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Effects of Osmolarity and Vitamins on the *In Vitro* Development of Bovine Embryo in a Chemically Defined, Protein-free Culture Medium

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무단백 한정배양액에서 삼투압 및 비타민이 소 수정란의 체외발생에 미치는 영향

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요 약

본 연구는 배양액의 삼투압과 비타민이 체외 배발생에 미치는 영향을 조사하기 위하여 소의 미성숙 난포란을 체외성숙 및 수정 후 무단백 한정배양액에서 체외배양하였다. 0.35mM phosphate와 19종 아미노산이 포함된 무단백 한정배양액의 삼투압을 NaCl농도에 의하여 달리하였을 때 소 수정란의 발생능력은 265mOsm과 290mOsm에서 유의적으로 증가하였다 ($p<0.05$). 수정 후 120시간에 5.56mM포도당이 포함된 새로운 배양액에서 배발생을 계속시켰을 때 상실배는 290mOsm과 315mOsm에서 유의적으로 증가하였으며, 배반포는 290mOsm배양액에서 유의적으로 증가하였다.

Glutamine과 아미노산이 첨가된 배양액에서 비타민은 소수정란의 발생에 영향을 미치지 않았다.

I. INTRODUCTION

In most mammals, *in vitro* culture of embryo does not lead to cleavage and development as occurred *in vivo* (Wright and Bondioli, 1981). The development of embryo *in vitro* is generally blocked at specific stage according to different species under conventional culture system (First and Barnes, 1989). Recently new culture system for *in vitro* matured/*in vitro* fertilization (IVM/IVF) bovine embryo has been developed by several worker (Kim et al., 1993; Rosenkrans et al., 1993)

However, biochemical requirement for normal *in vitro* development of preimplantation embryo of mammals have not been defined (Rexroad, 1989). A certain group of vitamins is reported to be necessary for blastocyst expansion and/or hatching in rabbit (Kane, 1988) and hamster (Kane et al., 1986) in the presence of amino acid.

Osmolarity of culture medium plays an important role in development of mouse (Whitten, 1971; Brinster, 1965; Barg, 1986), rabbit (Nalgee et al., 1969) and rat (Miyoshi et al., 1994) embryos *in vitro* at various stage.

In recent culture experiment with porcine zygote (Beckmann and Day, 1993; Galvin et al., 1993) detrimental effect of high NaCl concen-

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tration in the culture medium on developmental ability of embryo have also been reported. Culture media containing high inorganic concentration could be raise the intracellular concentration of ions to levels that alter the conformation of protein, causing the disruption of cellular process (Yancy et al., 1982; Arakawa and Timasheff, 1985; Chamberlin and Strange, 1989).

The present studies were undertaken to determined the effects of osmolarity and vitamins in chemically defined medium on the *in vitro* development of bovine embryos.

Ⅱ. MATERIAL AND METHOD

1. Media

The basic medium used for the treatment of spermatozoa and fertilization of oocytes was essentially the same as that used by Brackett and Oliphant (1975) for the fertilization of rabbit eggs *in vitro*, except that bovine serum albumin (BSA) and glucose were not added. The medium, designated as BO medium, was composed of 112.0 mM NaCl, 4.02 mM KCl, 2.25 mM $CaCl_2$, 0.83 mM NaH_2PO_4, 0.52 mM $MgCl_2$, 37.0 mM $NaHCO_3$, 1.25 mM sodium pyruvate and 31 μg sodium penicillin G /ml.

The basic medium used for maturation of oocytes was a glucose-free tissue culture medium (TCM)-199 (with Earle's salts) buffered with 25 mM N-2-hydroxy- ethylpiperazine N-2-ethane salphonic acid (HEPES), and designated as modified(m) TCM-199. The medium before being added HEPES was prepared from a powdered medium which was specially manufactured by Gibco Labs. (Grand Island, NY, USA) by eliminating glucose and reducing the concentration of NaCl to 6.1 mg /ml from the original product (Cat. No. 400-1100). A chemically defined medium was used for culture of embryos. The basic medium was a modified Tyrode's solution comprised 110.0-mM NaCl, 3.2 mM-KCl, 2.0 mM -$CaCl_2$, 0.5 mM-$MgCl_2$, 25.0 mM-$NaHCO_3$, 10.0 mM sodium-lactate, 0.5 mM-sodium pyruvate and 1 mg polyvinylalcohol (PVA) /ml and designated as modified(m) TLP-PVA.

