Effects of Glutamine, Glucosamine and Glutathione on the *In Vitro* Maturation of Porcine Oocytes

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ABSTRACT

This study was carried out to investigate the effects of the supplementation of glutamine, glucosamine and glutathione on the porcine oocytes on IVM rates. Cocs were incubated in NCSU-23 supplemented with at 2.0~10.0 mM glucosamine, 0.5~4.0 mM glutamine and 0.1~1.0 mM glutathione for 48 hrs. Oocytes were transferred to 50 ul drops of maturation medium covered with mineral oil and cultured in a CO_2 incubator (38℃, 5% CO_2, 95% air). The IVM rates of oocytes cultured in NCSU-23 supplemented with 0.5, 1.0, 2.0 and 4.0 mM glutamine for 48 hrs were 46.0± 4.5%, 52.0±4.8%, 50.0±4.2% and 44.0±4.5%, respectively. The IVM rates of oocytes cultured in NCSU-23 supplement with 2.0, 5.0, 7.0, 10.0 mM glucosamine for 48 hrs were 44.0±4.5%, 42.0±4.5%, 38.0±4.6% and 24.0±4.8%, respectively. The IVM rates of oocytes cultured in NCSU-23 supplemented with glucosamine were no significantly increased compare to the control (42.5±4.0%). The IVM rate of oocytes cultured in NCSU-23 supplemented with 3.0, 5.0, 7.0, 10.0 mM glutathione for 48 hrs were 40.0±3.2%, 54.0±4.2%, 48.0±4.5%, 44.0±4.8%, respectively. The IVM rate of oocytes cultured in NCSU-23 supplemented with glutamine and glutathione were significantly increased compared to those control (42.5±4.0%). Glucosamine did not affect the IVM rates of oocytes. IVM rates of oocytes cultured in NCSU-23 medium for 48 hrs were significantly increased compared to the cultured for 40 hrs.

(Key words : porcine oocytes, glucosamine, glutamine, glutathione, IVM rate)

INTRODUCTION

Despite of attempts to improve the meiotic resumption of nuclear and cytoplasm using various culture conditions, the efficiency of *in vitro* maturation (IVM) of porcine oocytes remains very low compared with that of other domestic animals.

The extent of glucose metabolism during maturation of the mammalian oocyte is known to influence several aspects of oocyte maturation and is related to oocyte developmental capacity. Glutamine also has important roles in bovine maturation, although the oocyte itself poorly utilizes glucose (Rieger and Loskutoff, 1994; Cetica *et al.*, 1999). Suzuki and Yoshioka (2006) report that the oocytes refreshing the medium and replacing polyvinyl alcohol with bovine serum albumin (BSA) on embryonic development were significantly increased by the addition of 2 mM glutamine to medium, as was blastocyst yields after supplementation with 0.25 to 4 mM glutamine (Sutton-McDowall *et al.*, 2004). Glucose is a major energy source for mammalian somatic cells, but the presence of glucose at the concentrations found in serum is detrimental for the embryos of several species during the early cleavage stages (Schini and Bavister, 1988; Larson *et al.*, 2001; Scott and Whittingham, 2002; Kwun *et al.*, 2003; Thompson *et al.*, 2007). Not only has a high concentration of glucose been found to be deleterious to bovine embryo development, it also selectively blocks development of female embryos during the morula to blastocyst transition (Larson *et al.*, 2001; Peippo *et al.*, 2001). However, Sutton-McDowall *et al.* (2006) reported the supplementation of glucosamine to bovine maturation medium unexpectedly impaired the developmental competence of bovine oocytes. Insufficient glucose during IVM leads to impaired completion of nuclear maturation (Hashimoto *et al.*, 2000), cumulus expansion and blastocyst development (Rose-Hellekant *et al.*, 1998), most likely due to insufficient substrates for hyaluronic acid synthesis. Although it is evident that glucose concentrations play a crucial role in oocyte maturation, the effects of culturing cumulus-oocytes complexes (COCs) in more physiological glucose concentrations have yet to be elucidated. Glutathione synthesis during IVM has an important role in embryo development. The increase in glutathione concentrations during IVM of cat-

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tle oocytes (Miyamura *et al.*, 1995) improved subsequent embryo development to blastocyst stage (de Matos *et al.*, 1995; de Matos *et al.*, 1996; Furnus *et al.*, 1998). Glutathione has an important role in cellular defense against hazardous agents of endogenous and exogenous origin (Meister and Anderson, 1983; Lafleur *et al.*, 1994). Glutathione is also important for chromatin decondensation and hence for male pronuclear formation, following sperm penetration (Perreault *et al.*, 1988; Yoshida *et al.*, 1992; Yoshida, 1993; Grupen *et al.*, 1995; Williams and Ford, 2005). Greater concentrations of intracellular glutathione enhance *in vitro* production of pig embryos (Whitaker and Knight, 2004) and IVM of buffalo oocytes (Gasparrini *et al.*, 2006). Investigating the effect of such an exposure on the developmental competence of oocytes could be an interesting next step in unravelling the pathways to subfertility.

