REPRODUCTION

Effects of follicle size and electrolytes and glucose in maturation medium on nuclear maturation and developmental competence of bovine oocytes

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Abstract

The concentrations of electrolytes (Na, K, Cl, Mg and Ca) and glucose in small follicle (SF) follicular fluid (SFF) and large follicle (LF) follicular fluid (LFF) from slaughterhouse-derived ovaries were studied. Oocytes were matured in medium based on synthetic oviductal fluid. The effects of various concentrations of electrolytes (Na, K, Ca and Mg) and glucose in the maturation medium on the progression of nuclear maturation and subsequent development were also studied. K in SFF was significantly greater than that in LFF. The Mg concentration in follicular fluid (FF) is 2.0–2.3 mM, which is greater than the concentration present in medium generally used for culture. The glucose concentration in FF is about 3.5–3.9 mM and rapidly decreases during the preservation of ovaries. LF oocytes resumed nuclear maturation and progressed to the M2 stage significantly faster than those collected from SF oocytes. In addition, more LF oocytes developed to blastocysts than did SF oocytes. Changing the Na/K ratio in the maturation medium from 16 to 24 did not affect either the progression of nuclear maturation or the rate of development. A low concentration of Mg (0.5 mM) combined with a low Ca concentration (0.5 mM) inhibited the rate of development, but did not affect the progression of nuclear maturation. On the other hand, increasing the Mg concentration to 2.0 mM from 0.5 mM hastened the progression of nuclear maturation and improved the rate of blastulation, irrespective of the Ca concentration. The progression of nuclear maturation was faster and the rate of development was greater with 5.56 mM glucose than with 1.5 mM glucose. The difference in time needed to progress to M2 among the experiment was about 2–3 h. Therefore, prolonging the maturation periods from 21 to 24 h did not change the rate of development. Our results show that the concentrations of Mg and glucose in the maturation medium and the follicle size enveloping the oocyte affect the progression of nuclear maturation and subsequent development. The time requirement for oocytes to reach M2 is strongly related to the developmental competence of the oocytes.

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Introduction

In vitro embryo development is known to be highly affected by oocyte quality. In the cow, growth of the oocyte is almost completed when its enveloping follicle reaches a diameter of 3 mm (Fair *et al.* 1995). Many studies have shown that oocytes collected from large diameter follicles (LFs) are more likely to develop to the blastocyst stage than oocytes collected from small diameter follicles (SFs) (Pavlok *et al.* 1992, Lonergan *et al.* 1994, Blondin & Sirard 1995, Yang *et al.* 1998, Hagemann 1999, Hagemann *et al.* 1999, Hendriksen *et al.*

2000) and these reports indicate that the prematuration of an oocyte which occurs during follicle growth is essential for subsequent development. Although some oocytes collected from SFs and LFs are already competent to develop *in vitro* into a blastocyst that can result in vital offspring after transfer, additional but essential development occurs *in vivo* during subsequent follicle growth, and this final maturation process (from luteinizing hormone surge to ovulation) strongly affects the developmental competence of oocytes (Blondin *et al.* 1997, Dieleman *et al.* 2002). These reports indicate that culture conditions that are

usually used for *in vitro* maturation (IVM) of bovine oocytes are suboptimal and that it is important to optimize the conditions for maturation to produce blastocysts more efficiently. The medium that is usually used for IVM is TCM-199 or synthetic oviductal fluid (SOF) (Takahashi & First 1992). Few studies have examined the effects of changing the basic elemental contents of the maturation medium.

The developmental rate and nuclear maturation are used as simple indexes of the ability of oocytes to undergo subsequent development. Dominko & First (1997) reported that the time it takes oocytes to resume meiosis and progress to M2 arrest is important for determining the subsequent developmental competence of oocytes. However, few reports have examined the relationship between the developmental competence of oocytes and the progression of nuclear maturation. In this study, the concentrations of Na, K, Cl, Mg, Ca and glucose in the follicular fluid (FF) from LFs (LFF) and the FF from SFs (SFF) were measured and then the effects of follicle size and electrolyte and glucose concentrations in the maturation medium on both the progression of nuclear maturation and the rate of development were studied.

