Effects of various nutrients and renewal of medium on *in vitro* development of bovine oocytes matured and fertilized *in vitro*

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Abstract

Effects of glutamine/amino acids, insulin, taurine/hypotaurine, glucose and renewal of culture medium on *in vitro* development of bovine oocytes matured, fertilized and cultured in synthetic oviduct fluid medium (SOFM) supplemented with human serum (HS) *in vitro* were investigated in five experiments. In each experiment, *in vitro*-matured and-inseminated oocytes (zygotes) were cultured for 10 days in SOFM supplemented with 10% HS containing 0.44mM glucose. Additions of glutamine (1 mM) and Eagle's essential amino acids (EAA) resulted in significantly higher rates of expanding (24.5%) and hatched (12.1%) blastocysts than those without the additives (12.3 and 7.7%). Insulin (10 μg/ml) did not improve either cleavage (2 – 8 cells) or development to blastocysts. The addition of taurine (10 mM) significantly increased cleavage rate, but the beneficial effect of taurine, hypotaurine (5 mM) or both on blastocysts formation was not observed. The addition of glucose (1.5mM) and the increase of glucose level (0.44 to 1.94mM) on Day 5 after insemination were not effective for embryo development. The reported beneficial effect of renewal of the medium every 48h was not found in the present culture system using SOFM supplemented with HS, regardless of the additional glucose.

Key Words : amino acid, glucose, *in vitro* development, oocyte, cattle

Introduction

In vitro bovine embryo production through *in vitro* maturation (IVM) and fertilization (IVF) has been improved tremendously during the past decade[1]. *In vitro* produced bovine embryos have been routinely utilized for research purposes and for large-scale embryo transfer[2]. However, the quality and survival rate of *in vitro* produced embryos after freezing and thawing is generally less than for embryos produced *in vivo*. Many different culture systems have been attempted to produce in vitro bovine embryos using co-culture with various somatic cells[3~7],

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conditioned medium[8], serum-and cell-free defined media such as synthetic oviduct fluid medium (SOFM)[9-10], CR 1[11], or other chemically defined media[12-15].

To investigate the metabolism of individual embryos during in vitro culture (IVC), a cell-serum-or protein-free, chemically defined medium should be developed. However, viability of embryos developed *in vitro* by the culture with completely protein-free, chemically defined media has not been confirmed and pregnancy following transfer has not been reported. Recently, Pugh et al.[16] reported that the use of SOFM supplemented with bovine serum albumin (BSA) and amino acids has showed satisfactory pregnancy rates after transfer the IVF -derived, fresh (71%) or frozen-thawed (31%) bovine embryos.

The most appropriate culture method for IVC in many laboratories is still a co-culture system with somatic cells and serum. At this time, a medium which is both chemically defined and produces optimal in vitro development has not been developed.

In the present study, to establish a better culture medium containing serum for IVC, we have investigated the effects of various nutrients (glutamine, amino acids, insulin, taurine, hypotaurine and glucose) and renewal of culture medium on *in vitro* development of IVM and IVF bovine oocytes in SOFM supplemented with human serum (HS).

