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Carbohydrate Requirements of Follicular Bovine Oocytes Cultured in a Chemically Defined, Hormone-, Amino Acid- and Protein-Free Medium

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호르몬, 아미노산 및 단백질이 첨가되지 않은 단순배양배지내 소 난포란의 탄수화물 요구량

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요 약

본 연구는 단순배양체계내 소 난포란의 성숙에 요구되는 탄수화물 (glucose, lactate와 pyruvate) 을 검토하기 위해 수행하였다. GV (germinal vesicle) 단계의 난구세포로 둘러싸인 소 난자를 단백질, 아미노산 및 호르몬이 첨가되지 않은 modified Tyrodes (mT)에서 24시간, 5% CO_2 배양기를 이용하여 성숙배양하였다. Glucose 무첨가군 (0~61%) 에 비해 5.6 mM의 glucose 첨가군 (71~74%) 이 유의적으로 높은 ($P<0.05$) M-Ⅱ 단계로의 난자 발육을 나타내었다. Glucose를 함유한 배지내에서는 lactate (10 mM)와 pyruvate (0.5 mM) 의 첨가에 따르는 M-Ⅱ 단계로의 발육에 있어 차이를 보이지 않았다. 그러나 glucose 무첨가 배지에서는 pyruvate와 lactate를 첨가하는 것이 첨가하지 않는 것에 비해 condensed GV (76% vs. 0~2%), M-Ⅱ, (43~61% vs. 0%) 단계에 이른 난자수가 유의적으로 높았다. Glucose 함유 mT 배지에 lactate와 pyruvate를 첨가하여 난자를 배양하였을 때, 동결-융해 정자와 24시간 정치한 후 난자중 87~93%가 정자침투되었고 39~44%가 전핵단계로 발육하였다. 침투난자의 26~30%는 다정자수정이었다. 결론적으로, GV 단계의 소 난포란은 에너지원으로 lactate, glucose와 pyruvate를 이용하지만, 감수분열 성숙을 유지하는 데 glucose가 가장 효과적이다. 이 결과들로 보아 단순배양배지는 소 난포란의 체외성숙에 영향을 미치는 다양한 물질들을 연구하는 데 잠재적으로 유용하다.

Key words: Bovine, Oocytes, *In vitro* maturation, *In vitro* penetration, Nutritional requirements, chemically defined medium.

INTRODUCTION

In most species, oocytes at the germinal vesicle (GV) stage spontaneously undergo the meiotic maturation to metaphase (M)-Ⅱ when they are freed from follicular fluid and culture in a variety of culture media (Biggers *et al.*, 1967). However, only small proportions of *in vitro*-maturation derived oocytes develop to the blastocysts following

in vitro-insemination (Xu *et al.*, 1992 ; Moor & Bondioli, 1993 ; Rosencrans *et al.*, 1993). One of the reasons for this developmental disorder may involve an incomplete oocyte maturation. However, since the culture systems used for oocyte maturation are not chemically defined condition, it is difficult to examine various factors affecting maturational process. It is, therefore, necessary to develop a chemically defined culture system for establishing an effective culture system for bovine oocytes.

When cultured *in vitro*, oocytes require carbohydrates since maturation is an energy consuming process (Gwatkin & Haidri, 1973). As a consequence, glucose and/or its metabolites are generally supplemented to culture media as energy substrates to support oocyte maturation. However, the carbohydrate requirement of oocytes varies according to developmental stages, and several carbohydrates such as glucose have a detrimental effect on oocyte development at specific stages (Takahashi & First, 1992 ; Lim *et al.*, 1993 ; Kim *et al.*, 1993). This study was, therefore, designed to examine carbohydrate (glucose, lactate and pyruvate) requirements of follicular bovine oocytes during maturation culture to formulate a chemically defined medium.

MATERIALS AND METHODS

1. Media

The basic medium assigned for the maturation of oocytes was modified Tyrodes (mT) medium, consisting of 89 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.35 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 1 mg polyvinylalcohol/ml. The composition of this medium is basically the same as that used in the previous study (Lim *et al.*, 1993) but lactate, pyruvate and amino acids were omitted. The basic medium used for the treatment of spermatozoa and the fertilization of oocytes was essentially the same as that used by Brackett and Oliphant (Brackett & Oliphant, 1975) for the fertilization of rabbit oocytes *in vitro*, except that bovine serum albumin (BSA) and glucose were not added. This medium was composed of 112 mM NaCl, 4.02 mM KCl, 2.25 mM $CaCl_2$, 0.52 mM $MgCl_2$, 0.83 mM NaH_2PO_4, 37 mM $NaHCO_3$, 1.25 mM sodium pyruvate and 31 g sodium penicillin G/ml.