2. Collection 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 to 35℃ within 2 hours. Follicular oocytes were aspirated from the follicles of 3~5 mm in diameter by means of a 18-gauge hypodermic needle attached to a 10-ml disposable syringe (Funahashi et al., 1991). The follicular contents were deposited to a little amount of a maturation medium in a watchglass. Oocytes were roughly selected according to the general appearance of the cytoplasm and cumulus cells under a dissecting microscope and washed four times with maturation medium. Only oocytes which are round with evenly pigmented cytoplasm and completely surrounded by dense layer of cumulus cells were matured.

3. Culture of oocytes for maturation

Thirty to 50 oocytes were transferred into 500 μl maturation medium, mTCM-199 supplemented with 5.56 mM glucose, 5 mM hemicalcium lactate, 0.4 mM sodium pyruvate, 10% (v /v) heat-treated (56℃, 30min) fetal bovine serum (FBS; Wheaton Scientific, Millville, NJ., USA), 60 μg sodium penicillin G /ml and 100 μg streptomycin sulfate /ml, which had been previously covered with warm paraffin oil (No. 261-17; Nacalai Tesque, Inc., Kyoto, Japan) in each well of a 4-well multidish (Nunc, Roskilde, Denmark). Oocytes were cultured for 22~24 hours at 39℃ under an atmosphere of 5% CO_2-95% air with high humidity.

4. Fertilization of oocytes *in vitro*

After maturation in culture, oocytes with black and evenly pigmented cytoplasm and surrounded by expanded cumulus were washed four times and placed in 50 ㎕ BO medium supplemented with 20 mg BSA (crystallized and lyophilized, essentially globulin-free, cat. No. A-7638, Sigma Chemical Co., St. Louis, MO, USA) /ml and 20 ㎍ porcine intestinal mucosal heparin (181 USP units /mg; Sigma Chemical Co.) /ml under paraffin oil in a polystyren culture dish (35×10 mm, Termo, Tokyo, Japan). The dishes were kept in a CO_2 incubator (5% CO_2 in air at 39℃) for about 30 minutes until spermatozoa were added.

One 0.5 ml straw of frozen semen obtained from Holstein bulls was thawed in a water bath at 37℃ for 1 minutes. Spermatozoa were washed twice by centrifugation at 833×g for 10 minutes each after dilution with BO medium supplemented with 10 mM caffeine-benzoate (3.383 mg /ml, Sigma Chemical Co.). The final sperm pellet was resuspended in the same medium as used for washing to give a sperm concentration of $2\sim4\times10^6$ spermatozoa /ml. A 50 ㎕ of the sperm suspension was introduced into 50 ㎕ of the medium that included the cumulus-oocyte complexes for fertilization (Niwa and Ohgoda, 1988). The mixture gave final concentrations of $1\sim2\times10^6$ spermatozoa /ml, 10mg BSA /ml, 10 ㎍ heparin /ml and 5 mM caffeine and incubated at 39℃ in 5% CO_2 in air with high humidity for 8 hours.

5. Culture of fertilized oocytes *in vitro*

At 8 hours post-insemination, oocytes were washed four times and 10~25 oocytes were placed into 500 ㎕ culture medium with various conditions according to the different experiments, under paraffin oil in each well of a 4-well multidish. In all experiments, the oocytes were cultured at 39℃ in an atmosphere of 5% CO_2-95% air with high humidity.

6. Experimental studies

In experiment 1, to examine the effect of osmolarity, embryos were cultured in mTLP-PVA supplement with 3.5 mM phosphate and 19 amino acids, and adjusted osmolarities to 215, 240, 265, 290, 315, 340 and 365mOsm by the concentration of NaCl. Embryo were transferred to fresh medium with glucose(5.56 mM) at 120hrs post-insemination in experiment 2. In experiment 3, the effects of vitamins in mTLP-PVA with 0.35 mM phosphate, glutamine and 19 amino acid on the development of embryos were examined.

7. Statistical analysis

The proportion of embryos developing to each stage was subjected to an arc-sine transformation, and the transformed values were assigned for one-way or two-way ANOVA. When ANOVA revealed a significant treatment effect, the treatments were compared by Duncan's multiple range test.

