (7%) both in the presence and absence of cysteine during fertilization. The MPN formation was observed in all the activated oocytes.

Table 1. Progress of meiotic maturation of cumulus enclosed pig oocytes cultured in maturation medium with or without 0.57 mM cysteine*

Time of examination (h of culture)	Presence (+) or absence (-) of cysteine	Number of oocytes examined	Number and (%) of oocytes at the stage of†				
			GV	Pro M-I	M-I	A-I~T-I	M-II
0		57	57(100)	0(0)	0(0)	0(0)	0(0)
12	+	57	57(100)	0(0)	0(0)	0(0)	0(0)
	-	60	59(98)	1(2)	0(0)	0(0)	0(0)
24	+	61	14(23)	11(18)	36(59)	0(0)	0(0)
	-	55	11(20)	8(15)	35(64)	1(2)	0(0)
36	+	61	3(5)	2(3)	15(25)	6(10)	35(57)
	-	52	5(10)	2(4)	11(21)	2(8)	32(62)
48	+	57	2(4)	1(2)	2(4)	2(4)	50(88)
	-	62	1(2)	1(2)	3(5)	1(2)	56(90)

*Experiments were repeated four times at each time of examination.

†GV: germinal vesicle; Pro M-I: prometaphase I; M-I: metaphase I; A-I: anaphase I; T-I: telophase I; M-II: metaphase II.

Table 2. Effect of cysteine during maturation on activation and male pronuclear formation of pig oocytes penetrated *in vitro**

Time of insemination (h after culture)	Presence (+) or absence (-) of cysteine during culture	Number of oocytes inseminated	Number of oocytes penetrated						
				At various stages† with decondensed sperm chromarinatin			With female pronucleus		
			Total (%)	GV (%)#	Pro M-I ~ T-I (%)#	M-II (%)#	Total (%)#	With decondensed sperm chromatin (%)‡	With male pronuclei (%)‡
24	+	85	63 (74)	2 (3)	42 (67)	5 (8)	14 (22)[a]	4 (29)[a]	10 (71)[a]
	-	51	41 (80)	1 (2)	30 (73)	1 (2)	9 (22)[a]	7 (78)[b]	2 (22)[b]
36	+	66	58 (88)	1 (2)	0 (0)	0 (0)	57 (98)[b]	9 (16)[a]	48 (84)[a]
	-	67	53 (79)	0 (0)	6 (11)	4 (8)	43 (83)[c]	33 (77)[b]	10 (23)[b]
48	+	69	61 (88)	0 (0)	0 (0)	0 (0)	61 (100)[b]	9 (15)[a]	52 (85)[a]
	-	71	66 (93)	0 (0)	1 (2)	1 (2)	64 (97)[b]	41 (64)[b]	23 (36)[b]

*Oocytes were examined 14 h after insemination; experiments were replicated four times.

†For abbreviation, see Table1.

#Percentages of the number of oocytes penetrated.

‡Percentages of the total number of oocytes penetrated with female pronucleus.

[abc]Values with different superscripts within each column differ significantly, $P < 0.05$.

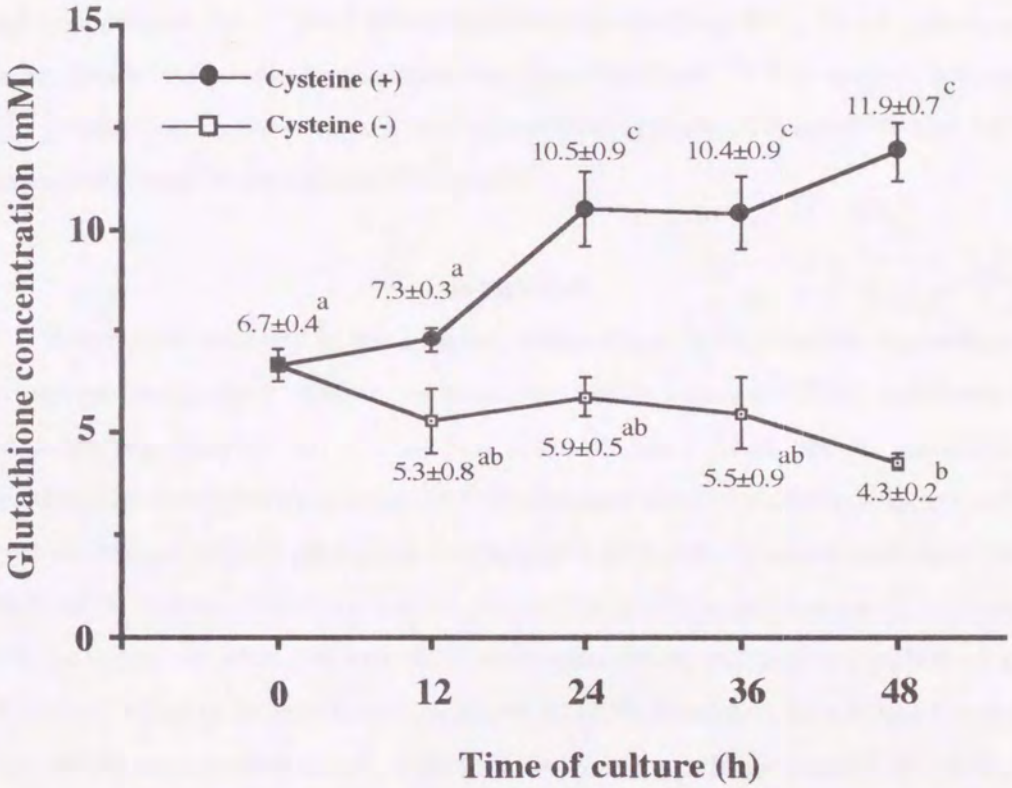

Fig. 1. Glutathione concentration (mean ± SEM) of pig oocytes cultured for various duration in maturation medium supplemented with (+) or without (-) 0.57 mM cysteine. The glutathione concentrations were determined by examining 90 oocytes in each group. Values with different superscripts means differ significantly ($P<0.05$).

Experiment 3

When culture of oocytes after insemination was prolonged, 45 (92%) and 44 (90%) of each 49 oocytes penetrated in the presence and absence of cysteine, respectively, reached M-II or were activated forming female pronucleus (Table 4). In high proportions (63-77%) of penetrated oocytes reaching M-II, transformation of sperm nuclei to metaphase chromosomes was observed. Of the oocytes activated after penetration in the presence and absence of cysteine, 87 and 61% had MPN, respectively, with no significant difference.

Discussion

The results obtained in this chapter indicate that 1) the addition of cysteine to serum-free maturation medium is associated with increased GSH synthesis by immature pig oocytes but it dose not promote nor inhibit oocyte maturation, penetrability of oocytes by spermatozoa *in vitro*, and decondensation of sperm nuclei in penetrated oocytes; 2) premature oocytes (24 h after maturation culture) have a less ability to be activated forming female pronucleus after sperm penetration compared with maturing oocytes (36 and 48 h after maturation culture) regardless of the addition of cysteine to maturation medium; 3) MPN formation in activated oocytes after sperm penetration at any stages of maturation is largely accelerated when cysteine was added to maturation medium; and 4) the lower ability of premature oocytes to be activated is not improved by neither the addition of cysteine in fertilization medium nor prolonged culture period after insemination which induces transformation of sperm nuclei to metaphase chromosomes.

The proportion of pig oocytes reaching the M-II stage *in vivo* markedly increases 36 h after hCG injection (Hunter and Polge, 1966). When pig oocytes at the GV stage are cultured in media containing FCS, a majority of oocytes can also mature to M-II by 36 h after the start of culture (Yoshida *et al.*, 1989; Wang *et al.*, 1994). It seems therefore that inclusion of FCS in medium produces an appropriate

Table 3. Effect of cysteine during *in vitro* fertilization (IVF) on activation and male pronuclear formation of pig oocytes*

Presence (+) or absence (-) of cysteine during IVF	Number of oocytes inseminated	Number of oocytes penetrated						
			At various stages† with decondensed sperm chromatin			With female pronucleus		
		Total (%)	GV (%)#	Pro M-I ~ T-I (%)#	M-II (%)#	Total (%)#	With decondensed sperm chromatin (%)‡	With male pronuclei (%)‡
+	51	41 (80)	0 (0)	35 (85)	3 (7)	3 (7)	0 (0)	3 (100)
-	54	46 (85)	0 (0)	40 (87)	3 (7)	3 (7)	0 (0)	3 (100)

*Oocytes cultured for 24 h in maturation medium with cysteine were inseminated and examined 14 h after insemination. Experiment were replicated five times.

†For abbreviation, see Table. 1.

#Percentages of the number of oocytes penetrated.

‡Percentages of the total number of oocytes penetrated with female pronucleus.