Materials and Methods

IVM, in vitro *fertilization (IVF) and* in vitro *culture (IVC)*

Bovine ovaries were collected from carcasses at a slaughterhouse. They were preserved in physiological saline at approximately 35 °C and transported to the laboratory within 3 h. Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (3–6 mm in diameter) or large antral follicles (6–12 mm in diameter) using a 20-G needle connected to a 10 ml syringe. COCs surrounded by compact and thick cumulus cells were randomly selected and cultured in a 100 μl drop of maturation medium (15 oocytes/drop) under paraffin oil at 39 °C in an atmosphere of 5% CO_2 in air with maximum humidity. The media used for IVM, IVF and IVC were based on SOF supplemented with 5 mM taurine and 1 mM glutamine (Nacalai Tesque, Kyoto, Japan) and the medium was modified for each usage as described in Table 1. FF was collected from pooled small or large antral follicles from five pairs of ovaries that were stored for 1–2 h at 35 °C. After centrifugation, each FF was transferred to a test tube and stored at −35 °C until examination.

IVF

Frozen-thawed (37 °C) semen from a Japanese Black bull was washed with a 45–60% discontinuous Percoll (Pharmacia Co., Ltd, Uppsala, Sweden) gradient solution by centrifugation (500 *g*) for 10 min. After centrifugation, the semen was diluted to a concentration of 2×10^6 sperm/ml with IVF medium. Oocytes cultured for 21 h in maturation medium were washed with IVF medium and were

Table 1 Composition of control SOF used for IVM, IVF and IVC.

Ingredient	Source	IVM	IVF	IVC1*	IVC2**
Glucose (mM)	—	5.56	−	1	1.5
Pyruvic acid (mM)	a	0.5	0.5	0.5	0.5
Na lactate	a	−	+	+	+
NEAA	b	+	−	+	+
EAA	c	+	−	−	+
Fetal calf serum (%)	d	10	−	1	5
BSA(mg/ml)	e	−	7	−	−
Heparin (units/ml)	a	−	10	−	−

*In vitro culture medium from 18–48 h after fertilization.
**In vitro culture medium from 48–196 h after fertilization.
a, Sigma Chemical Co., St. Louis, MO, USA.
b, Non-essential amino acids (NEAA) basal Eagle's medium.
c, Essential amino acids (EAA) Eagle's minimal essential medium.
d, Gibco–BRL, Rockville, MD, USA.
e, Nacalai Chemicals co., Tokyo, Japan.

transferred to 100 μl drops of sperm suspension for fertilization (15 oocytes/drop), and then cultured for 18 h. Incubation was carried out at 39 °C in an atmosphere of 5% CO_2 in air with maximum humidity.

IVC

Eighteen hours after fertilization, oocytes were denuded by vortexing (30 s) and cultured in a 100 μl drop of IVC1 medium (Table 1) (15 zygotes/drop). Forty-eight hours after fertilization, the number of cleaved embryos (greater than four cells) was counted and these cleaved embryos were transported to a 100 μl drop of IVC2 medium. Forty-five oocytes were taken for experiment and each experiment was repeated four times and the rate of blastulation was assessed at days 7 and 8 after fertilization. Embryos were cultured at 39 °C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 with maximum humidity. To study nuclear maturation, about 60 oocytes were randomly selected for each experimental group. Oocytes that had been cultured for 5, 18 and 21 h in the IVM medium were denuded by treatment with 0.2% hyaluronidase for 6 min followed by vortexing for 6 min. The denuded oocytes were mounted on a slide, fixed with acetic acid/ethanol (1:3 v/v) for 24 h and stained with aceto-orcein before examination under a phase-contrast microscope (× 400).

Experimental design

In experiment 1, FF was thawed just before examination. The concentrations of Mg, Ca and glucose were measured with an automatic analyzer (Accute TBA-40 FR; Toshiba Co., Tokyo, Japan) and the concentration of Na, K and Cl were measured using a Spotchem-SE electrolyte analyzer (Arkray Inc., Kyoto, Japan).

In experiment 2, the effect of follicle size on the developmental competence and the nuclear maturation of oocytes was studied.

In experiment 3, the effects of Na and K concentrations in the maturation medium on developmental competence and nuclear maturation were studied. Based on the results

Table 2 Elemental contents in FF collected from SFs and LFs ($n = 7$).