In order to obtain insights into how to improve oocyte IVM in the porcine, the purpose of this study was to investigate the effects of glutamine, glucosamine and glutathione on IVM rates of porcine oocytes.

MATERIALS AND METHODS

1. Collection and Incubation of Oocytes

All chemicals, unless noted otherwise, were purchased from Sigma Chemical Company (St. Louis, USA). Porcine ovaries were collected at a local slaughterhouse and transported to the laboratory in sterile physiological saline containing 100 IU/ml penicillin G and 100 ug/ml streptomycin sulfate at 25~30℃. Oocytes were aspirated from medium size follicles with an 18 gauge fixed to a 10 ml disposable syringe. The COCs were cultured with NCSU-23 (North Carolina State University-23, U.S.A.) medium supplement with 10% (v/v) FCS (Gibco. U.S.A.), 1 μg/ml FSH, 10 IU/ml hCG and 1 μg/ml β-estradiol. Oocytes were transferred to 50 ul drops of maturation medium covered mineral oil and cultured at 38℃ in 5% CO_2, 95% air.

2. *In Vitro* Maturation of Oocytes

Then groups of each 50 COCs were cultured in 500 μl of NCSU-23 medium supplement with 2 IU/ ml hCG and 10% FCS which had previously been covered with mineral oil and equilibrated in a humidified atmosphere of 5% CO_2 and 95% air at 38℃. COCs were incubated in NCSU-23 medium supplement with at 0.5 1.0, 2.0 and 4.0 mM glutamine 2, 5, 7 and 10 mM glucosamine and 0.1, 0.3, 0.5 and 1.0 mM glutathione for 48 hrs.

3. Assessment of Meiotic Stage

Oocytes were fixed in acetic acid : ethanol (1 : 3) solution for 24 hs then stained with 1% acetoorcein or 10 μg/ml bisbenzimide (Hoechst 33342) and observed under an fluorescence microscope. The judgement of oocytes maturation *in vitro* was carried out depending on the criteria of maturation by cell and nuclear division.

4. Statistical Analysis

In each experimental group, oocytes were randomly distributed. Each experiment was repeated four times. The One-way ANOVA were used to determine the statistical significance of differences between values for the experimental and control groups. *P* values of 0.05 or less were considered as statistically significant.

RESULT AND DISCUSSION

1. Effect of Glutamine on IVM Rates

This experiment was carried out to investigate the effect of glutamine concentration on IVM rate of porcine oocytes was shown in Table 1. The IVM rate of oocytes cultured in NCSU-23 supplemented with 0.5, 1.0, 2.0 and 4.0 mM glutamine for 48 hrs were 46.0±4.5%, 52.0±4.8%, 50.0±4.2% and 44.0±4.5%, respectively. The IVM rates of oocytes cultured in NCSU- 23 supplemented with 1.0 and 2.0 mM glutamine were significantly higher than that of control (42.5±4.0%). These result was lower than that of Suzuki and Yoshioka (2006)'s report that the oocytes refreshing the medium and replacing PVA with BSA on embryonic development were significantly increased by

Table 1. IVM of oocytes after incubation for 48 hrs in glutamine containing media

Glutamine (mM)	No. of oocytes examined	No. of at the stage of			Rate of IVM (%)
		GV	GVBD	MⅡ	
Control	40	8	15	17	42.5±4.0[a]
0.5	50	9	18	23	46.0±4.5
1.0	50	8	16	26	52.0±4.8[b]
2.0	50	10	15	25	50.0±4.2[b]
4.0	50	15	13	22	44.0±4.5

[a,b] Values within column with different superscript differ ($p<0.05$).

the addition of 2 mM glutamine to PZM, as was blastocyst yields after supplementation with 0.25 to 4 mM glutamine. Also, Rieger and Loskutoff (1994) report that glutamine metabolism was unchanged from 0 to 12 hrs and then increased significantly at 18 hrs. Also, this suggest that oxidative metabolism increases, and is the major site of cellular energy production, during maturation of the cattle oocytes *in vitro*.

2. Effect of Glucosamine on IVM Rates

This experiment was carried out to investigate the effect of glucosamine concentration on IVM rate of porcine oocytes was shown in Table 2. The IVM rates of oocytes cultured in NCSU-23 supplemented with 2.0, 5.0, 7.0 and 10.0 mM glucosamine for 48 hrs were 44.0±4.5%, 42.0±4.5%, 38.0±4.6% and 24.0±4.8%, respectively. The IVM rates of oocytes cultured in NCSU-23 supplement with glucosamine were no significantly increased compare to the control (42.5±4.0%). The experimental animal was different but, the above result was similar to Sutton-McDowall *et al.*, (2005) reported that the GVBD rate of bovine oocytes cultured in glucosamine containing with TCM-199. Also, glucosamine supplementation has no effect on the nuclear maturation of bovine oocytes regardless of the glucosamine concentration. Despite the addition of glucosamine has no effect on oocytes nuclear maturation, the developmental capacity of oocytes to form blastocysts stage embryos was severely diminished in both bovine and porcine oocytes when matured in concentrations of 2.5 mM GlcN or more. This is manifested as a detrimental effect on developmental competence following early embryo development, because cleavage rates were comparable to those of oocytes matured without glucosamine in both species. Glucosamine is an alternative substrate to glucose for extracellular matrix synthesis during cumulus expansion (Salustri *et al.*, 1989; Chen *et al.*, 1990) and its supplementation during IVM reduces glucose consumption by bovine COCs (Sutton-McDowall, 2004).