Table 4. Effect of cysteine during *in vitro* fertilization (IVF) on activation and male pronuclear formation of pig oocytes cultured for a longer period after insemination*

Presence (+) or absence (-) of cysteine during IVF	Number oocytes inseminated	Number of oocytes penetrated									
			At metaphase - II				With female pronucleus				
		Total (%)	Total (%)#	With decondensed sperm chromatin (%)†	With recondensed sperm chromatin (%)†	With sperm metaphase chromosomes (%)†	Total (%)#	With decondensed sperm chromatin (%)†	With recondensed sperm chromatin (%)†	With sperm metaphase chromosomes (%)†	With male pronuclei (%)†
+	55	49(89)	30(61)	10(33)	1(3)	19(63)	15(31)	1(7)	1(7)	0(0)	13(87)
-	55	49(89)	26(53)	6(23)	0(0)	20(77)	18(37)	5(28)	1(6)	1(6)	11(61)

*Oocytes cultured for 24 h in the presence of cysteine were inseminated. After culturing with spermatozoa for 14 h, they were additionally cultured for 24 h in maturation medium without hormones. Experiments were replicated four times.

#Percentages of the total number of oocytes penetrated.

†Percentages of total number of oocytes at metaphase - II or with female pronucleus.

condition at least regarding the time required for pig oocyte maturation *in vitro*. Although, in the present study using serum-free medium, nuclear maturation of pig oocytes did not complete until 48 h after the start of culture, high proportions of premature oocytes were penetrated by spermatozoa *in vitro* as reported previously in oocytes maturing in FCS containing medium (Wang *et al.*, 1994). Therefore, excluding of FCS, which contains many undefined factors and is reported to be one of the detrimental factors for cytoplasmic maturation of pig oocytes (Naito *et al.*, 1988; Funahashi and Day, 1993c), from maturation medium makes it possible to examine the direct effects of cysteine on MPN formation in penetrated oocytes.

The first step in the induction of sperm nuclear decondensation is a reduction of sperm protamine disulfide bonds (Perreault *et al.*, 1984; Zirkin *et al.*, 1985). Since GSH seems to be associated with a reduction of sperm protamine disulfide bonds (Perreault, 1990), a high level of oocyte GSH may be required for decondensation of penetrating sperm nuclei. In the present study, the concentration of GSH in pig oocytes were maintained at relatively low levels (4.3-6.7 mM) during maturation when they were cultured for 48 h in medium without cysteine. However, when oocytes were inseminated *in vitro*, high proportions of oocytes were penetrated and sperm nuclear decondensation or MPN formation was observed in all penetrated oocytes irrespective of the culture periods. It is reported that immature hamster oocytes at the GV stage contain about 4 mM GSH (Perreault *et al.*, 1988) but do not support decondensation of fertilizing or micro-injected sperm nuclei (Usui and Yanagimachi, 1976; Perreault *et al.*, 1984). Since, in pig oocytes, sperm nuclear decondensation can easily be induced even at the GV stage (Wang *et al.*, 1994) or at premature stages before reaching M-II (present study), pig spermatozoa may be very sensitive to GSH for reducing sufficient disulfide bonds. High incidence of nuclear decondensation in immature oocytes is also reported in bull spermatozoa (Niwa *et al.*, 1991). These results indicate that protamine removal from boar and bull spermatozoa may occur in immature oocytes. Indeed, there are evidences indicated

that, even in mature oocytes, their activation is not required for decondensation of sperm nuclei in the ooplasm (Uehara and Yanagimachi, 1977; Borsuk and Manaka, 1988; Wright and Longo, 1988; Schatten *et al.*, 1989).

In the present study, when premature oocytes 24 h after maturation culture were inseminated *in vitro*, the proportions (7-22%) of activated oocytes after sperm penetration was very low. This is apparently due to the low proportions of oocytes reaching M-II during fertilization. Fertilization medium containing caffeine used in the present study may not be suitable for maturation of pig oocytes. However, although prolonged culture of oocytes in maturation medium after sperm penetration made it possible to reach M-II in most penetrated oocytes, the incidence of activation was still low (31-37%). In contrast to the cattle in which immature oocytes reached M-I can complete maturation and easily activate after sperm penetration (Chian *et al.*, 1992), it is possible that premature pig oocytes have less ability to be activated by penetrated sperm nuclei compared with mature oocytes even after maturation has been completed. It is reported in the mice (Clarke and Masui, 1986; 1987), humans (Schmiady *et al.*, 1986; Schmiady and Kentenich, 1989; Tesarik and Kopecny, 1989), and cattle (Abeydeera and Niwa, 1992) that the lower ability of activation in penetrated immature oocytes induces transformation of sperm nuclei to recondensation forming a small mass of chromatin and then chromosome like structures. The same phenomena were also observed in the present study; the formation of the metaphase chromosomes was observed in 63-77% of premature pig oocytes which reached M-II after penetration by prolonged culture after insemination.

Oocyte activation is required for transformation of decondensed sperm nucleus into a male pronucleus (Yanagimachi, 1994). The MPN formation was observed only in the activated pig oocytes also in the present study. However, when cysteine was added to maturation medium, MPN formation in activated oocytes after sperm penetration at the premature stages was greatly promoted. This result indicates that an

increased concentration of GSH in maturing pig oocytes could induce full decondensation of sperm nuclei removing sperm protamine and replacing it with somatic histones, but that, for MPN formation, intimate synchronization with oocyte activation is essential.

In conclusion, these results indicate that the synthesis of GSH by maturing pig oocytes greatly increased when they were cultured in serum-free maturation medium containing cysteine and the increased level of GSH may induce full decondensation of sperm nuclei in penetrated oocytes ensuring transformation of the decondensed sperm nuclei to male pronuclei when the oocytes were activated.

Summary

Pig immature oocytes were cultured for 12, 24, 36 and 48 h in serum-free maturation medium with or without 0.57 mM cysteine. The addition of cysteine to the medium was associated with increased GSH synthesis by oocytes but did not promote nor inhibit nuclear maturation, sperm penetration *in vitro*, and decondensation of sperm nuclei in penetrated oocytes. The incidence of activation of penetrated oocytes 14 h after insemination *in vitro* was lower in those cultured both in the presence and absence of cysteine for 24 h than 36 and 48 h. The lower ability of oocytes cultured for 24 h to be activated was not improved by neither the addition of cysteine (0.57 mM) in fertilization medium nor prolonged culture time after insemination. However, MPN formation in activated oocytes after sperm penetration at any stages of maturation was largely accelerated when cysteine was added to maturation medium. An increased concentration of GSH may induce full decondensation of sperm nuclei in immature pig oocytes penetrated *in vitro* ensuring transformation of the decondensed sperm nuclei to male pronuclei only in synchronization with oocyte activation.

CHAPTER 3

STAGE-SPECIFIC REQUIREMENT OF CYSTEINE DURING *IN VITRO* MATURATION OF PIG OOCYTES FOR GLUTATHIONE SYNTHESIS ASSOCIATED WITH MALE PRONUCLEAR FORMATION

It has been believed that the reduction of sperm nuclear disulfide bonds is required to induce sperm nuclear decondensation as the first step of MPN formation (Perreault *et al.*, 1984; Zirkin *et al.*, 1985; Perreault, 1990), and that GSH, the major intracellular free thiol, is an important factor for the reduction (Griffith and Meister, 1979; Perreault *et al.*, 1984; Calvin *et al.*, 1986). The GSH concentration of mature oocytes is higher than that of immature oocytes in the hamster (Perreault *et al.*, 1984). In pig oocytes, a relatively high concentration of cysteine during *in vitro* maturation appears to be required to increase oocyte GSH concentration at the end of culture for maturation, and consequently to promote MPN formation following *in vitro* fertilization (IVF; Yoshida *et al.*, 1992b, 1993b; Yoshida, 1993; Chapter 2). However, it is unknown whether cysteine is required throughout or at the specific stage of meiotic maturation. By using a specific inhibitor of GSH synthesis, buthionine sulfoximine, it has been demonstrated that GSH synthesis occurred throughout maturation of hamster (Perreault *et al.*, 1988) and pig oocytes (Yoshida, 1993) and that the synthesis during initial and mid phase of maturation may be related to the acquisition of sperm nuclear decondensing ability of pig oocytes (Yoshida, 1993). When a specific inhibitor was used, however, inhibition of GSH synthesis does occur in oocytes only after the stage of maturation at which the inhibitor was added. This makes it unreliable to determine the specific stage of oocyte maturation at which GSH synthesis regulates directly MPN formation. The

study in this chapter was designed to clarify this point in pig oocytes by adding or removing cysteine at various times after the start of culture for maturation.

Materials and Methods

The basic methods for the manipulation of oocytes were exactly the same as described in Chapters 1 and 2 except that cysteine was not added in fertilization medium.