2. Maturation of oocytes

Ovaries isolated from Holstein heifers or cows at a local slaughterhouse were brought to the laboratory in 0.9% NaCl solution at 30~32℃ within 2 h. Cumulus-enclosed oocytes were aspirated from follicles of 3~5 mm in diameter with a 18-gauge hypodermic needle attached to a 10-ml disposable syringe containing maturation medium. Cumulus-enclosed oocytes were then placed into a 60×15 mm Falcon polystyrene culture dish (No. 3002, Becton & Dickinson, Lincoln Park, NJ, USA) and washed four times with maturation medium. Oocytes with an evenly granulated cytoplasm and which were completely surrounded by dense layers of cumulus cells were selected under a dissecting microscope (SMZ-1, Nikkon, Tokyo, Japan). Eighteen to 22 oocytes were then transferred to 100μl of maturation medium which had been previously covered with warm mineral oil (M-8410, Sigma Chemical, St. Louis, MO, USA) in 35×10 mm Falcon polystyrene culture dish (No. 1008,

Becton & Dickinson). Oocytes were cultured at 39℃ in 5% CO_2 in air for 24 h.

3. Fertilization of oocytes

Following 24 h of maturation culture, oocytes were washed four times and 8~12 oocytes were introduced into 50μl droplets of fertilization medium supplemented with 20 mg fatty acid-free BSA (A-6003, Sigma Chemical)/ml and 20μg porcine intestinal mucosal heparin (181 USP units/ml, H-3125, Sigma Chemical)/ml. The dishes were kept in a CO_2 incubator (5% CO_2 in air) for about 30 min until spermatozoa were added for fertilization.

One 0.5-ml straw of frozen semen collected from a fertile Holstein bull was thawed in a 39℃ water bath and, after dilution with fertilization medium, spermatozoa were washed twice by centrifugation at 200×g for a period of 6 min each. The final sperm pellet was resuspended in the same medium to give a concentration of $2\sim3\times10^6$ spermatozoa/ml. A 50-ml sperm suspension was then introduced into the droplets that contained the oocytes. The mixture was incubated at 39℃ in 5% CO_2 in air.

4. Preparations for assessments of maturation and sperm penetration of oocytes

At 22~24 h after maturation culture or 24 h after insemination, oocytes were freed from cumulus cells by vigorous pipetting. Denuded oocytes were then placed in the centre of four vaseline :paraffin (1:9) spots on a slide glass. After being compressed gently with a coverslip, the oocytes were fixed for at least 96 h at room temperature in 25% (v/v) acetic alcohol and stained with 1% (w/v) orcein in 45% (v/v) acetic acid as described by Ohgoda *et al.* (1988).

5. Classification of maturation and sperm penetration

The morphological changes of the nucleus in oocytes were classified into six categories: GV, condensed GV (cGV), M-Ⅰ, anaphase (A)-I, telophase (T)-Ⅰ and M-Ⅱ as descried in porcine oocytes by McGaughey and Polge (Mcgaughey & Polge, 1971). The morphological changes observed in penetrated oocytes were divided into two categories: oocytes with enlarged sperm head and female pronuclei, and with expanded male and female pronuclei.

6. Experimental design

In Experiment 1, combined effects of the addition of glucose (5.6 mM), lactate (10 mM) and/or pyruvate (0.5 mM) to mT medium on nuclear maturation of oocytes were examined in a 2×2×2 factorial design. In Experiment 2, combined effects of the addition of lactate and/or pyruvate to mT medium supplemented with 5.6 mM glucose on sperm penetration were examined in a 2×2 factorial design.

7. Statistical analysis

Each experiment was replicated four times. The proportions of oocytes at each stage were subjected to an arcsine transformation. The transformed values were assigned for three-way (Experiment 1) and two-way (Experiment 2) ANOVA. When ANOVA revealed a significant treatment effect, each treatment was compared by Tukeys advanced test.

RESULTS

As a preliminary experiment, oocytes were fixed immediately after collection and observed nuclear maturation stage. 100% of fixed oocytes (22/22) were GV stage with distinct nuclear membrane and dispersed chromosomes.