Element	Concentration (mM)	
	SFs	LFs
Na	127 ± 9.6	128 ± 10.8
K	9.7 ± 1.5[a]	6.3 ± 0.8
Cl	100 ± 6.3	99.4 ± 6.1
Mg	2.3 ± 0.2	2 ± 0.5
Ca	1.9 ± 0.2	2.1 ± 0.1
Glucose	1.9 ± 0.2[a]	2.9 ± 0.3
Glucose*	3.5 ± 0.5	3.9 ± 0.4

[a]Different superscripts within the same row differ significantly ($P < 0.05$).
*FFs were collected just after ovary collection.

of experiment 1, Na and K concentrations were adjusted to 133 and 5.4 mM (Na/K: 24) in order to resemble the concentrations present in TCM-199, or 133 and 8.3 mM (Na/K: 15.9) in order to resemble the concentrations present in SOF.

In experiment 4, the effects of two different concentrations of Mg (0.5 and 2 mM) and two different concentrations of Ca (0.5 and 1.8 mM) in the maturation medium on the developmental competence and the nuclear maturation were examined in a 2×2 factorial design. Concentrations of Mg and Ca in the IVM medium were adjusted to 0.5 and 1.8 mM, 0.5 and 0.5 mM, 2 and 1.8 mM, and 2 and 0.5 mM.

In experiment 5, the effect of two glucose concentrations in the maturation medium (1.5 or 5.56 mM) on developmental competence and nuclear maturation were studied. Based on the glucose concentration measured in experiment 1, 1.5 and 5.56 mM were selected as the low and high concentrations.

In experiment 6, the maturation periods of the experimental groups in which the progression of nuclear maturation was slow were prolonged from 21 to 24 h. The culture medium was IVM medium containing 1.5 or 5.56 mM glucose. And then the effect of the maturation period on the developmental competence of the oocyte was studied.

Statistical analysis

To analyze the electrolytes and glucose concentrations in FF and the rate of blastulation, the frequency of dates were compared using Fisher's protected least significant difference test followed by ANOVA. All developmental data were subjected to an arcsine transformation before statistical analysis. To analyze the progression of nuclear maturation, data were compared by a chi-square test. P values less than 0.05 were considered to be significant.

Results

The concentrations of electrolytes and glucose in FF are shown in Table 2. The K concentration was significantly greater in SFF than in LFF. Glucose concentration in SFF (1.9 mM) was significantly less than that in LFF (2.9 mM), although when the FFs were collected just after slaughter, the concentrations were the same. The concentration of Mg in FF was 2–2.3 mM, which was greater than the concentrations in SOF (0.5 mM) or TCM-199 (0.8 mM).

In experiment 2, oocytes collected from LFs resumed nuclear maturation and progressed to the M2 stage significantly faster than SF oocytes (Table 3). However, at the end of the maturation period, the developmental rate of oocytes to the M2 stage was the same. In addition, more of the LF oocytes developed to the blastocyst stage than did

Table 3 The progression of meiotic maturation and developmental rate of oocytes collected from LFs or SFs.

Follicle size	Hours after culture			No. of oocytes examined	Rate (%) of development		
	5 h (no. of oocytes at GV (%))	18 h (no. of M2 (%))	21 h (no. of M2 (%))		Day 2 (cleaved (>4 cells))	Day 7* (blastocyst)	Day 8* (blastocyst)
Small	32/60 (53)	22/65 (34)	53/61 (87)	180	66	33	37
Large	14/60 (23)[a]	40/62 (65)[a]	53/60 (88)	60	82[a]	43[a]	47[a]

GV, geminal vesicle stage.
[a] Different superscripts within the same column differ significantly ($P < 0.05$).
*Days after insemination.

Table 4 The progression of meiotic maturation and developmental rate of oocytes cultured in medium containing various Na/K ratios.

Ratio of Na/K	Hours after culture			No. of oocytes examined	Rate (%) of development		
	5 h (no. of oocytes at GV (%))	18 h (no. of M2 (%))	21 h (no. of M2 (%))		Day 2* (cleaved (>4 cells))	Day 7* (blastocyst)	Day 8* (blastocyst)
16	27/60 (45)	16/63 (25)	49/61 (80)	180	67	21	26
24	34/60 (57)	17/62 (27)	49/60 (82)	178	67	20	24

GV, geminal vesicle stage.
*Days after insemination.

Table 5 The progression of meiotic maturation and developmental rate of oocytes cultured in medium containing various concentrations of Mg and Ca.