Materials and Methods

In vitro Maturation

Ovaries were obtained from Holstein cows and heifers killed at a local slaughterhouse and were transported in saline (9 g NaCl/l) at 30−35℃ to the laboratory within 1 h. The cumulus-oocyte complexes were collected from follicles of 2 − 5 mm in diameter with an 18-gauge needle attached to a 5 −ml disposable syringe. only oocytes with an unexpanded cumulus oophorus and evenly granulated cytoplasm were selected and cultured in a 4 −well plate (Nunclon, Roskide, Denmark) containing 0.5ml of Tissue Culture Medium (TCM)−199 (Earle's salts with L-glutamine and without sodium bicarbonate ; Flow Laboratories Inc., Scotland, pH 7.4) supplemented with 10% (v/v) heat-inactivated (56℃, 30 min) fetal calf serum (FCS) and 25 mM $NaHCO_3$. The medium was also supplemented with 2.5 μg FSH−B− 1 /ml (U. S. Department of Agriculture, Baltimore, ML, USA), 5 μg LH−B− 5 /ml (National Hormone and Pituitary Program, Baltimore, ML, USA) and 1 μg estradiol /ml (Sigma chemical Co., St. Louis, MO, USA) and with 2×10^6 granulosa cells/ml. Granulosa cells were collected from antral follicles of about 10 mm in diameter after dissection by the method of Moor and Trounson[17] and washed (500× g, 10 min) twice with TCM−199 (pH 7.4) containing 10 mM Hepes, 2 mM $NaHCO_3$ and 0.3% (w/v) BSA (fatty acid free, Fraction V ; Sigma). The oocytes were statically cultured for 24 h at 39℃ under an atmosphere of 5 % CO_2 in air and > 95% humidity.

In vitro Fertilization

Each 0.5 ml frozen straw of semen from three Holstein bulls was thawed at 37℃ and was prepared for sperm capacitation. The thawed semen was pooled and layered (0.25 ml) under 1 ml of a modified Tyrode's calcium-free medium (pH 7.4 : capacitation medium) in conical tubes (Becton, Dikinson Labware, Lincoln park, NJ, USA) for a swim - up procedure[18-19]. The top 0.8 ml medium was then collected after incubation for 1 h at 39℃. The pooled medium containing spermatozoa was washed (500×g, 10 min) twice with the capacitation medium. The final pellet of semen was re-suspended with the medium to a concentration of 50×10^6 spermatozoa/ml. An equal volume of the medium containing 200μg heparin/ml (Grade I for porcine intestinal mucosa : Sigma) was added to the

semen suspension to yield sperm and heparin concentrations of 25×10^6 cells/ml and 100 μg/ml, respectively. The heparin-treated spermatozoa were incubated for 15 min at 39℃ in 5 % CO_2 in air and > 95% humidity.

The oocytes cultured for 24 h were gently washed three times with a modified Tyrode's medium (pH 7.4 : washing medium) containing 2 mM $CaCl_2$ and 2 mM $NaHCO_3$. An aliquot (3 μl) of the washing medium containing 5 expanding cumulus-enclosed oocytes was placed into 43 μl drops of a modified Tyrode's fertilization medium (pH7.8)[19] under mineral oil (E. R. Squibb & Sons Inc., Princeton, NJ, USA). Then, 4 μl of the heparin-treated semen suspension was added to give a final sperm concentration of 2×10^6 cells/ml. Oocyte and spermatozoa were incubated for 18－20 h at 39℃ under 5 % CO_2 in air.

In vitro Culture

After the culture, all oocytes were denuded from cumulus cells by repeated pipetting and 5－10 oocytes (zygotes) were cultured in 24－well plates (Becton) containing 0.5 ml of SOFM supplemented with 10% HS for 10 days in experiments 1 to 5. SOFM was made using deionized, 17 MQ water (Mili－Q Lab., Japan) according to the method of Tervit et al.[20]. The HS was obtained from ten healthy young men and heat-inactivated (56℃, 30 min) after pooling. During the culture, embryonic development was examined at Days 3 (2－8 cell stage), 8 (expanding blastocysts) and 10 (hatched blastocysts) after insemination (Day of insemination＝0).

Experiment 1

Effect of glutamine (1 mM) and Eagle's essential amino acids (EAA : 1 % v/v, Basal Medium Eagle Amino Acids Solution, Gibco BRL : Life Technologies Inc., Grand-island, NY, USA) added to SOFM＋10% HS was investigated. Replicates were run 5 times in different days, and in each replicate about half numbers of inseminated oocytes were cultured in SOFM + HS supplemented with glutamine and EAA, and the remaining oocytes were cultured in the medium without glutamine and EAA. During the culture for 10 days, the embryos were moved to fresh medium every 48 h. The moving embryos to fresh medium every 48 h was also performed in the following experiments.