1. Experiment 1

Overall, significantly ($P<0.05$) higher percentages of oocytes developed to M-Ⅱ stage when cultured in glucose-containing (71~74%) than in glucose-free (0~61%) mT medium. When oocytes were cultured in glucose-containing medium, there were no significant differences in the percentages of M-Ⅱ stage oocytes in the presence of lactate and/or pyruvate. When cultured in glucose-free medium, however, significantly higher percentages of oocytes at the GV stage were obtained in no addition (24%) than in the presence of lactate and/or pyruvate (0~2%). Furthermore, higher percentages of oocytes were developed to M-Ⅱ stage by lactate and/or pyruvate addition (40~61%) compared with no addition (0%).

2. Experiment 2

When oocytes were cultured in glucose-containing mT medium supplemented with lactate and/or pyruvate, there were no differences in the percentages of penetrated oocytes (87~93%) among oocytes treated with lactate and/or pyruvate. 39~44% and 26~30% of penetrated oocytes developed to the pronuclear stage and were polyspermic fertilization, respectively.

DISCUSSION

The results of this study clearly demonstrate that oocytes utilize lactate, glucose and pyruvate during maturation but that glucose acts as a main energy substrate to support meiotic maturation. In a chemically defined medium supplemented with glucose, follicular bovine oocytes at GV stage are capable of developing to M-Ⅱ stage and being penetrated by frozen-thawed spermatozoa.

As seen in murine oocytes (Biggers *et al.*, 1967), maturation of follicle-free oocytes takes place in a simple medium such as Krebs-linger solution containing pyruvate under 5% CO_2 in air atmosphere. However, hamster (Gwattkin & Haidri, 1973) oocytes at GV stage require a lower partial O_2 pressure or addition of amino acids to culture medium. In our study, the initiation of maturation in cumulus-enclosed bovine oocytes was independent of exogenous amino acids, proteins, and hormones, as long as oocytes were cultured in the medium with glucose, lactate and/or pyruvate. However, optimal concentrations of energy substrates, inorganic ions (H^+, Na^+, Cl^-, Ca^{2+}, Mg^{2+}, HCO^{3-}, PO^{4-}), pH (7.3~7.5), osmolarity (287~295 mOsm) and 5% CO_2 in air atmosphere are necessary for resuming meiotic maturation of cumulus-enclosed oocyte. This result was partly supported by the hypothesis that oocyte maturation is not necessarily induced by hormonal stimulation (Masui & Clarke, 1979).

In our study, it was not clear whether resumption of meiotic maturation was independent of endogenous substances derived from amino acids, hormones and proteins, since oocytes were surrounded by cumulus cells. Anderson and Albertini

(Anderson & Albertini, 1976) reported that processes extruded from cumulus cells traverse the zona pellucida and communicate with oocyte cytoplasm via gap junction. Furthermore, it has been reported that heterogous cell contact and metabolic coupling between cumulus cells and oocytes are quite important for normal growth of the oocytes (Brower & Schultz, 1981 ; Moor & Cran, 1981 ; De Loos *et al.*, 1991). Since cumulus granulosa cells can take up and incorporate with various substrates from hormone-, amino acid- and protein-rich follicular environment, metabolic products and signal transducers produced by cumulus cells may affect spontaneous maturation of oocytes in defined medium.

The importance of glucose for oocyte maturation in the bovine has been reported by Zeulke and Brackett (Zuelke & Bracckett, 1992). Rieger and Loskutoff (Rieger & Loskutoff, 1994) confirmed that oxidative metabolism of glucose is the major site of cellular energy production and that metabolic activity of this pathway increases during maturation of bovine oocyte *in vitro*. On the other hand, development of 1-cell embryos to the blastocyst stage is significantly inhibited by the presence of 5.56 mM glucose in culture medium (Kim *et al.*, 1993 ; Lim *et al.*, 1993). Conversely, addition of 2.78 or 5.56 mM glucose to culture medium 120 h post-insemination significantly promotes blastocyst formation (Kim *et al.*, 1993 ; Lim *et al.*, 1994). The addition of lactate and/or pyruvate to culture medium stimulates the development of 1-cell embryos to the blastocyst stage (Kim *et al.*, 1993). The results of this study demonstrate that requirement patterns of lactate, glucose and pyruvate in maturing oocytes are partly different from those requirements in oocytes at further developmental stages.