In Experiment 1, to determine the meiotic stage at which cysteine affects on oocyte maturation, cumulus-enclosed oocytes were cultured in maturation medium supplemented with and without 0.57 mM cysteine and transferred to newly prepared medium without and with cysteine, respectively, at various times after the start of culture. At the end of culture for a total period of 48 h, cumulus-enclosed oocytes were inseminated *in vitro* to examine the ability to form male pronuclei. Oocytes for assessment of GSH concentration were prepared from cumulus-enclosed oocytes which were cultured separately from those used for IVF. Two groups of oocytes were used in separate experiments each for removing and adding cysteine at different times after the start of culture.

In Experiment 2, on the results obtained in experiment 1, to clarify the precise timing and duration of cysteine requirement for maturation of oocytes, maturation medium was supplemented with 0.57 mM cysteine for only last 0, 3, 6, 9, and 12 h of culture (Experiment 2a) or for only 3 and 6 h from 42 or 45 h after the start of culture (Experiment 2b). Glutathione concentration and the ability to form male pronuclei were determined as described above.

RESULTS

Experiment 1

Very high and stable penetration rates (84-95%) were obtained in any times of adding or removing cysteine (Table 5). There were no differences in the incidence of

MPN formation among different times of adding cysteine (85-92%). When cysteine was removed at 12 and 24 h of culture, the incidence of MPN formation (31-39%) was not different with the value in negative control (36%) in which cysteine was not added throughout culture for 48 h. The value increased ($P < 0.01$) when cysteine was present until 36 h of culture (75%) and further increased ($P < 0.05$) when it was present throughout culture for 48 h (90%).

There were no differences in oocyte GSH concentration among different times both of adding (10.2 ± 0.4 to 11.9 ± 1.3 mM; Fig. 2) and removing (4.0± 0.6 to 7.2 ± 1.5 mM; Fig. 3) cysteine until 36 h of culture. The former values were higher ($P < 0.01$) than the value (3.7 ± 0.4 mM) in oocytes cultured for 48 h without cysteine. In contrast, the latter values were lower ($P < 0.05$) than the value (11.5 ± 1.6 mM) in oocytes cultured for 48 h with cysteine.

Experiment 2

As shown in Table 6, when maturation medium was supplemented with cysteine 36-42 h after the start of culture, the incidence (86-90%) of MPN formation was higher ($P < 0.05$) than the values when cumulus-enclosed oocytes were exposed to cysteine for only last 3 h of culture (60%) or not exposed (45%).

As shown in Table 7, the highest incidence (92%) of MPN formation was obtained when oocytes were exposed to cysteine for 6 h from 42 h after the start of culture ($P < 0.01$). The GSH concentrations (8.9 ± 0.4 - 11.4 ± 1.5 mM) of oocytes cultured with cysteine from 36-42 h of culture were higher ($P < 0.05$) than the values (3.4 ± 0.4 - 3.8 ± 0.4 mM) of oocytes exposed to cysteine for only 3 h from 42-45 h after the start of culture or not exposed (Fig. 4).

DISCUSSION

Maturation medium used in the present study basically contains 0.0008 mM cysteine. However, since cysteine is rapidly metabolized (Meister, 1983) and it is

Table 5. Effect of cysteine (0.57 mM) added to or removed from medium changed at various times after the start of culture on male pronuclear formation of pig oocytes after *in vitro* fertilization*

Time of changing medium (h of culture)	Adding (+) or removing (-) cysteine§	Number of oocytes inseminated	Number of oocytes penetrated			
			Total (%)‡	With swollen sperm head at various stages†		With male and female pronuclei (%)#
				M-II (%)#	FP (%)#	
0	+	59	52(88)	2(4)	2(4)[a]	47(90)[a]
	-	50	45(90)	0(0)	29(64)[b]	16(36)[b]
12	+	56	52(93)	2(4)	2(4)[a]	48(92)[a]
	-	60	57(95)	0(0)	35(61)[b]	22(39)[b]
24	+	56	47(84)	1(2)	5(11)[a]	41(87)[a]
	-	67	59(88)	0(0)	41(69)[b]	18(31)[b]
36	+	62	58(94)	4(7)	4(7)[a]	50(85)[ac]
	-	63	56(89)	6(11)	7(13)[b]	42(75)[c]

*Experiments were replicated four times.

†M-II: metaphase II, FP: female pronucleus.

§The oocytes cultured in maturation medium without (with) additional cysteine were transferred to newly prepared medium with (without) cysteine at each time after the start of culture. The oocytes were cultured for a total period of 48 h.

‡Percentage of the number of oocytes inseminated.

#Percentage of the total number of oocytes penetrated.

[abc]Values with different superscripts within each column differ significantly, $P < 0.05$.

Table 6. Effect of cysteine added to medium on and after 36 h of culture for maturation on pronuclear formation of pig oocytes after *in vitro* fertilization*

Time of adding cysteine (h of culture)	Number of oocytes inseminated	Number of oocytes penetrated			
		Total (%)‡	With swollen sperm head at various stages†		With male and female pronuclei (%)#
			M-II (%)#	FP (%)#	
36	63	59(94)	1(2)	4(7)[a]	53(90)[a]
39	61	58(95)	4(7)	4(7)[a]	50(86)[a]
42	57	53(93)	0(0)	6(11)[a]	47(89)[a]
45	57	53(93)	1(2)	19(36)[b]	32(60)[b]
48	60	56(93)	1(2)	27(48)[b]	25(45)[c]

* Experiments were replicated four times.

† For abbreviation, see Table 5.

‡ Percentage of the number of oocytes inseminated.

\# Percentage of the total number of oocytes penetrated.

[abc] Values with different superscripts within each column differ significantly, $P < 0.05$.

indeed reported that such a low concentration of cysteine does not stimulate both GSH synthesis (Yoshida *et al.*, 1993b) and MPN formation (Yoshida *et al.*, 1992b) of pig oocytes, the original content of cysteine may be ignored when the results are discussed.

The results of the present study indicate that, although GSH is synthesized throughout maturation of pig oocytes in medium with cysteine (0.57 mM) as already reported by Yoshida *et al.* (1993b), the presence of cysteine in medium for 6 h of the last phase of maturation, when oocytes reached the late M-I to M-II stages (Chapter 2), is essential for oocyte GSH synthesis enough to induce MPN formation with the same efficiency as in oocytes matured in the presence of cysteine

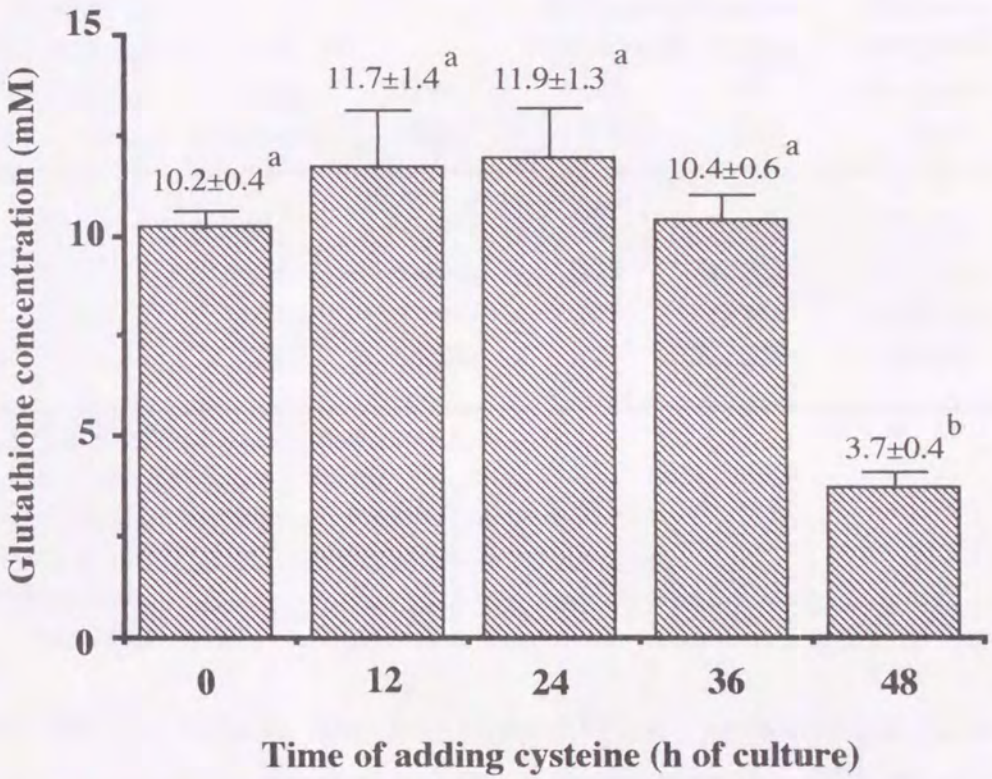

Fig. 2. Glutathione concentration (mean ± SEM) of pig oocytes transferred from maturation medium without cysteine to that supplemented with 0.57 mM cysteine at various time of culture and then continued to culture until 48 h after the start of culture. The glutathione concentration were determined by examining 90 oocytes in each group. Values with different superscripts differ significantly ($P<0.01$).