Experiment 2

Effect of insulin (10 μg/ml : bovine pancreas, Sigma) added to SOFM supplemented with 10 %HS, glutamine (1 mM) and EAA solution (1 %, v/v) was investigated. Replicates were run 3 times in different days, and in each replicate the numbers of inseminated oocytes were divided into two groups with or without insulin.

Experiment 3

Effects of taurine (T : 10 mM, Sigma), hypotaurine (H : 5 mM, Sigma) or both added to SOFM supplemented with 10% HS, glutamine (1 mM) and EAA solution (1 %, v/v) were investigated. Replicates were run 3 times in different days, and in each replicate the numbers of inseminated oocytes were divided into 4 groups (control, T, H, and both T and H).

Experiment 4

Effect of change of glucose levels during IVC was investigated. In this experiment, SOFM with (1.5mM) or without glucose were prepared and supplemented with 10% HS containing 0.44 mM glucose, glutamine (1 mM), EAA (1 %, v/v) and taurine (10 mM). Four replicates were carried out in different days, and in each replicate the numbers of inseminated oocytes were divided into 3 groups ; no glucose addition throughout the culture (Group1), no glucose addition from Day 1 to Day 4 and the addition of glucose (1.5mM) from Days 5 to 10 after insemination (Group 2) and with glucose

(1.5mM) throughout the culture duration (Group 3).

Experiment 5

Effects of glucose addition and renewal of culture medium every 48 h during IVC were investigated. The two glucose levels (Groups 1 and 3 in Experiment 4) were compared, and in each group the medium of about half numbers of inseminated oocytes was changed to fresh medium every 48 h and the medium for the remaining oocytes was not changed through out the culture duration. Three replicates were carried out in different days, and in each replicate the numbers of inseminated oocytes were divided into 4 groups (two glucose levels with or without renewal of medium).

Statistical Analysis

In all experiments, frequencies in the proportions of cleavage, expanding and hatched blastocysts were expressed by mean ± SEM. After an arc-sine transformation, the proportions were analyzed by analysis of variance using general linear model of Statistical Analysis Systems.

Results

Experiment 1

The addition of glutamine and EAA did not improve cleavage rate (51.0 and 47.3%), but the mean proportions of expanded (24.5%) and hatched (12.1%) blastocysts were significantly ($P < 0.01$) higher in SOFM + 10% HS supplemented with glutamine and EAA than those in the medium without the additives (12.3 and 7.7%) (Table 1).

Experiment 2

Additional effect of insulin in SOFM supplemented with 10% HS, glutamine and EAA did not find in either cleavage (76.1 and 67.2%) or development to expanding (18.7 and 20.8%) and hatched (9.0 and 10.8%) blastocysts (Table 2).

Table 1. Effect of addition of glutamine and EAA in SOFM supplemented with 10% HS on in vitro development of bovine oocytes matured and fertilized in vitro (mean±SEM)

With(+)or without(−) glutamine and EAA	No. oocytes cultured (No. trials)	% oocytrs cleaved (2–8cell)	% oocytes develped to	
			Expanding BL[a]	Hatched BL[a]
+	123 (5)	51.0±5.4	24.5±6.0[b]	12.1±2.9[b]
−	121 (5)	47.3±3.1	12.3±2.1[c]	7.7±1.4[c]

[a]Blastocyst.
[b, c]Values with different superscripts differ ($P < 0.01$)

Table 2. Additional effect of insulin in SOFM supplemented with 10% HS, glutamine and EAA on in vitro development of bovine oocytes matured and fertilized in vitro (mean±SEM)

With(+)or without(−) insulin	No. oocytes cultured (No. trials)	% oocytrs cleaved (2–8cells)	% oocytes develped to	
			Expanding BL	Hatched BL
+	172 (3)	76.1±4.1	18.7±3.0	9.0±0.7
−	164 (3)	67.2±0.5	20.8±2.3	10.8±1.9

Experiment 3

Addition of taurine to SOFM supplemented with 10% HS, glutamine and EAA significantly ($P<0.05$) increased cleavage rate as compared with control (81.0 and 70.9%, respectively). Although the additions of taurine, hypotaurine or both tended to improve the development rates of expanding and hatched blastocysts, significant differences were not revealed among the four groups (Table 3).