Ⅲ. RESULTS

1. Experiments 1

As shown in Table 1, the first cleavage was significantly ($p<0.05$) inhibited when 1-cell embryos were cultured in the medium of 340~365 than 215~315 mOsm. Significantly ($p<0.05$) higher proportions of embryos developed to >8-cell (50~51%), morula (32~35%) and blastocyst (24~28%) stages at 96, 144 and 192 hours post-insemination, respectively, in the medium of 265~290 mOsm than 215~240 or 315~365 mOsm.

Table 1. Effects of different osmolarities of mTLP-PVA with 0.35 mM phosphate and 19 amino acids on development of bovine oocytes fertilized *in vitro**

Osmolarity (mOsm)	No. of oocytes inseminated	No. and (%)† of embryos developed to			
		≤2-cell [48]‡	≤8-cell [96]‡	Morula [144]‡	Blastocyst [192]‡
215	97	64(66)[a]	7(7)[a]	3(3)[a]	0(0)[a]
240	102	73(72)[ab]	25(25)[b]	7(7)[ab]	3(3)[b]
265	98	76(78)[b]	50(51)[c]	31(32)[c]	27(28)[c]
290	100	73(73)[ab]	50(50)[c]	35(35)[c]	24(24)[c]
315	96	68(71)[ab]	37(39)[d]	24(25)[d]	14(15)[d]
340	90	50(56)[c]	18(20)[b]	5(6)[ab]	0(0)[a]
365	101	32(32)[d]	1(1)[e]	0(0)[d]	0(0)[a]

* Experiments were repeated 7 times.

† Percentage of the number of oocytes inseminated.

‡ Numbers in parenthesis indicate the time of examination (hours after insemination).

[a~e] Within each column, values with different superscripts are significantly different ($p<0.05$ at least).

Table 2. Effects of addition of glucose (5.56mM) at 120 hours post-insemination on development of bovine oocytes fertilized *in vitro* and cultured in mTLP-PVA with 0.35 mM phosphate and 19 amino acids, and with different osmolarities*

Osmolarity (mOsm)	No. of oocytes inseminated	No. and (%)† of embryos developed to			
		≥2-cell [48]‡	≥8-cell [96]‡	Morula [144]‡	Blastocyst [192]‡
215	100	66(66)[a]	10(10)[a]	2(2)[ab]	0(0)[a]
240	102	68(67)[ab]	27(26)[b]	5(5)[b]	3(3)[b]
265	99	73(72)[b]	47(47)[c]	30(30)[c]	15(15)[c]
290	92	64(70)[ab]	45(49)[c]	37(40)[d]	29(32)[d]
315	101	71(70)[ab]	51(50)[c]	35(35)[cd]	22(22)[e]
340	96	52(54)[c]	24(25)[b]	5(5)[b]	0(0)[a]
365	99	34(34)[d]	3(3)[d]	0(0)[a]	0(0)[a]

* Experiments were repeated 7 times.

† Percentage of the number of oocytes inseminated.

‡ Numbers in parenthesis indicate the time of examination (hours after insemination).

[a~e] Within each column, values with different superscripts are significantly different ($p<0.05$ at least).

2. Experiments 2

When embryos were transferred to fresh medium additionally supplemented with glucose (5.56 mM) at 120 hours post-insemination, higher osmolarities (340~365 mOsm) again inhibited the first cleavage ($p<0.05$) (Table 2). Although significantly ($p<0.05$) higher proportions of embryos developed to >8-cell (47~50%) and morula (30~40%) stages at 265~315 than 215~240 and 340~365 mOsm, the highest proportion of blastocysts (32%) were obtained at 290 mOsm ($p<0.05$).

3. Experiments 3

As shown in Table 3, vitamins added to the

Table 3. Effects of vitamins in mTLP-PVA with 0.35 mM phosphate, glutamine and amino acids on development of bovine oocytes fertilized *in vitro**

With(+) or without(−) vitamins	No. of oocytes inseminated	No. and (%)† of embryos developed to			
		≤2-cell [48]‡	≤8-cell [96]‡	Morula [144]‡	Blastocyst [192]‡
+	68	53(78)	36(53)	28(41)	19(28)
−	64	48(75)	31(48)	25(39)	20(31)

* Oocytes were transferred into the medium with 5.56 mM glucose 120 hours post-insemination. Osmolarity of the medium was adjusted to 290 mOsm. Experiments were repeated 4 times.