Table 7. Effect of exposure duration of cumulus enclosed pig oocyte to cysteine added to medium on and after 42 h of culture for maturation on pronuclear formation of oocytes after *in vitro* fertilization*

Duration of the presence of cysteine (h of culture)	Number of oocytes inseminated	Number of oocytes penetrated			
		Total (%)‡	With swollen sperm head at various stages† M-II (%)#	FP (%)#	With male and female pronuclei (%)#
42-48	68	62(91)	1(2)	3(5)[a]	57(92)[a]
42-45	65	61(94)	1(2)	16(26)[b]	43(70)[b]
45-48	61	57(93)	4(7)	15(26)[b]	37(65)[b]
-	63	54(86)	1(2)	36(67)[c]	15(28)[c]

* Experiments were replicated four times.

† For abbreviation, see Table 5.

‡ Percentage of the number of oocytes inseminated.

Percentage of the total number of oocytes penetrated.

[abc] Values with different superscripts within each column differ significantly, $P < 0.01$.

from the start of culture. It has been reported by many workers that an increased GSH level at the end of maturation plays an important role for MPN formation of oocytes after sperm penetration (Griffith and Meister, 1979; Perreault *et al.*, 1984; Calvin *et al.*, 1986; Perreault *et al.*, 1988; Yoshida, 1993; Yoshida *et al.*, 1993b; Funahashi *et al.*, 1994b). It appears, however, that the integration of GSH during the whole culture period is not necessary to keep the final level of concentration. The importance of GSH synthesis at the last phase of maturation for inducing MPN formation was also demonstrated from the results of Experiment 1, which indicated that, when cysteine was removed from medium at 36 h of culture, both the incidence of MPN formation and oocyte GSH concentration were lower than when cysteine was present for 48 h of culture.

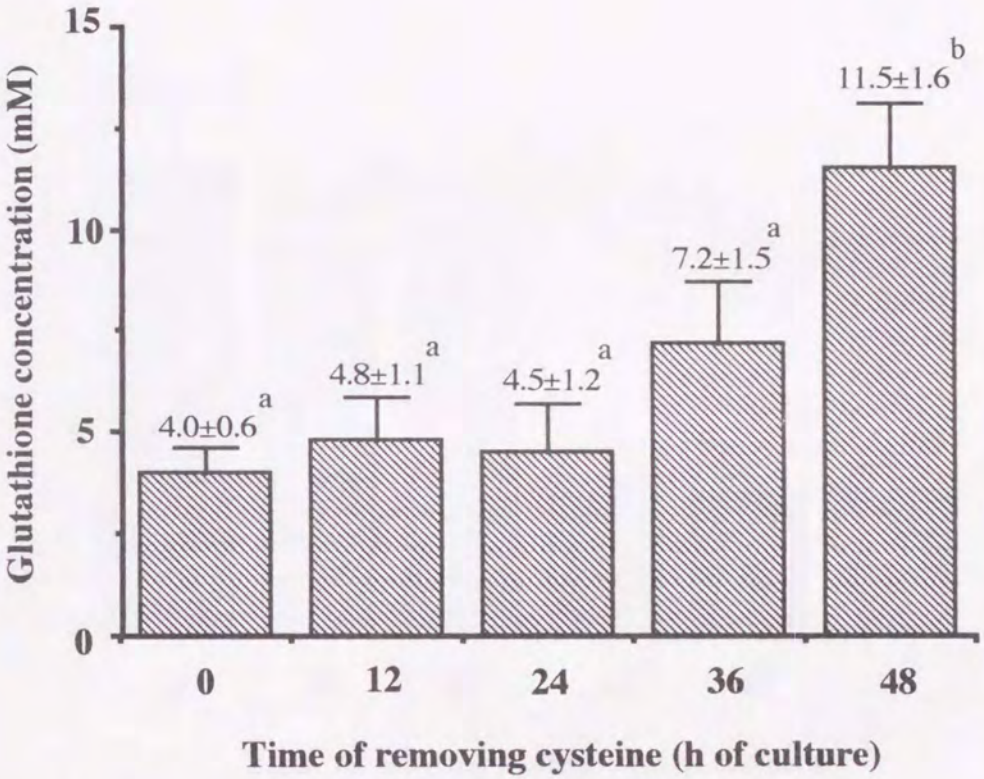

Fig. 3. Glutathione concentration (mean ± SEM) of pig oocytes transferred from maturation medium supplemented with 0.57 mM cysteine to that without cysteine at various time of culture and then continued to culture until 48 h after the start of culture. The glutathione concentration were determined by examining 90 oocytes in each group. Values with different superscripts differ significantly ($P<0.05$).

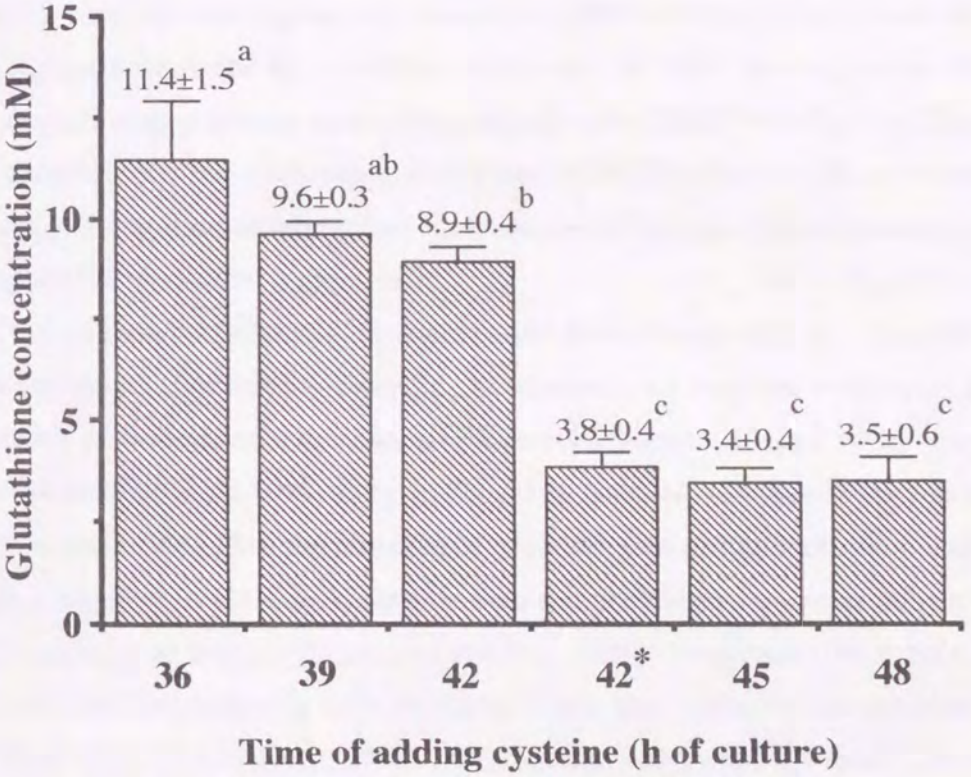

Fig. 4. Glutathione concentration (mean ± SEM) of pig oocytes cultured in the presence of 0.57 mM cysteine on and after 36 h of culture for a total period of 48 h. *Oocyte-cumulus complexes were cultured for only 3 h in the presence of 0.57 mM cysteine and then for another 3 h in the absence of cysteine. The glutathione concentrations were determined by examining 90 oocytes in each group. Values with different superscripts differ significantly ($P<0.05$).

However, as indicated in hamsters by Perreault *et al*. (1984), the absolute amount of GSH in pig oocytes is not likely to be the sole determinant of MPN formation. For example, when cysteine was present in medium for only 3 h of the last phase of maturation (42-45 h or 45-48 h of culture), the incidence (65-70%) of MPN formation was higher than the value (28%) when cysteine was absent throughout culture for 48 h, whereas there were no differences in oocyte GSH concentration among these three groups of treatment (Table 7 and Fig. 1 in Chapter 2). These results may indicate that cysteine itself also plays a role in reducing protamine disulfide bonds, which induces sperm nuclear decondensation and thereby MPN formation is promoted.