Experiment 4

Glucose levels during IVC of inseminated oocytes did not affect cleavage and development rates, although the Group 1 without the additional glucose had higher rates of expanding (24.1%) and hatched (14.1%) blastocysts as compared with the Group 2 (16.4 and 11.9%) which increased glucose levels (0.44 to 1.94 mM) on Day 5 after insemination and with Group 3 (17.1 and 9.3%) containing the additional glucose (1.5 mM) throughout the culture duration (Table 4).

Experiment 5

Advantages of renewal of culture medium at 48 h intervals were not observed in either cleavage or development to blastocysts. (Table 5). The proportion of hatched blastocysts in the group changing the medium every 48 h was lower than the group without the renewal of medium (8.2 and 15.8%), but these values were not significantly different. Addition of glucose (1.5mM) to SOFM + 10% HS had no effects on cleavage rate (77.1 and 79.9%), and development to expanding (19.8 and 19.4%) and hatched (8.9 and

Table 3. Effects of taurine (T), hypotaurine (H) and their combination added to SOFM supplemented with 10% HS, glutamine and EAA on in vitro development of bovine oocytes matured and fetrtilized in vitro (mean±SEM)

Additives	No.oocytes cultured (No.trials)	%oocytes cleaved (2–8cells)	% oocytes developed to	
			Expanding BL	Hatched BL
Control	119 (3)	70.9±1.3[a]	8.9±1.5	5.7±0.3
T	119 (3)	81.0±1.2[b]	19.5±7.8	15.7±4.9
H	122 (3)	73.8±4.5	14.4±4.5	7.0±3.2
T+H	122 (3)	75.2±0.2	13.7±4.1	7.6±2.5

[a, b] Values with different superscripts differ ($P<0.05$)

Table 4. Effect of glucose addition during IVC on in vitro development of bovine oocytes matured and fertilized in vitro (mean±SEM)

Glucose addition (Group)[a]	No. oocytrs cultured (No. trials)	% oocytes cleaved (2–8cells)	% oocytes developed to	
			Expanding BL	Hatched BL
−(1)	209 (4)	67.2±3.6	24.1±3.7	14.1±3.7
±(2)	201 (4)	69.3±3.4	16.4±2.0	11.9±2.5
+(3)	194 (4)	71.7±4.0	17.1±4.5	9.3±4.2

[a] Group 1: glucose was not added to the medium (SOFM+10% HS),
Group 2: glusose was not added until Day 4 and 1.5 mM glucose was added on Day 5 after insemination,
Group 3: 1.5mM glucose was added to the medium.

Table 5. Effects of renewal of medium added with or without glucose on in vitro development of bovine oocytes matured and fertilized in vitro (mean±SEM)

Medium change[a]	Glucose addition[b]	No. oocytes cultured (No. trials)	% oocytes cleaved (2–8cell)	% oocytes developed to	
				Expanding BL	Hatched BL
+	−	159 (3)	80.8±5.2	17.6±6.5	8.8±4.4
+	+	154 (3)	82.8±5.3	21.2±9.0	7.6±2.2
Sub−total		313 (3)	81.8±4.0	19.4±7.6	8.2±3.3
−	−	168 (3)	79.3±1.0	21.0±9.7	12.2±6.2
−	+	167 (3)	71.7±3.5	19.2±4.3	10.2±3.9
Sub−total		335 (3)	75.5±1.7	20.1±6.8	11.2±1.4

[a]Medium was renewed(+)every 48h or not (−) for the culture duration.
[b]Glucose (1.5mM) was added (+) or not (−) to SOFM supplemented with 10% HS, glutamine and EAA.

