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Effect of Phosphate, Amino Acid, and BSA on in vitro Development of Mammalian Embryo in Chemically Defined Culture Medium

Jong-Heung Kim and Byung-Ki Kim*†

*Department of Biology, Dongeui university, Pusan 614-714, Korea
Saewha Infertility Institute, Pusan, Korea

Abstract

The aim of this study was to evaluate the effects of phosphate, amino acid, and BSA on in vitro development of mammalian embryos. In vitro-matured and -fertilized(IVM/IVF) bovine embryos were cultured in simple, chemically defined, protein-free medium(mTLP-PVA). When the phosphate concentration of mTLP-PVA supplemented with 19 amino acid were adjusted to 0.0, 0.10, 0.35, 1.05 and 2.10mM by the concentration of sodium phoshpate, there were no significant different in developmental ability of IVM/IVF bovine embryos cultured in the medium containing from 0.00 to 1.05mM phosphate until 48 hours post- insemination. However, proportion of embryos developing to ≧8-cell and morula at 96 and 144 hours post-insemination, respectively, was significantly increased in the medium with 0.35 mM phosphate($p<0.05$). There was significant difference between 0.10(18%)-0.35(24%)mM phosphate and 1.05(13%)-2.10(1%) mM phosphate in supporting development to blastocyst($p<0.05$). When IVM/IVF bovine embryos were cultured in the medium supplemented with 19 amino acids, significant different was observed in the proportion of embryos reaching ≧8-cell(49-50%), morula(38-40%) and blastocyst (29-32%) stages at 96, 144, and 192 hours post-insemination, respectively($p<0.05$). Glutamine alone had no benefit on embryo development. When BSA was added to mTLP-PVA with 0.35mM phosphate, glutamine and 19 amino acids at 8, 48, 120 hours post-insemination, BSA significantly enhanced the developmental ability of embryos reaching ≧2-cell (74-77%), ≧8-cell (49-53%), morula(43-47%), and blastocyst(38-42%) stages at 48, 96, 144, and 192 hours post-insemination, respectively, regardless of the time of BSA addition.

Key words :

Introduction

Bovine oocyte isolated from ovary have been spontaneously matured[1] and fertilized in vitro[2]. In many labolatories, bovine oocyte matured/fertilized in vitro can develop into morulae/blastocyst in chemically defined, protein-free culture medium[3]. However, the developmental ability of in vitro matured oocytes was lower than those matured in vivo[4]. Biochemical requirement for in vitro development of mammalian embryos have not been defined[5]. Energy substrates and embryo

† Corresponding author

metabolism have attracted the attention of recent investigator. Glucose is determined to be deterimental to development of preimplantation hamster[6-7], mouse[8], and bovine[9-10] embryo. Pregnant mouse oviduct secrete first pyruvate and then lactate at high levels during the first 5 days after ovulation[11]. This suggests that lactate and pyruvate are normally preferred as metabolic substrate for the early embryo[12].

The effects of amino acid on in vitro development have been studied. Glutamine has been implicated as important amino acid in the maturation of hamster oocytes[13], and development in vitro of pig[14], hamster[15], mouse[8] and bovine[9]embryos. Glutamine is five-carbon amino acid. This amino acid is converted into glutamate, which is then oxidatively deaminated to yield α-ketoglutarate[16]. Bovine embryos can utilize glutamine as energy substrate in place of glucose via its oxidation to CO_2 through TCA cycle[17]. Glutamine is also required for the synthesis of purine and pyrimidine base necessary for nucleic acid synthesis[18-19]. The additions of glycine, alanine, or glycine and alanine combined enhence development of in vitro matured and fertilized bovine embryos[12]. However, the contribution of protein and/or amino acid to development of preimplantation mammalian embryos has not been clearly defined. BSA enhence the development in vitro of preimplantation mouse embryo only when media is prepared from relatively impure water that have the potential of contributing embryo toxic substance to the media[20]. Both fration V-BSA and fatty acid free BSA support high incidence of fertilization and first cleavage in hamster embryos[21]. Preimplantation mouse embryos take up whole ^{125}I-labelled BSA via an endocytotic process from their environment[22]. Glucose and inorganic phosphate(P_i) are responsible for the cell block in hamster[6,23] and rat[24] embryos.

The present studies were undertaken to determined the effects of phosphate, glutamine and BSA the development in vitro of bovine embryos in chemically defined culture medium.

Material and Method

Culture media

The basic oocyte maturation medium was a glucose-free tissue culture medium(TCM)-199 (with Earl's salts) buffered with 25mM 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 6.1mg/ml from the original product(Cat. no. 400-1100).