It is unknown whether GSH is synthesized directly in the oocyte or transferred into the oocyte after synthesization in cumulus cells via oocyte-cumulus junction. Cumulus cells are known to synthesize high concentration of GSH when hamster (Zuelke and Perreault, 1994) and pig (Funahashi and Day, 1995) cumulus-enclosed oocytes were cultured in the presence of gonadotropins or cysteine. Since there is high correlation in GSH concentration between oocytes and cumulus cells at the early stage of pig oocytes (Funahashi and Day, 1995), it is hypothesized that GSH may be transferred directly from cumulus cells to the oocyte via oocyte-cumulus junction (Day and Funahashi, 1996). In the present study, when cysteine was added to medium with an interval for 3 h on and after 36 h of culture, oocyte GSH concentration gradually reduced as the time of cysteine addition delayed. Since it has been demonstrated that the degree of intercellular coupling between the oocyte and cumulus cells reaches minimal level before oocyte maturation completed (Mattioli *et al*., 1988a), the lower levels of oocyte GSH concentration during the last stage of maturation may indicate that GSH synthesized in cumulus cells is transferred into the oocyte.

In conclusion, the presence of cysteine (0.57 mM) in serum-free medium during the last phase of oocyte maturation appears to be critical for synthesization of GSH

enough to MPN formation of pig oocytes.

Summary

The present study was designed to clarify the duration of maturation of pig oocytes at which cysteine promotes MPN formation through oocyte GSH synthesis. When cysteine was added to medium at 0, 12, 24, and 36 h of culture and cumulus-enclosed oocytes were cultured for a total of 48 h, both oocyte GSH concentration at the end of culture and the incidence of MPN formation after IVF were higher than the values in oocytes cultured for 48 h without cysteine. In contrast, the removal of cysteine from medium at every 12 h of culture resulted in increased incidence of MPN formation in the presence of cysteine until 36 and 48 h than for only 0-24 h of culture; oocyte GSH concentrations also changed similarly. When cysteine was added to medium at every 3 h from 36 h of culture, higher incidence of MPN formation were obtained in oocytes cultured in the presence of cysteine from 36-42 than 45 h of culture although GSH concentrations were higher in oocytes cultured with cysteine from 36 than 39-42 h of culture. These results suggest that the presence of cysteine in maturation medium only between 42 and 48 h of culture when pig oocytes reached to the late M-I to M-II stages can promote oocyte GSH synthesis and thereby MPN formation after IVF is promoted.

CHAPTER 4

EFFECTS OF CUMULUS CELLS ON GLUTATHIONE SYNTHESIS AND MALE PRONUCLEAR FORMATION OF PIG OOCYTES CULTURED IN MEDIUM WITH CYSTEINE OR CYSTINE

In Chapters 2 and 3, it was indicated that culture of cumulus-enclosed pig oocytes in the presence of a high concentration (0.57 mM) of cysteine in a maturation medium greatly increased oocyte GSH synthesis at the end of maturation culture and consequently improved MPN formation following sperm penetration. The GSH content of oocytes has been demonstrated to be highly correlated with that of cumulus cell mass in the hamster (Zuelke and Perreault, 1994) and pig (Funahashi and Day, 1995). However, since GSH synthesis in many kinds of cells are dependent on the availability of cysteine in the medium (Tateishi *et al.*, 1974; Meister and Tate, 1976), it is postulated that cumulus-free pig oocytes also may directly utilize cysteine for GSH synthesis. On the other hand, it is well known that cysteine in medium is oxidized under the usual culture conditions and form cystine (Toohey, 1975; Mohindru *et al.*, 1985). However, it has been demonstrated that mouse lymphocytes cannot utilize cystine for the synthesis of GSH (Ishii *et al.*, 1981). Therefore, there may be another possibility that cystine formed by oxidization of added cysteine is reduced to cysteine by cumulus cells and utilized by oocytes for GSH synthesis. The present studies were undertaken to clarify these possibilities by culturing cumulus-enclosed or cumulus-free pig oocytes in the medium supplemented with cysteine or cystine at various times after the start of culture for maturation.

Materials and Methods

The basic methods for the manipulation of oocytes were exactly the same as described in Chapter 1. Ten cumulus-enclosed oocytes were transferred to 100 μl of the maturation medium with or without 0.57 mM cysteine or 0.57 mM cystine (Sigma) and cultured for totally 48 h. After the culture, cumulus-enclosed oocytes (Experiment 1) or oocytes freed from cumulus cells by repeated passage through a fine pipette at various times after the start of culture or at the end of culture (Experiments 2 and 3) were washed twice with fertilization medium. Some oocytes were assayed for determination of GSH concentration as described in Chapter 1.

In Experiment 1, to examine the effects of cysteine or cystine present in medium throughout culture on GSH concentration of matured oocytes and MPN formation of penetrated oocytes *in vitro*, cumulus-enclosed oocytes were cultured for 48 h in maturation medium supplemented with 0.57 mM cysteine or 0.57 mM cystine or without both chemicals. Since, when cumulus-free pig oocytes were cultured, nuclear maturation is greatly inhibited in preliminary observation, only cumulus-enclosed oocytes were used in this experiment. Oocytes used for assessment of GSH concentration were cultured separately from those used for *in vitro* fertilization.

In Experiment 2, to examine the effects of cysteine or cystine associated with cumulus cells during the latter half of culture for oocyte maturation on GSH concentration of matured oocytes and MPN formation of penetrated oocytes *in vitro*, cumulus-enclosed oocytes were first cultured for 24 h in medium without cysteine and cystine and some oocytes were freed from cumulus cells. Cumulus-enclosed and cumulus-free oocytes were transferred to newly prepared medium supplemented with 0.57 mM cysteine or 0.57 mM cystine or without both chemicals and continued to culture for a further 24 h. At the end of culture (a total period for 48 h), cumulus-enclosed oocytes were denuded, and the denuded oocytes were fertilized *in vitro*. Oocytes used for assessment of GSH concentration were cultured separately from those used for *in vitro* fertilization.

In Experiment 3, since it has been shown that the presence of cysteine in maturation medium between 36 and 48 h of culture can promote oocyte GSH synthesis and thereby MPN formation after *in vitro* fertilization in cumulus-enclosed pig oocytes in Chapter 3, the effects of the presence of cysteine or cystine associated with cumulus cells during this period on MPN formation of oocytes were examined. Experimental procedures were completely the same as in experiment 2 except that the time of treatment of oocytes or changing medium was 36 h instead of 24 h after the start of culture and that oocyte GSH concentration was not determined.

Results

Experiment 1

As shown in Fig. 5, GSH concentration was higher ($P < 0.01$) in oocytes matured in the presence of cysteine (7.6 ± 0.5 mM) or cystine (8.2 ± 0.5 mM), without difference between these values, compared with value (3.0 ± 0.8 mM) in oocytes without these compounds. As shown in Table 8, very high proportions (95 to 98%) of oocytes were penetrated *in vitro* regardless of the presence of cysteine or cystine in maturation medium. However, the incidence of MPN formation were increased ($P < 0.01$) in oocytes matured in the presence of cysteine (86%) or cystine (81%) as compared with value (38%) in oocytes matured without these compounds (38%). There was no difference in MPN formation between groups added cysteine and cystine.

Experiment 2

When cysteine was added to maturation medium 24 h after the start of culture, oocyte GSH concentration (8.2 ± 0.6 to 8.4 ± 1.0 mM, Fig. 6) and the incidence of MPN formation (79 to 90%, Table 9) were higher ($P < 0.05$) compared with the comparable values (3.5 ± 0.7 to 3.6 ± 1.0 mM and 22 to 25%, respectively) when cysteine and cystine were not added to medium, regardless of denuding oocytes at