Mg (mM)	Ca (mM)	5 h (no. of oocytes at GV (%))	18 h (no. of M2 (%))	21 h (no. of M2 (%))	No.of oocytes examined	Day 2* (cleaved (>4 cells))	Day 7* (blastocyst)	Day 8* (blastocyst)
		Hours after culture				**Rate (%) of development**		
0.5	1.8	27/60 (45)[a]	16/63 (25)[a]	49/60 (82)	179	77	22[a]	29[a]
0.5	0.5	28/60 (47)[a]	15/62 (24)[a]	49/60 (82)	180	72	20[a]	21[b]
2	1.8	19/61 (31)[ab]	35/60 (58)[b]	55/60 (92)	180	70	36[b]	39[c]
2	0.5	16/60 (27)[b]	29/60 (48)[b]	54/60 (90)	180	76	35[b]	37[ac]

GV, geminal vesicle stage.
[a–c] Different superscripts within the same column differ significantly ($P < 0.05$).

Table 6 The progression of meiotic maturation and developmental rate of oocytes cultured in medium containing 5.56 or 1.5 mM glucouse.

Glucose (mM)	5 h (no. of oocytes at GV (%))	18 h (no. of M2 (%))	21 h (no. of M2 (%))	No. of oocytes examined	Day 2* (cleaved (>4cells))	Day 7* (blastocyst)	Day 8* (blastocyst)
	Hours after culture				**Rate (%) of development**		
5.56	14/60 (23)	22/62 (35)	52/60 (87)	180	66	29	35
1.5	19/60 (32)	6/61 (10)[a]	51/60 (85)	180	60	22[a]	27[a]

GV, geminal vesicle stage.
[a] Different superscripts within the same column differ significantly ($P < 0.05$).

the SF oocytes (Table 3). In experiment 3, the progression of nuclear maturation and the rate of development were the same for the two different Na/K ratios (Table 4). In experiment 4, increasing the Mg concentration to 2 mM from 0.5 mM hastened the progression of meiotic maturation and improved the rate of blastulation, irrespective of the Ca concentration. On the other hand, low concentrations of Mg and Ca inhibited the rate of development, although the progression of nuclear maturation was the same (Table 5). In experiment 4, the progression of meiotic maturation was faster and the rate of development was greater (Table 6) with a high concentration of glucose (5.56 mM) than with a low concentration of glucose (1.5 mM), although the rates of M2 oocytes at the end of maturation were the same. In experiment 5, prolonging the maturation periods from 21 to 24 h did not affect the rate of development, regardless of the glucose concentration (Table 7).

Table 7 Developmental rate of oocytes cultured for 21 or 24 h in medium containing 5.56 or 1.5 mM glucose.

Glucose (mM)	Maturation period (h)	No. of oocytes	Rate (%) of cleaved*	Day7**	Day8**
				Rate (%) of blastocysts	
1.5	21	180	68	23	26
1.5	24	180	65	22	27
5.56	21	180	76	31	35
5.56	24	180	75	26	31

* > 4 cells.
**Days after insemination.

Discussion

Many studies have found that the blastocyst rate is greater for LF oocytes than for SF oocytes (Pavlok *et al.* 1992, Lonergan *et al.* 1994, Blondin & Sirard 1995, Yang *et al.* 1998, Hagemann 1999, Hagemann *et al.* 1999, Hendriksen *et al.* 2000). This difference appears to be due to the different degree of prematuration of oocytes. Our results also showed that (i) the blastocyst rate was higher for LF oocytes than for SF oocytes; (ii) the progression of nuclear maturation occurred 2–3 h sooner for LF oocytes than for SF oocytes, although at the end of culture the rate of oocytes at the M2 stage was the same; and (iii) prolonging the maturation period of SF oocytes for 3 h did not change the rate of development (Table 7) These results show that high developmental competence may be associated with fast progression of nuclear maturation but not with the timing of fertilization. Therefore, only oocytes collected from SFs were used in the following experiments.