The basic medium used for the treatment of spermatozoa and fertilization of oocytes was essentially the same as that used by Brackett and Oliphant[25] 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 112.0mM NaCl, 2.25mM $CaCl_2$, 0.83mM NaH_2PO_4, 0.52mM $MgCl_2$, 37.0mM $NaHCO_3$, 1.25mM sodium pyruvate and 31μg sodium penicillin G/ml.

A chemically defined medium was used for culture of embryos. The basic medium was a modified Tyrode's solution comprised 110.0mM NaCl, 3.2mM KCl, 2.0mM $CaCl_2$, 0.5mM $MgCl_2$, 25.0 mM $NaHCO_3$, 10.0mM sodium lactate, 0.5mM sodium pyruvate and 1mg polyvinyalcohol(PVA) /ml and designated as modified(m) TLP -PVA.

In vitro maturation(IVM)

Cow ovaries from random breed were collected

immediately post mortem at a local slaughterhouse and transported to the laboratory in 0.9% NaCl solution at 30 to 35℃ within 2 hours. The ovaries were pooled regardless of stages of estrous cycle of donors. Follicular oocytes were aspirated from small follicles(3−5mm) using a 18-gauge hypodermic needle attached to a 10-ml disposable syringe, then pooled and allowed to settle in 10-ml test tube. The supernant was discarded and sediment were deposited to a little amount of a maturation medium in a watchglass. By use of a low-power(40×), only cumulus-intact oocytes with evenly granulated and pigmented cytoplasm were selected from follicular fluid with a fine-tip pipette. The oocyte-cumulus complexes(OCCs) were washed three time in maturation media.. A group of 30 to 50 randomly selected oocytes were transferred into 50μl maturation medium, mTCM−199 supplemented with 5.56mM glucose, 5mM hemicalcium lactate, 0.4mM sodium pyruvate, 10%(v/v) heat-treated(56℃, 30min) fetal bovine serum(FBS ; Wheaton Scientific, Millville, NJ., USA), 60μg sodium penicillin G/ml and 100μg 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.

In vitro fertilization(IVF)

The OCCs contained in each drop of maturation medium were removed, washed four times and introduced into 50μl drop of BO medium supplemented with 20mg BSA(crystallized and lyophilized, essentially fatty acid-free, Sigma Chemical Co., St. Louis, MO, USA)/ml and 20μg porcine intestinal mucosal heparin(181 USP units/mg ; Sigma Chemical Co.)/ml under paraffin oil in a polystyrene culture dish(35×10mm, 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.

For each experiment, a 0.5ml 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 10mM caffeine-benzoate(3.383mg/ml, Sigma Chemical Co.). The final sperm pellet was resuspended in the same medium as used for washing to give a sperm concentration of $2-4\times10^6$ spermatozoa/ml. A 50μl of the sperm suspension was introduced into 50μl of the medium that included the OCCs for fertilization. The mixture gave final concentrations of $1-2\times10^6$ spermatozoa/ml, 10mg BSA/ml, 10μg heparin/ml, and 5mM caffeine and incubated at 39℃ in 5% CO_2 in air with high humidity for 8 hours.

In vitro embryo development

At 8 hours post-insemination, the oocytes in each drop were stripped of cumulus cell by passing them through a fine-tip pipette and then were washed four times and 10−25 oocytes were placed into 100μl 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.

Experimental studies

In experiment 1, to examine the effect of phosphate on the development of bovine IVM/IVF embryos, embryos were cultured in mTLP-PVA supplement with 19 amino acids and no glucose. The phosphate concentration of mTLP-PVA were

adjusted to 0.0, 0.10, 0.35, 1.05, and 2.10mM by the concentration of sodium phoshpate. In experiment 2, effects of glutamine and 19 amino acid in mTLP-PVA with 0.35mM phosphate on supporting bovine embryo development were examined in complete 2×2 factorial experiment. During bovine embryos are developing, effect of protein in mTLP-PVA were also studied in experiment 3. Embryos were firstly cultured in mTLP-PVA with 0.35mM phosphate, glutamine and 19 amino acid and then transferred into the medium additionally supplemented with 3 mg fatty acid-free BSA/ml at 8, 48, and 120 hours post-insemination.

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.