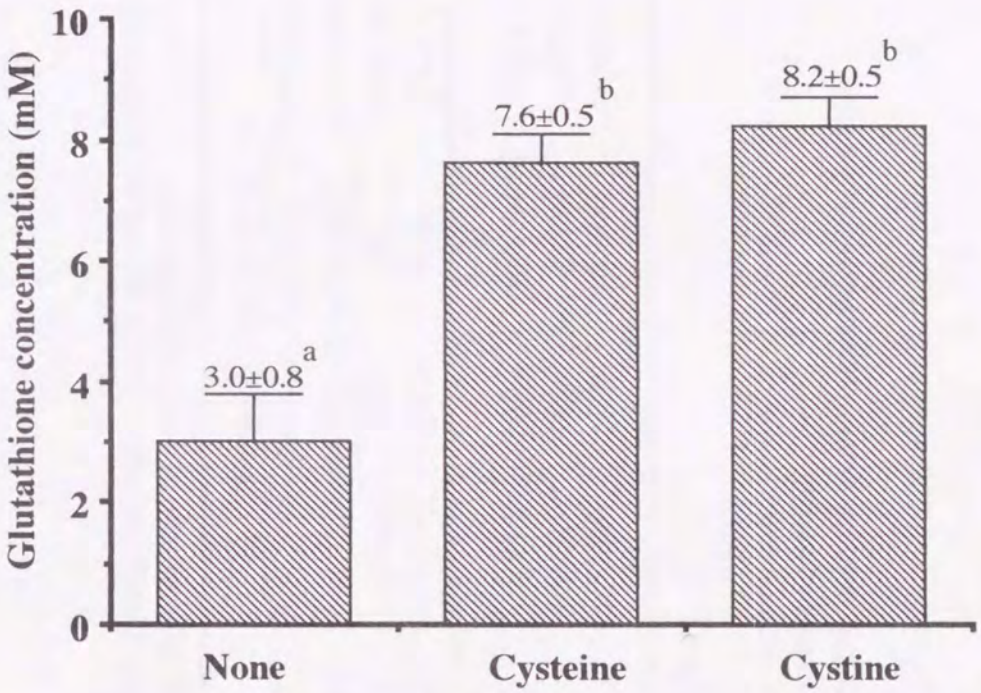

Fig. 5. Glutathione concentration of pig oocytes after cumulus-enclosed oocytes were cultured in maturation medium with 0.57 mM cysteine or 0.57 mM cystine for 48 h for meiotic maturation. The results represent the mean ± SEM of a group of 30 oocytes in each experiment. Separated experiments were replicated 4 times. Values with different superscripts differ ($P < 0.01$).

Table 8. Effect of cysteine and cystine during maturation on male pronuclear formation of cumulus-enclosed pig oocytes penetrated by spermatozoa *in vitro**

Supplements	Number of oocytes inseminated	Number of oocytes penetrated			
		Total (%)‡	With swollen sperm head at various stages†		With male and female pronuclei (%)#
			M-II (%)#	FP (%)#	
—	58	55(95)	0(0)	34(62)[a]	21(38)[a]
Cysteine (0.57 mM)	62	59(95)	1(2)	7(12)[b]	51(86)[b]
Cystine (0.57 mM)	58	57(98)	1(2)	10(18)[b]	46(81)[b]

*Experiments were replicated four times.

†For abbreviation, see Table 5.

‡Percentage of the number of oocytes inseminated.

#Percentage of the total number of oocytes penetrated.

[ab]Values with different superscripts within each column differ significantly, $P < 0.01$.

the time of the addition. In contrast, when cystine was added to maturation medium and cumulus cells were freed from oocytes, GSH concentration (7.2 ± 0.5 mM) and the incidence of MPN formation (90%) were comparable to the values in oocytes matured with cysteine but when cumulus cells were freed from oocytes, the values (2.4 ± 1.0 mM and 16%, respectively) greatly reduced ($P < 0.05$) to the same levels as in oocytes matured in cysteine- and cystine-free medium.

Experiment 3

When cysteine was added to maturation medium 36 h after the start of culture, there was no difference in the incidence of MPN formation between cumulus-enclosed (92%) and cumulus-free oocytes (79%), but the values were much higher ($P < 0.01$) than those when cystine was added (14 to 29%) or when both compounds were not added (6 to 20%) to medium, regardless of denuding oocytes at 36 h of culture (Table 10).

Discussion

Maturation medium used in the present studies basically contains 0.0008 mM cysteine and 0.06 mM cystine. However, it has been reported that such a low concentration of cysteine and cystine does not stimulate both GSH synthesis and MPN formation of pig oocytes (Wang *et al.*, 1991; Yoshida *et al.*, 1992b; Funahashi and Day, 1993c; Funahashi *et al.*, 1994a). Therefore, the original content of cysteine and cystine may be ignored when the results obtained in the present studies are discussed. Furthermore, in the preliminary experiments, removing cumulus cells from cumulus-enclosed pig oocytes at 0 or 12 h after the start of culture drastically decreased the incidence of meiotic maturation to the M-II stage at 48 h of culture, whereas removing them on and after 24 h of culture did not do so. In the present study, therefore, cumulus cells were removed only on and after 24 h of culture in the experiments in which cumulus-free oocytes were used.

Table 9. Effect of cysteine and cystine associated with cumulus cells during 24 to 48 h of maturation culture on male pronuclear formation of pig oocytes penetrated by spermatozoa *in vitro**

Culture condition			Number of oocytes inseminated	Number of oocytes penetrated			
					With swollen sperm head at various stages†		With male and female pronuclei
Cysteine (0.57 mM)	Cystine (0.57 mM)	Cumulus cells		Total (%)‡	M-II (%)#	FP (%)#	
+	-	+	53	42(79)	2(5)	2(5)[a]	38(90)[a]
+	-	-	53	47(89)	1(2)	9(19)[a]	37(79)[a]
-	+	+	54	51(94)	1(2)	3(6)[a]	46(90)[a]
-	+	-	56	55(98)	1(2)	45(82)[b]	9(16)[b]
-	-	+	55	49(89)	5(10)	33(67)[b]	11(22)[b]
-	-	-	52	48(92)	0(0)	35(73)[b]	12(25)[b]

*Cumulus-enclosed oocytes were cultured for first 24 h in medium without cysteine and cystine. Experiments were replicated four times.

†For abbreviation, see Table 5.

‡Percentage of the number of oocytes inseminated.

#Percentage of the total number of oocytes penetrated.

[ab]Values with different superscripts within each column differ significantly, $P<0.05$ at least.

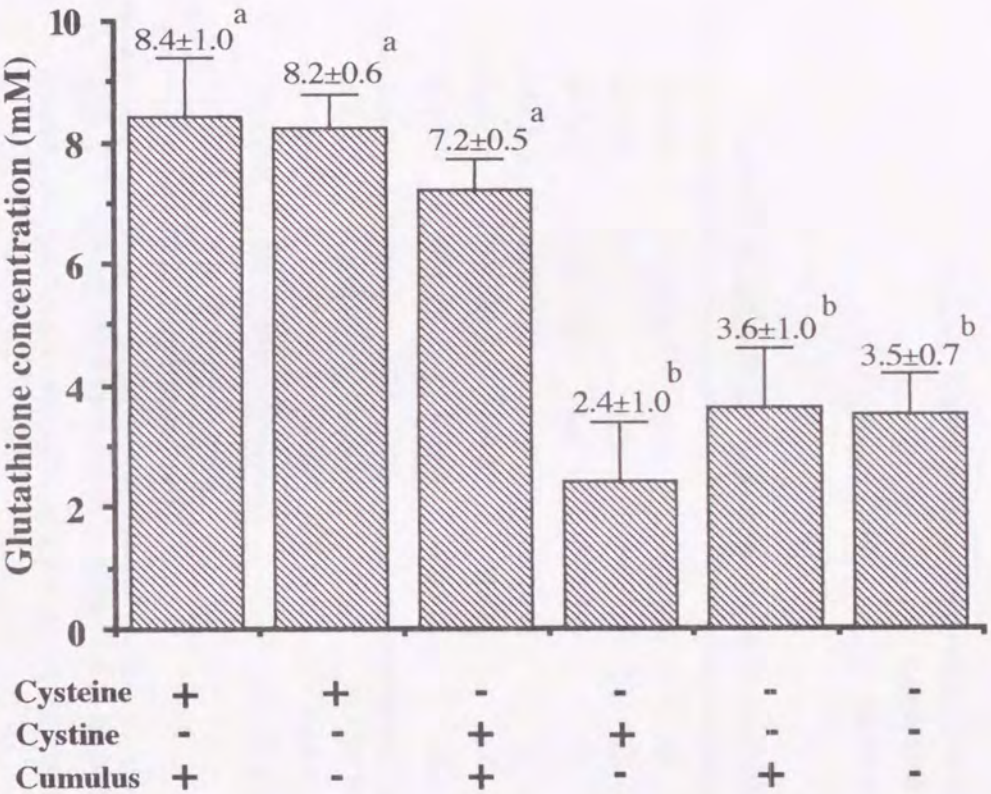

Fig. 6. Effect of cysteine and cystine associated with cumulus cells during 24 to 48 h of maturation culture on glutathione synthesis of pig oocytes. After cumulus-enclosed oocytes were cultured in maturation medium without both cysteine and cystine for 24 h and some oocytes were freed from cumulus cells. Then, cumulus-enclosed and cumulus-free oocytes were cultured in maturation medium with 0.57 mM cysteine or 0.57 mM cystine or without both compounds for a further 24 h. The results represent the mean ± SEM for 4 groups of 30 oocytes collected from separate experiments. Values with different superscripts are different ($P < 0.01$).