In this study, FF was collected from ovaries stored for 1–2 h as described above. During ovary preservation, the occlusion of blood flow reduced the oxygen and energy supply and placed ovaries under ischemic conditions. Under ischemic conditions, ATP depletion and the inability to re-establish high-energy phosphate damages cell function and causes cell death. In this study, the glucose concentration in SFF rapidly decreased during ovary preservation. We previously showed that long preservation of ovaries (7 h) under warm (30 °C) conditions impairs the developmental competence of the oocytes (Iwata *et al.* 2003). Furthermore, there is an efflux of ions, primarily K, from the cell before its death (Bortner *et al.* 1997, McCarthy & Cotter 1997, Bortner & Cidlowski 1999, James *et al.* 2000), and ion concentrations in FF change

post-mortem (Knudsen *et al.* 1979). These results and the results of the present study indicate that ion concentrations measured in slaughterhouse-derived ovaries may not directly reflect those *in vivo*. However, measurement of the elemental contents in FF provides useful information for improving maturation conditions.

In this study, the Na/K ratio was greater in LFF than in SFF. Knudsen *et al.* (1979) reported that the K concentration in porcine SFF is greater than that in LFF and the Na/K ratio affected embryo development (Jin *et al.* 1994). However, in this study, the Na/K ratio in the maturation medium did not affect either the developmental competence or the progression of nuclear maturation.

The concentration of Mg in FF (2–2.3 mM) was found to be higher than that in the media generally used for maturation such as TCM-199 and SOF (0.5–0.8 mM), but the Ca concentration in FF was not different from that in these media. Mg has important roles in mammalian cells. For example, it acts as a co-factor in enzymatic reactions, and induces meiotic maturation of *Xenopus* oocytes (Belle *et al.* 1986). Furthermore, low concentrations of divalent cations (Mg and Ca), inhibit the resumption of meiosis, and the inhibitory effect of low Mg concentration is greater than that of low Ca (Leibfreid & First 1979, Jagiello *et al.* 1982). In the present study, low Mg and Ca concentrations in the IVM medium impaired developmental competence, but did not affect the progression of nuclear maturation. On the other hand, a high Mg concentration (2 mM) and high Mg/Ca ratio (2:1) improves early embryo development because Mg regulates Ca homeostasis in zygotes (Lane *et al.* 1998). However, increasing Mg to 2 mM from 0.5 mM improved developmental competence and hastened the progression of nuclear maturation, irrespective of the Ca concentration. This indicates that the optimal Mg concentrations in IVM are probably higher than the concentrations in TCM-199 and SOF. However, it is unclear why Mg has a beneficial effect.

Glucose has been reported to be an essential energy substrate for maturation of bovine oocytes (Rieger & Loskutoff 1994, Lim *et al.* 1999). And adding glucose to the culture medium enhanced glycolysis of oocytes, and a higher glycolysis rate in *in vitro*-matured oocytes reflects a higher developmental competence (Krisher & Bavister 1999). Cumulus and oocyte complexes metabolize glucose through glycolysis and the pentose phosphate pathway, and metabolites of glucose are thought to regulate meiotic maturation (Rose-Hellekant *et al.* 1995, Downs *et al.* 1996, Krisher & Bavister 1999). The present results indicate that sufficient glucose is required for oocytes to obtain developmental competence during the maturation period, although the reason for the beneficial effect of high glucose concentration in maturation remains unclear.

The time required for oocytes to resume meiosis and progress to M2 arrest is closely associated with developmental competence of the oocytes (Dominko & First 1997). However, little is known about the underlying causes of this relationship. On the other hand, when oocytes are inseminated shortly after M2 arrest, their developmental competence is very low (Johnston *et al.* 1986, Kubiak 1989). In this study, high Mg and glucose hastened the progression of nuclear maturation and improved the developmental competence of oocytes. However, prolonging the maturation period from 21 to 24 h did not change the rate of blastulation (Table 7). In addition, the rate of normal fertilization was the same between these experimental groups (data not shown). The present results, together with these previous results, indicate that the beneficial effects of high Mg and glucose in the IVM medium are due to their ability to speed up the progression of nuclear maturation.

Recently, Ali & Sirard (2002) reported that during bovine oocyte maturation serum was successfully replaced with polyvinylpyrrolidone-40. In this study, we used serum as a protein source, which may change the final concentration of elements. To clearly understand the effects of ion concentration on oocyte maturation, further study is required with fully chemically defined conditions.

In conclusion, the progression of meiotic maturation is affected by the oocyte source (LFs are better than SFs), and by the Mg and glucose concentrations in the IVM medium. Furthermore, a fast progression of nuclear maturation is closely associated with the high developmental competence of oocytes.

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