Results

The effect of phosphate in presense of 19 amino acid was evaluated. As shown in table 1, we examined five concentration of sodium phosphate in glucose-free mTLP-PVA : 0.00, 0.10, 0.35, 1.05 and 2.10mM. Although there were no significant different in developmental ability of IVM/IVF bovine embryos cultured in the medium containing from 0.00 to 1.05 mM phosphate until 48 hours post-insemination, the embryos in 2.10 mM phosphate were significantly inhibited($p<0.05$). Development to ≥8-cell and morula in medium with 0.35 mM phosphate at 96 and 144 hours post-insemination, respectively, was significantly higher than other concentration of phosphate($p<0.05$). There was no significant difference between 0.10 (18%) and 0.35 mM phosphate (24%) in supporting development to blastocyst, but all other concentration decreased the proportion of embryos reaching the blastocyst stage(1−13%)($p<0.05$).

The role of glutamine and 19 amino acids to support blastocyst development from the zygote is shown in table 2. No significant different were observed in the proportion of embryos reaching ≥ 2-cell stages among the medium with glutamine and/or amino acid. However, the proportion of

Table 1. Effects of different concentrations of phosphate in mTLP-PVA with amino acid on development of bovine IVM/IVF embryos in vitro*

Concentration of phosphate (mM)	No. of oocytes inseminated	No. and (%)† of embryos developed to			
		≥2-cell [48]‡	≥8-cell [96]‡	Morula [144]‡	Blastocyst [192]‡
0.00	68	49(72)[a]	31(46)[a]	20(29)[a]	7(10)[a]
0.10	68	51(75)[a]	31(46)[a]	23(34)[a]	12(18)[bc]
0.35	71	52(73)[a]	40(56)[b]	31(44)[b]	17(24)[b]
1.05	72	50(69)[a]	32(44)[a]	22(31)[a]	9(13)[c]
2.10	69	39(57)[b]	23(33)[c]	11(16)[c]	1(1)[d]

* Experiments were repeated 7 times.

† Percentage of the number of oocytes inseminated.

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

a-b Within each column, values with different superscripts are significantly different ($p<0.05$ at least).

Table 2. Effects of glutamine(1.0 mM) and/or 19 amino aicd in mTLP-PVA with 0.35 mM phosphate on development of bovine IVM/IVF embryos in vitro*

Medium with(+) or without(−)		No. of	No. and(%)† of embryos developed to			
Amino acid	Glutamine	ooctyes inseminated	≥2-cell [48]‡	≥8-cell [96]‡	Morula [144]‡	Blastocyst [192]‡
+	+	72	53(74)	35(49)[a]	29(40)[a]	23(32)[a]
−	+	73	51(70)	26(36)[b]	14(19)[b]	2(3)[b]
+	−	72	52(72)	36(50)[a]	27(38)[a]	21(29)[a]
−	−	70	49(70)	24(34)[b]	12(17)[b]	3(4)[b]

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

† Percentage of the number of oocytes inseminated.

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

a-b Within each column, values with different superscripts are significantly different ($p<0.05$ at least).

Table 3. Effects of bovine serum albumin (3mg/ml) added to mTLP-PVA with 0.35mM phosphate, glutamine and amino acids at various times post-insemination on development of bovine IVM/IVF embryos in vitro*

Time of BSA addition(hours post-insemination)	No. of oocytes inseminated	No. and (%)† of embryos developed to			
		≥2-cell [48]‡	≥8-cell [96]‡	Morula [144]‡	Blastocyst [192]‡
8	69	51(74)	34(49)	31(45)	26(38)[a]
48	72	54(75)	38(53)	34(47)	30(42)[a]
120	73	54(74)	37(51)	33(45)	29(40)[a]
No addition	69	53(77)	35(51)	30(43)	20(29)[b]

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

† Percentage of the number of oocytes inseminated.

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

a-b Within each column, values with different superscripts are significantly different ($p<0.05$ at least).

embryo developing ≧8-cell(49−50%), morula (38−40%) and blastocyst(29−32%) stages at 96, 144, and 192 hours post-insemination, respectively, were significantly higher in the presence than absence of 19 amino acids regardless of presence or absence of glutamine($p<0.05$).

When BSA was added to mTLP-PVA with 0.35 mM phosphate, glutamine, and 19 amino acids at 8, 48, 120 hours post-insemination, the developmental ability of IVM/IVF bovine embryos is shown in table 3. According to the time of BSA addition, there were no significant different in the proportion of embryos developing ≧2-cell (74−77x), ≥8-cell (49−53%) and morula(43−47%) stages at 48, 96, and 144 hours post-insemination, respectively. However, the proportion of embryo developed to blastocyst stage was significantly inceased when embryos were cultured in

the medium with BSA regardless of the time of addition($p<0.05$).