10.6%) blastocysts as compared with the medium without glucose addition. There was no interaction in cleavage and development rates between renewal of medium and glucose addition.

Discussion

The present study was carried out to establish an appropriate culture medium based with SOFM +10% HS for in vitro development of bovine oocytes matured and fertilized in vitro. HS has been utilized successfully instead of FCS, BSA or other proteins in various culture media including SOFM for the culture of sheep[21, 22], goat[23] and cattle[9, 24] embryos.

The additions of glutamine and EAA to the medium significantly improved the proportions of expanding and hatched blastocysts (Experiment 1) and have shown the advantage even with the presence of serum (HS). The present result has accorded with the reports in pig[25], sheep[26] and cattle[10, 11]. Glutamine and amino acids are major nutritional source for many somatic cells[27] and metabolized by mouse, sheep and cattle embryos in vitro[28–30]. supplementation of glutamine and/or specific amino acids (essential : EAA and non-essential : NEAA amino acids) has been demonstrated to increase blastocysts formation in mouse[30, 31], sheep[22, 26] and cattle[9, 10, 32]. However, it has not been identified which amino acid (EAA, NEAA or certain amino acids) is the most important and how they or it contribute (s) to enhance the embryo development in the culture. Gardner and Lane[30] described that embryos at the early and late stages may have different amino acid requirements. Recently, Lane and Gardner[31] demonstrated in mouse that only EAA significantly improved fetal development and weight over the control after transfer of in vitro cultured embryos.

Furthermore, as glutamine and amino acids are metabolized by embryos and break down at 37 ℃ to produce ammonium in the culture medium which is detrimental to embryo development *in vitro*[30], it is necessary to renew the medium every 48 h if glutamine and amino acids are included in the culture medium[16, 31, 32]. However, the requirement for media replacement is controversial. The renewal of culture medium every 48 h is labor-consuming and may be reversely detrimental for embryo development by changing pH and gas atmosphere and by lowing the temperature of culture medium. As an alternative method, Lane and Gardner[33] developed a method

of enzymatical removing ammonium from the culture medium. More researches are needed to maximize the significant role of amino acids on in vitro development of IVM-and IVF-derived bovine zygotes. From the positive result in Experiment 1, we added both glutamine and EAA to SOFM supplemented with 10% HS throughout the present study. Insulin enhances cleavage and morphological development of preimplantation mouse embryos[34] by stimulating amino acids transport[35] and protein synthesis[35]. However, in the present study a positive effect of insulin addition was not found in the culture with SOFM containing HS, glutamine and EAA. Zhang and Armstrong[37] reported that the addition of insulin alone to serum-free medium had little effect on rat embryo development *in vitro*, but insulin with amino acids acted synegistically to improve the rate of development. From the fact that the present culture medium has contained both HS and EAA, the clear reasons for not improving the development of IVF-derived bovine zygotes are unknown.

Beneficial effects of supplementation of taurine and /or hypotaurine in culture medium for embryo development have been reported in mouse[38], hamster[39], pig[40, 41] and cattle[42]. In the present study, a positive effect was only found in cleavage rate with the addition of taurine (10 mM) alone. Although the proportions of developed blastocysts tended to be higher in the culture medium supplemented with taurine and/or hypotaurine than that without the both, there was no significant difference in the present study in contrary to the reports in pig[40, 41] and cattle[42]. The effect of hypotaurine on development of hamster zygotes is dependent on the time of post-fertilization[39]. Furthermore, the addition of hypotaurine has been shown to increase the frequencies of *in vitro* fertilization in bovine and hamster oocytes and the subsequent cleavage[43, 44]. Taurine is present at a high concentration in the fluids of the reproductive tract and represents 50, 17 and 39% of the total amino acids pool in the mouse egg, morula and blastocysts, respectively[45]. It seems from the present result and the report of Lim et al.[42] that taurine probably enhances the *in vitro* development of IVF-derived bovine oocytes, but the beneficial effect in a combination with hypotaurine is still questionable. However, cysteamine which is a precursor of hypotaurine and is converted to hypotaurine by oxidation *in vitro* [46], facilitates the development of IVF-derived bovine 6 – 8 cell embryos to blastocysts when it is included in culture media[47].