Table 10. Effect of cysteine and cystine associated with cumulus cells during 36 to 48 h of maturation culture on male pronuclear formation of pig oocytes penetrated by spermatozoa *in vitro**

Culture condition			Number of oocytes inseminated	Number of oocytes penetrated			
					With swollen sperm head at various stages†		With male and female pronuclei
Cysteine (0.57 mM)	Cystine (0.57 mM)	Cumulus cells		Total (%)‡	M-II (%)#	FP (%)#	(%)#
+	-	+	57	50(88)	1(2)	3(6)[a]	46(92)[a]
+	-	-	59	52(88)	3(6)	8(15)[a]	41(79)[a]
-	+	+	61	51(84)	4(8)	32(63)[b]	15(29)[b]
-	+	-	56	49(88)	1(2)	40(82)[bc]	7(14)[bc]
-	-	+	52	44(85)	1(2)	34(77)[bc]	9(20)[bc]
-	-	-	56	49(88)	2(4)	44(90)[c]	3(6)[c]

*Cumulus-enclosed oocytes were cultured for first 36 h in medium without cysteine and cystine. Experiments were replicated four times.

†For abbreviation, see Table 5.

‡Percentage of the number of oocytes inseminated.

#Percentage of the total number of oocytes penetrated.

[abc]Values with different superscripts within each column differ significantly, $P<0.01$.

Cysteine is an essential amino acid for many types of mammalian cells in culture (Ishii *et al.*, 1981). Yoshida *et al.* (1993b) reported that oocyte GSH concentration and the incidence of MPN formation increased when pig oocytes were matured in medium supplemented with cysteine. The results in the present study confirmed these stimulating effects of cysteine on oocyte GSH synthesis and MPN formation. In the present study, further, intracellular GSH concentration of matured oocytes and the incidence of MPN formation after *in vitro* fertilization were very high even when cumulus-enclosed oocytes were denuded at 24 h of culture and exposed to cysteine to the end of culture for maturation. Although about 90% of cysteine when it exists in medium appears to be oxidized to cystine within a few hours under the usual culture conditions (Mohindru et al., 1985; Ishii et al., 1981), the present results demonstrated that oocyte GSH concentration and the incidence of MPN formation were lower when cumulus-enclosed oocytes were denuded at 24 h of culture and exposed to cystine instead of cysteine. These evidences indicate that pig oocytes can directly utilize, without cumulus cells, cysteine to synthesize GSH and consequently increase the ability to form a male pronucleus. Furthermore, the incidence of MPN formation was increased even when cumulus-enclosed oocytes were denuded at 36 h of culture and exposed to cysteine in the present study. In Chapter 3, oocyte GSH concentration and the incidence of MPN formation were greatly reduced to a basal level when cysteine was removed at 36 h of culture of cumulus-enclosed pig oocytes. Therefore, direct utilization of cysteine by oocytes on and after 36 h of culture appears to be critical for GSH synthesis associated with MPN formation following *in vitro* fertilization.

It has been reported that maturation medium with cysteine at a concentration as low as 0.08 mM stimulates glutathione synthesis and MPN formation in cumulus-enclosed pig oocytes (Yoshida *et al.*, 1993b). If cysteine is oxidized to cystine in medium within a few hours, it would be hypothesized that cystine in medium might also be used for GSH synthesis and MPN formation of pig oocytes. The results of

the present study demonstrated that the presence of a high concentration of cystine in maturation medium from 24 h of culture was effective in promoting the ability of cumulus-enclosed oocytes to synthesize GSH and MPN formation, but that these effects were not observed when cumulus-enclosed oocytes were freed from cumulus cells. It has been reported that promoted growth of mouse lymphoma L1210 cells in co-culture with feeder cells is due to cysteine which is continuously provided by feeder cells and that the ability to form cysteine in medium by feeder cells is due to about 15 times greater capacity of feeder cells to take up cystine into the cells compared with L1210 cells (Ishii *et al.*, 1981). Therefore, it may be postulated that cumulus cells have greater capacity to take up cystine compared with oocytes and thus GSH synthesis and MPN formation are promoted in cumulus-enclosed pig oocytes in the presence of cystine. However, the stimulating effects of cystine were not observed when cumulus-enclosed oocytes were exposed to cystine from 36 h of culture. This may be due to decreased ability of cumulus cells to take up cystine and to form cysteine at the time of culture. The promotion of cystine uptake to chinese hamster ovary cells appears to be induced by extracellular thiols (Meier and Issels, 1995). Exposure of cumulus-enclosed oocytes to cystine from 36 h of culture in Experiment 3 might be too short to form an enough concentration of cysteine and/or other thiols by cumulus cells. Alternatively, loosened intercellular coupling between the oocyte and cumulus cells may be associated with failure in utilization of cystine to synthesize oocyte GSH because the coupling is known to reach minimal level before completion of oocyte maturation (Mattioli *et al.*, 1988a). Further investigations are required to clarify these hypotheses.

In conclusion, the results of the present study indicated that cumulus cells have an important role for pig oocytes to utilize cystine for glutathione synthesis associated with MPN formation after *in vitro* fertilization; whereas, cysteine is directly incorporated into pig oocytes *in vitro* and utilized for GSH synthesis.

Summary

The present study examined the effect of cysteine and cystine associated with cumulus cells in maturation medium on oocyte GSH concentration at the end of maturation culture (for 48 h) and MPN formation following *in vitro* fertilization of pig oocytes. When cumulus-enclosed oocytes were cultured in a serum-free medium added 0.57 mM cysteine or cystine, GSH concentration and incidence of MPN formation were higher than when they were cultured without cysteine and cystine. Supplementation with cysteine from 24 of culture resulted in a higher incidence of MPN formation and an increased GSH concentration of oocytes regardless of removing cumulus cells at 24 h of culture than in those cultured without cysteine and cystine. The same high incidence of MPN formation was observed even when oocytes were denuded at 36 h of culture and exposed to cysteine. In contrast, when oocytes were denuded at 24 h of culture and exposed to cystine, both GSH synthesis and MPN formation were not improved, but improved when oocytes were not denuded. Exposure of cumulus-enclosed oocytes to cystine from 36 h of culture did not promote MPN formation. These results indicate that cumulus-free pig oocytes can synthesize glutathione in the presence of cysteine but that the presence of cumulus cells is essential for oocyte GSH synthesis in the presence of cystine.

CONCLUSION

Although the pig oocytes matured *in vitro* can be penetrated *in vitro* by spermatozoa under appropriate conditions, incomplete oocyte maturation results in low rates of male pronuclear (MPN) formation. The incidence of MPN formation is positively associated with oocyte glutathione (GSH) content at the end of maturation, and oocyte GSH content increased during oocyte maturation when a high concentration of cysteine was added to the maturation medium. The present study was carried out to clarify the effects of cysteine and cystine, an oxidized form of cysteine, associated with the presence of cumulus cells on GSH synthesis and MPN formation of pig oocytes maturing *in vitro*.

The first series of experiments was designed to clarify the effects of cysteine in maturation medium on MPN formation of maturing pig oocytes penetrated *in vitro*. Cumulus-enclosed pig immature oocytes were cultured for 12, 24, 36 and 48 h in serum-free maturation medium with or without 0.57 mM cysteine. The addition of cysteine to the medium was associated with increased GSH synthesis by oocytes but did not promote nor inhibit nuclear maturation, sperm penetration *in vitro*, and decondensation of sperm nuclei in penetrated oocytes. The incidence of activation of penetrated oocytes 14 h after insemination *in vitro* was lower in those cultured both in the presence and absence of cysteine for 24 h (22%) than 36 (83-98%) and 48 h (97-100%). The lower ability of oocytes cultured for 24 h to be activated was not improved by neither the addition of cysteine (0.57 mM) in fertilization medium nor prolonged culture time after insemination. However, MPN formation in activated oocytes after sperm penetration at any stages of maturation was largely accelerated when cysteine was added to maturation medium.

The second series of experiments was designed to clarify the duration of maturation of pig oocytes at which cysteine promotes MPN formation through

oocyte GSH synthesis. When cysteine was added to medium at 0, 12, 24, and 36 h of culture and oocyte-cumulus complexes were cultured for a total of 48 h, both oocyte GSH concentrations (10.4-11.7 mM) at the end of culture and the incidence (85-92%) of MPN formation after *in vitro* fertilization were higher than the values (3.7 mM and 36%, respectively) in oocytes cultured for 48 h without cysteine. In contrast, the removal of cysteine from medium at every 12 h of culture resulted in increased incidence of MPN formation in the presence of cysteine until 36 and 48 h (75-90%) than for only 0-24 h (31-39%) of culture; oocyte GSH concentrations also changed similarly. When cysteine was added to medium at every 3 h from 36 h of culture, higher incidence of MPN formation were obtained in oocytes cultured in the presence of cysteine from 36-42 (86-90%) than 45 h (60%) of culture although GSH concentrations were higher in oocytes cultured with cysteine from 36 (11.4 mM) than 39-42 h (8.9-9.6 mM) of culture.