† Percentage of the number of oocytes inseminated.

‡ Numbers in parenthesis indicate the time of examination (hours after insemination).

medium had no significant effects on the development of 1-cell embryos to the blastocyst stage.

Ⅳ. DISCUSSION

The results of present experiments confirm thaty NaCl concentration in medium can profoundly influences the development *in vitro* of bovine preimplantation embryo. The development of bovine one cell embryos beyond the two cell stage is largely dependent on the osmolarity of the medium.

The similar results have been reported that development *in vitro* of mouse embryo (Barg, 1986), rabbit(Naglee et al., 1969), and rat(Miyoshi, 1994) are largely affected by osmolarity in medium. The developmental ability of bovine embryo is decreased in the embryos developed in medium containing higher NaCl concentration than optimal range.

It has also been reported that use of low salt culture medium was effective for mouse(Lawitts and Biggers, 1991, 1991, 1992 ; Anbrai and Schultz, 1993 ; Pomp et al., 1988), porcine(Beckmann and Day, 1993 ; Galvin et al., 1993), and rat embryo(Myoshi, 1994) overcoming the *in vitro* developmental arrest, the so-called cell block and *in vitro* maturation of porcine(Funahashi, 1994) hamster oocyte(Gwatkin and Haidri, 1973).

Large proportion of morolae and blastocyst are obtained at 265 mOsm of culture medium without glucose supplement (Table 1). When glucose is added at 120hrs post-insemination into medium, optimal osmolarity for blastocyst is increased to 290~315 mOsm (Table 2).

The possible explanation of the protective effect of glucose may be found in the way glucose metabolism of blastocyst increase higher than early embryo(Rieger, 1992 ; Javed and Wright, 1991) and glucose has role as an organic osmolyte. When cell are exposed to hypertonic environment, solute are actively moved into cell from its environment to eliminate the activity gradient. The transported solute are either ions or osmotically active organic solute known as organic osmolyte. When cells respond to osmotic stress (an increase or decrease in the osmotic pressure of the surrounding environment of organism or cell), the organic solute are adjusted to prevent large changes in intracellular ionic strength that could adversely affect protein function(Csonka, 1989).

We observed in the current studies that the addition of soluble vitamins into medium containing glutamine and amino acid did not affect the *in vitro* development of bovine embryo. There is

very limited amount of comparative information on the culture of preimplantation embryo in medium with vitamin.

Our finding is in agreement with previous study(Bavister et al., 1983) in which vitamin supplementation was neither beneficial nor deleterious to hamster embryo cultured from the eight-cell to blastocyst stage. However, Kane et al.(1986) demonstrated that the addition of vitamin in presence of glutamine or F-10 amino acid significantly enhanced *in vitro* development of hamster embryo to blastocyst. The addition of vitamins to simple medium containing energy source and one or more amino acid is essential in allowing hamster(Kane et al., 1986, 1988), rabbit(Kane, 1988) to hatch *in vitro*.

In conclusion, this study has shown that osmolarity of medium is important factor for the *in vitro* development to blastocyst stage. The addition of glucose at 120 hrs after postinsemination adjusts bovine embryo to the inhibitory effect of high salt. Vitamins in culture medium do not affect the *in vitro* development of bovine embryo in chemically defined medium.

V. SUMMARY

The purpose of this study was to evaluate effects of osmolarity and vitamins on the *in vitro* development of embryos. Bovine embryo that had been matured and fertilized *in vitro* were cultured in simple, chemically defined, protein-free medium (mTLP-PVA). When the osmolarity of the medium supplemented 0.35 mM phosphate and 19 amino acid was changed by NaCl concentration, significantly($p<0.05$) higher proportion of 1-cell embryos in the medium of 265 or 290 mOsm developed to the morula(32~35%) and blastocyst(24~28%) stage. When embryos were transferred to fresh medium containing 5.56mM glucose at 120hrs post-insemination, the highest proportion of embryos developed to morula(40%) and blastocyst(32%) stages at 290 mOsm($p<0.05$), although the value in morulae was not significantly different with that(35%) at 315 mOsm.

Vitamins in presence of glutamine and amino acids had no beneficial effects on the development of 1-cell embryos to the blastocyst.

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