3. Effect of Glutathione on IVM Rates

The effect of glutathione concentration on IVM rate of porcine oocytes was shown in Table 3. The IVM rate of oocytes cultured in NCSU-23 supplemented with 3.0, 5.0, 7.0 and 10.0 mM glutathione for 48 hrs were 40.0±3.2%, 54.0±4.2%, 48.0±4.5% and 44.0±4.8%, respectively. The IVM rates of oocytes cultured in NCSU-23 supplement with glutathione were significantly increased compare to the control (42.5±4.0%). The experimental animal was different but, these result was similar than that of Luciano *et al.*, (2006) reported that the glutathione concentration was significantly higher in MII than immature germinal vesicle stage equine oocytes. Glutathione are responsible for the limited early *in vitro* developmental capability of equine oocytes. Because of the metabolic and communication link between the cumulus and the oocyte, glucose availability and metabolism within the cumulus can have a significant impact on oocyte meiotic and developmental competence (Thompson *et al.*, 2007). IVM involves the removal of COCs from antral follicles and culturing them in standard cell culture conditions until they reach MⅡ, but a small proportion of these mature oocytes have full developmental potential to term (Schroeder and Eppig, 1984; Gilchrist and Thompson, 2007). Also, Funahashi *et al.* (1995) suggested that intracellular glutathione content of porcine oocytes at the end of IVM appears to re-

Table 2. IVM of oocytes after incubation for 48 hrs in glucosamine containing media

Glucosamine (mM)	No. of oocytes examined	No. of at the stage of			Rate of IVM (%)
		GV	GVBD	MⅡ	
Control	40	8	15	17	42.5±4.0[a]
2.0	50	10	18	22	44.0±4.5[b]
5.0	50	13	16	21	42.0±4.5
7.0	50	12	19	19	38.0±4.6
10.0	50	19	19	12	24.0±4.8

[a,b] Values within column with different superscript differ ($p<0.05$).

Table 3. IVM of oocytes after incubation for 48 hrs in glutathione containing media

Glutathione (mM)	No. of oocytes examined	No. of at the stage of			Rate of IVM (%)
		GV	GVBD	MⅡ	
Control	40	8	15	17	42.5±4.0[a]
3.0	50	13	17	20	40.0±3.2
5.0	50	5	18	27	54.0±4.2[b]
7.0	55	10	16	24	48.0±4.5[b]
10.0	50	14	14	22	44.0±4.8

[a,b] Values within column with different superscript differ ($p<0.05$).

Table 4. Effect of culture tine and medium with supplement on IVM rates

Medium with supplement (mM)	No. of oocytes examined	IVM rates during the culture time (hrs)	
		40	48
Control	40	40.5±5.0	42.5±4.0[a]
Glutamine	50	48.0±4.2	54.0±4.8[b]
Glucosamine	50	44.0±4.8	46.0±4.5
Glutathione	50	46.0±5.0	50.0±4.2[b]

[a,b] Values with different alphabets (a, b) within columns are significantly different ($p<0.05$).

Glutamine : 0.3 mM, glucosamine : 2.0 mM, glutachione 3 mM.

flect the degree of cytoplasmic maturation.

4. Culture Ttime and Medium with Supplements on IVM Rate

The effect of glutamine, glucosamine and glutathione following culture time on IVM rate of oocytes and the results was shown in Table 4. The IVM rates of oocytes cultured for 40 and 48 hrs in NCSU-23 medium supplemented with glutamine, glucosamine and glutathione were 48.0±4.2%, 54.0±4.8% and 44.0±4.8%, 46.0±4.5% and 46.0±5.0%, 50.0±4.2%, respectively. The IVM rates of oocytes in NCSU-23 medium supplemented with 0.3 mM glutamine and 3 mM glutathione for 48 hrs were increased compared to the control (40.5±5.0 *vs* 42.5±4.0%). These result was similar to Suzuki and Yoshioka (2006) and reported that the embryonic development were significantly increased by the addition of 2 mM glutamine to PZM although the experimental animal was different. Luciano *et al.* (2006) reported that the glutathione concentration was significantly higher in MⅡ than immature germinal vesicle stage equine oocytes. Also, glutathione are responsible for the limited early *in vitro* developmental capability of equine oocytes.

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