When murine oocytes were cultured in serum-free medium, their zona pellucida become resistant to chymotrypsin digestion (zona hardening) after undergoing *in vitro* maturation (Zhang *et al.*, 1991). Furthermore, the modification of the zona pellucida significantly blocked sperm entry into oocyte cytoplasm (Ducibella *et al.*, 1990). In our study, however, approximately 90% of oocytes cul-

Table 1. Combined effects of the addition of glucose, lactate and/or pyruvate to mT medium on *in vitro*-maturation of cumulus-enclosed bovine oocytes at the GV stage

Medium with			No. of oocytes cultured	No. and (%)[a] of oocytes at the stage of				
Glucose (5.6 mM)	Lactate (10 mM)	Pyruvate (0.5 mM)		GV	cGV	M-I	T-I	M-II
+	+	+	42	0(0)[b]	0(0)	12(29)	0(0)	30(71)[b]
+	+	−	47	0(0)[b]	2(4)	9(20)	1(2)	35(74)[b]
+	−	+	43	1(2)[b]	0(0)	11(26)	0(0)	31(72)[b]
+	−	−	42	1(2)[b]	1(2)	10(25)	0(0)	30(71)[b]
−	+	+	44	0(0)[b]	0(0)	17(39)	0(0)	27(61)[c]
−	+	−	40	0(0)[b]	2(5)	21(53)	1(3)	16(40)[d]
−	−	+	46	1(2)[b]	1(2)	24(52)	0(0)	20(43)[d]
−	−	−	41	10(24)[c]	16(39)	15(37)	0(0)	0(0)[e]

[a]Percentage of the number of oocytes cultured.
[b,c,d,e]Different superscripts within each column are significantly different, $P<0.05$.

Table 2. *In vitro*-penetration of cumulus-enclosed bovine oocytes following a 24 h culture in 5.6 mM glucose-containing mT medium supplemented with lactate and/or pyruvate

Medium with		No. of	No. of oocytes penetrated			No. of
Lactate (10 mM)	Pyruvate (0.5 mM)	oocytes cultured	Total (%)[a]	With enlarged sperm head	With male and female pronuclei (%)[b]	polyspermic oocytes (%)[b]
+	+	38	33(87)	20	13(39)	9(27)
+	−	35	34(89)	20	14(41)	9(26)
−	+	45	40(89)	23	17(43)	12(30)
−	−	46	43(93)	24	19(44)	11(26)

[a] Percentage of the number of oocytes cultured.
[b] Percentage of the number of oocytes penetrated.

tured in serum-free medium were penetrated by spermatozoa (Table 2). The results of this study clearly demonstrate that *in vitro*-matured bovine oocytes can be penetrated by spermatozoa without any disturbances following 24 h of serum-free culture. Also, the presence of neither lactate nor pyruvate in glucose-containing mT medium affects the sperm receptivity of the oocyte.

When oocytes were matured in TCM-199 with 10% fetal bovine serum, more than 90% of inseminated oocytes developed to the pronuclear (1-cell) stage following 24 h post-insemination (Lim *et al.*, 1993). However, only 39~44% of penetrated oocytes matured in the chemically defined medium could develop to the pronuclear stage. This result demonstrates that, although oocytes fully underwent nuclear maturation in defined culture condition, their cytoplasmic maturation is not complete. We are further examining the effects of various substances on *in vitro*-maturation of bovine oocytes to investigate various maturation promoting factors in defined condition.

ABSTRACT

This study was undertaken to examine carbohydrate (glucose, lactate and pyruvate) requirement of follicular bovine oocytes during maturation for developing a chemically defined culture system. Cumulus-enclosed bovine oocytes at the germinal vesicle (GV) stage were cultured in a modified Tyrodes (mT) medium with no protein, amino acid and hormone for 24 h under 5% CO_2 in air. Significantly ($P<0.05$) higher percentages of oocytes developed to M-II stage when cultured in glucose (5.6 mM)-containing (71~74%) than in glucose-free (0~61%) media. In glucose-containing media, there were no significant differences in percentages of metaphase (M)-II stage oocytes in the presence of lactate (10 mM) and/or pyruvate (0.5 mM). In glucose-free media, however, significantly higher percentages of oocytes developed to the condensed GV (76% vs. 0~2%) and M-II (43~61% vs. 0%) stages were obtained in the presence of pyruvate and/or lactate compared with no addition. When oocytes were cultured in glucose-containing mT medium supplemented with lactate and/or pyruvate, 87~93% and 39~44% of oocytes were penetrated by spermatozoa and developed to the pronuclear stage, respectively, following a 24 h incubation with frozen-thawed spermatozoa 26~30% of penetrated oocytes were polyspermic fertilization. In conclusion,

follicular bovine oocytes at GV stage utilize lactate, glucose and/or pyruvate as an energy substrate but glucose is the most effective to support meiotic maturation. Based on these results, a chemically defined medium is potentially useful for studying the effects of various substances on *in vitro*-maturation of follicular bovine oocytes.

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