Discussion

The result of present study clearly indicates that an adequate concentration(0.35mM) of phosphate supplementation in mTLP-PVA with amino acid and no glucose is essential for the development of IVM/IVF bovine embryos to the 8-cell, morula and blastocyst stages. The similiar results have been reported that development of bovine[3] one-cell and porcine[14] one and two-cell embryos are not affected by the presence of phoshpate alone, but are inhibited by glucose alone[10,26]. There have been no comparable reports about the effect of phosphate concentration on development of IVM/IVF bovine embryo in the chemically defined condition. It is quite different from other species embryos. In hamster, two6) and four-cell [23,26] embryos are highly sensitive to the presence of phosphate, but eight-cell embryos are not inhibited by the presence of phosphate[7]. In rat, the development of rat one-cell embryo is completely blocked at the two-cell stage by phosphate[24], but phosphate does not affect the development of rat eight-cell embryo[27], regardless of glucose.

It has been suggested that glucose/phosphate inhibition is due to enzymic reation of glycolysis competing with Krebs cycle and stimulate cellular glycolysis[6,7]. Possible explanation about the effect of phosphte may be found in the way phosphate alone can not enhance glycolysis in bovine embryos and bovine embryo can utilize phosphate in vitro. It is worth noting that change in sensitivity of mammalian embryos to phosphate may be related not only to species but also to the developmental stages of embryos.

Amino acids(BME essential amino acids, MEM non essential amino acid)stimulated bovine embryo development. Oviductal and uterine fluids of mammals contain significant level of amino acids which are relatively high level of the amino acid analine, asparate, asparagine, glycine, glutamate, glutamine and taurine[28-31]. All of these amino acids is the nonessential amino acid except taurine and glutamine. Present study revealed that maximal development to the blastocyst occured in the presence of all of amino acids. It has been determined that the earlier 2- and 4-cell embryos require more extensive amino acid supplements 23,32) while fewer amino acids are necessary for the development of the later embryos[31].

It has been reported that glutamine is also required for the synthesis of purine and pyrimidine base necessary for nucleic acid synthesis[18-19] and the enregy substrate in place of glucose via its oxidation to CO_2 through TCA cycle[17] and the addition of glutamine has proven benefical to the maturation of hamster oocytes[13], and development in vitro of pig[14], hamster[15], mouse[8], and bovine[9] embryos. However, glutamine alone could not support the development of bovine 1-cell to blastocyst.

The results of this study indicate that success of blastocyst formation was significantly inceased when embryos were cultured in the medium with BSA regardless of the time of addition($p<0.05$). Two-cell mouse embryos, morulae and blastocysts all can take up BSA via an endocytotic process. Uptake by blastocyst is 7 times greater than that by morulae and 2-cell embryos[22]. BSA enters into embryo as an acid-insoluble macromolecule which is rapidly degraded by lysosomal activity[33] and released as an acid-soluble product to the medium [22]. The catabolism of BSA may be a considerable potential source of amino acids to blastocyst. Since the medium used in this study has 19 amino acids, it is not possible to determine whether the

stimulatory effect of BSA is a potential source of amino acids or not. When media is prepared from TAP water(purified only by the city's municipal water treatment plant), supplementation of the media with BSA cause a significant enhancement of embryo development but when media are prepared from water samples purer than TAP water, BSA cause no significant improvement in embryo devlopment over that seen in BSA-free media[20]. It suggests that role of BSA in culture medium is protection of embryo from the effects of potentially embryotoxic substances in the medium.

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초록 : 한정 배양액에서 포유동물 수정란의 체외 발생에 인, 아미노산 및 BSA가 미치는 영향

김종홍 · 김병기†(동의대학교 생물학과)

본 연구는 포유동물 수정란의 체외발생에 인, 아미노산과 BSA가 미치는 영향을 조사하기 위하여 체외성숙 및 수정된 소의 수정란을 한정 무단백 배양액에서 배양하였다. 배양액에 포함된 인의 농도를 0.00, 0.10, 0.35, 1.05와 2.10mM로 조정하였을 때 0.00부터 1.05mM 농도에서는 수정후 48시간 까지 수정란 발생에 영향을 미치지 않았고, 수정후 96시간과 114에서의 8세포기와 상실배 발생이 0.35mM에서 유의적($P<0.05$)으로 증가하였다. 19종 아미노산의 첨가는 수정후 96, 144, 192시간에 각각 8세포기(49－50%), 상실배(38－40%), 배반포(29－32%)발생을 유의적($P<0.05$)으로 증가하였으나, glutamine단독 첨가는 영향이 없었다. BSA첨가는 첨가시간에 관계없이 수정후 48, 96, 144, 192시간에서 각각 2세포기, 8세포기, 상실배와 배반포의 발생을 증가시켰다.