The third series experiments examined the effect of cysteine and cystine associated with cumulus cells in maturation medium on oocyte GSH concentration at the end of maturation culture (for 48 h) and MPN formation following *in vitro* fertilization of pig oocytes. When cumulus-enclosed oocytes were cultured in a serum-free medium added 0.57 mM cysteine or cystine, GSH concentration (7.6-8.2 mM) and incidence (81-86%) of MPN formation were higher than when they were cultured without cysteine and cystine (3.0 mM and 38%, respectively). Supplementation with cysteine from 24 of culture resulted in a higher incidence (79-90%) of MPN formation and an increased GSH concentration (8.2-8.4 mM) of oocytes regardless of removing cumulus cells at 24 h of culture than in those cultured without cysteine and cystine (22-25% and 3.5-3.6 mM). The same high incidence (79-92%) of MPN formation was observed even when oocytes were denuded at 36 h of culture and exposed to cysteine. In contrast, when oocytes were denuded at 24 h of culture and exposed to cystine, both GSH synthesis (2.4 mM) and MPN formation (16%) were not improved, but improved when oocytes were not denuded

(7.2 mM and 90%). Exposure of cumulus-enclosed oocytes to cystine from 36 h of culture did not promote MPN formation (29%).

Taken together, these results indicate that 1) An increased concentration of GSH may induce full decondensation of sperm nuclei in cumulus-enclosed pig immature oocytes penetrated *in vitro* ensuring transformation of the decondensed sperm nuclei to male pronuclei only in synchronization with oocyte activation, 2) the presence of cysteine in maturation medium only between 42 and 48 h of culture when cumulus-enclosed pig oocytes reached to the late M-I to M-II stages can promote oocyte GSH synthesis and thereby MPN formation after *in vitro* fertilization is promoted, and 3) cumulus-free pig oocytes can synthesize GSH in the presence of cysteine but that the presence of cumulus cells is essential for oocyte GSH synthesis in the presence of cystine.

It seems that these findings are useful not only for elucidation of the mechanism(s) of MPN formation in mammalian oocytes but also for practical production of pig embryos *in vitro*.

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ABSTRACT IN JAPANESE

ブタ体外受精卵子の雄性前核形成に関する研究

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家畜における初期胚の体外生産技術の確立は、優良な遺伝形質を持つ個体を効率的に増産する手段として極めて重要である。しかし、ブタでは体外成熟卵子を体外受精した場合、多精子侵入が高率に起こり、さらに卵子内に侵入した精子の雄性前核 (MPN) 形成率が低いため、正常な1-細胞期胚を体外で作出することは困難である。最近、高濃度 (0.57 mM) のシステインを添加した成熟培地でブタ卵子を培養した場合、受精後のMPN形成の促進されることが報告されたが、その作用機序の詳細は知られていない。そこで本研究では、MPN形成に対するシステインの作用機構を明かにする目的で、成熟過程におけるシステインと他の幾つかの要因がブタ体外受精卵子のMPN形成におよぼす影響について検討し、次のような新しい知見を得た。

I. 成熟途上ブタ卵子のMPN形成におよぼすシステインの影響

ブタでは卵子成熟の各段階で精子侵入の可能なことが報告されているが、このような未熟侵入卵子ではMPNはほとんど形成されない。そこで本実験では、システインの存在下で各成熟段階に到達した卵子のMPN形成能について検討した。ブタ卵子をシステイン (0.57 mM) 添加または無添加の修正TCM-199B (mTCM-199B) で0-48時間培養した。培養開始後12時間ではほとんど全ての卵子 (98-100%) が卵核胞期にとどまっていたが、その後、培養の継続にともなって核成熟は進行し、培養開始後48時間では88-90%の卵子が第二減数分裂中期に到達した。卵子内のグルタチオン (GSH) 濃度は、システインを添加した場合、培養後24時間の卵子 (10.5 mM) において培養開始前の卵子 (6.7 mM) よりも有意に高い値

を示し、その値は培養開始後48時間まで維持されたが、システイン無添加では、GSH濃度の増加はみられず、逆に培養開始後48時間でのGSH濃度 (4.3 mM) は有意に減少した。培養開始後24、36および48時間の卵子を体外受精 (IVF) した結果、培養開始後24時間で侵入した卵子の活性化率 (22%) は低かったが、活性化された卵子におけるMPN形成率はシステイン添加 (71%) において無添加 (22%) よりも高かった。一方、培養開始後36および48時間における侵入卵子の活性化率は83-100%と高く、MPN形成率はシステイン添加 (84-85%) において無添加 (23-36%) と比較して有意に高かった。システインを添加した成熟培地で24時間培養した卵子をシステイン添加または無添加の培地でIVFした結果、卵子の活性化およびMPN形成率に差はみられなかった。授精14時間後にこれらの卵子をシステイン無添加の成熟培地に移し24時間追加培養した結果、卵子の活性化は促進されなかったが、中期像染色体を形成した精子核が観察された。

II. システイン添加の時期および期間がブタ卵子のGSH合成およびMPN形成におよぼす影響

本実験では、MPN形成の重要な要因と考えられているGSH合成に影響をおよぼすシステイン添加の種々の条件について検討した。システイン (0.57 mM) を添加したmTCM-199Bで0-36時間培養した後、無添加培地に移し48時間後まで培養を継続した卵子のMPN形成率 (31-75%) およびGSH濃度 (4.0-7.2 mM) は、全培養期間中システインが存在していた場合 (それぞれ、90%および11.5 mM) と比較して有意に低かった。一方、培養開始後36時間に至るいずれの時間にシステインを添加しても、MPN形成率 (85-92%) およびGSH濃度 (10.2-11.9 mM) は高かった。システインの添加時期が培養開始後36-42時間の範囲では、添加時期の相違によるMPN形成率 (86-90%) に差はみられなかったが、培養開始後45時間でシステインを添加するとMPN形成率 (60%) は有意に低下した。培養開始後42および45時間から3時間のみシステインを添加した場合、MPN形成率 (65-70%) は、培養開始後42時間から培養終了までシステインが存在した場合 (92%) と比較して有意に低かったが、全培養期間無添加の場合 (28%) と比較して高かった。また、卵子のGSH濃度は、培養開始後

36、39および42時間から培養終了までシステインを添加することによって (8.9-11.4 mM)、42および45時間から3時間のみシステインを添加した場合 (3.4-3.8 mM) と比較して高くなった。

III. システィンあるいはシスチンの存在下で成熟したブタ卵子のGSH合成およびMPN形成におよぼす卵丘細胞の影響

本実験では、システインおよびシスチン (酸化型システイン) のブタ卵子による利用性と卵丘細胞との関連性について検討した。システイン (0.57 mM) もしくはシスチン (0.57 mM) を添加または無添加のmTCM-199Bで卵丘細胞付着卵子を48間培養した結果、IVF後のMPN形成率および卵子内のGSH濃度は、システイン (それぞれ、86%および7.6 mM) もしくはシスチン (それぞれ、81%および8.2 mM) を添加した場合、無添加 (それぞれ、38%および3.0 mM) と比較して有意に高かった。卵丘細胞付着卵子をシステインおよびシスチン無添加の培地で24時間培養後、一部の卵子から卵丘細胞を除去し、それらをシステインもしくはシスチン添加または無添加の培地に移し48時間まで培養した結果、システイン添加培地においては卵丘細胞の付着の有無にかかわらずMPN形成率 (79-90%) は高かった。シスチン添加培地では卵丘細胞付着卵子のMPN形成のみ促進され (90%)、卵丘細胞除去卵子のMPN形成率 (16%) は全培養期間システインおよびシスチン無添加の卵子におけるそれ (22%) と差がなかった。これらの卵子のGSH濃度はMPN成率に比例した。システインもしくはシスチンの添加および卵丘細胞の除去を培養開始36時間に行なった結果、MPN形成率は、システインを添加した場合においては卵丘細胞付着の有無にかかわらず高かったが (79-92%)、シスチン添加では卵丘細胞付着および除去卵子のいずれにおいても低かった (14-29%)。

これらの結果から、1)ブタ卵子内のGSH濃度が高くなることによって、侵入した精子核の十分な脱濃縮が誘起され、それによってMPN形成が促進されるが、これらの過程は卵子の活性化と密接に関連していること、2)卵子核成熟が第一成熟分裂中期から第二成熟分裂

中期に至る成熟過程の後半時期 (培養開始後42-48時間) にのみシステインが存在することによって、MPN形成に十分なGSHが合成されること、3)卵丘細胞除去卵子においてもシステインによるGSH合成は可能であるが、シスチンによるGSH合成には卵丘細胞の存在が必要である、ことが示唆された。