The metabolism of glucose increases markedly from the 2 –cell to the blastocysts stage in mammalian embryos[48-50]. For bovine embryos, glucose is essential for blastocysts formation[14, 15, 24, 51, 52] and hatching process[53] as increased glucose uptake and metabolism. However, the requirement for glucose is controversial and is dependent on embryonic stages. It has been demonstrated that a high (3 – 5 mM) glucose in culture media inhibits the development of hamster[53], sheep[22], pig[48] and cattle[24] embryos. On the other hand, glucose (<3 mM) is necessary at the later stage of embryo development in sheep[22] and cattle[14, 15, 24]. The addition of glucose (2.78 or 5.56 mM) at Day 5 after insemination increased the development of blastocysts and their expansion when a simple chemically defined, protein-free medium (mTALP)[14] or the medium supplemented with either BSA or calf serum[15]. However, in contrary to our previous study[24] and the other studies[14, 15], the increase of glucose level (from 0.44 to 1.94 mM) at Day 5 after insemination was not beneficial for the development to expanding and hatched blastocysts in the present study. Also, the addition of glucose (1.5 mM) originally included in SOFM did not inhibit cleavage and development rates. In the present study, SOFM was supplemented with 10% HS containing 0.44 mM glucose, glutamine, and taurine, and the

inhibitory effect of glucose appears to be prevented by the presence of amino acids[32] including taurine.

When culture medium was supplemented with glutamine and/or amino acids, the renewal of medium every 48 h during the culture duration is necessary, as discussed earlier. In the present study, however, the beneficial effect of renewing culture medium has not confirmed. Some reasons considered for this controversy would be different culture methods in the present and other studies. Most workers[16, 28, 31, 32] have cultured 4 – 10 embryos per drop (20 – 30 μl) covered with oil using SOFM supplemented with BSA (8 mg/ml) and amino acids. On the other hand, in the present study, 10 – 15 zygotes were cultured in 24 – well plate containing 0.5ml SOFM containing HS, glutamine, EAA and taurine without oil. Gardner et al.[55] measured the level of ammonium in a 20 – μl drop of medium containing all of the Eagle's amino acids and found a liner increase in ammonium concentrations with culture time. Although the ammonium levels in the medium during IVC was not determined in the present study, a large volume of the culture medium containing HS and an open system (24 – well plate without oil) in the group culture of embryos would be some reasons which had not been found the advantage of renewal of culture medium, regardless of the additional glucose. Embryos cultured in groups appears to be stimulated by an autocrine and/or paracrine factors produced in the culture[26, 31, 55]. These factors may be beneficial to embryos when ammonium concentration in the medium is increasing[50, 55].

The purpose of the present study was to develop an optimal culture medium for IVC of IVF-derived bovine zygotes in proceeding Experiments 1 to 5, so that we have not investigated interactions of nutrients examined (glutamine and / or amino acids × insulin, glucose levels × taurine and/or hypotaurine, and others). Further detailed roles of each energy source and their interactions should be examined in a chemically defined medium in future research.

Viability of blastocysts developed in the present study was investigated by the proportions of expanding and hatched blastocysts. The ultimate proof of the viability of embryos developed *in vitro* is pregnancy following transfer[1], but this has not been easy all the time to organize a large scale of transfer trials. Greve et al.[1] suggested that the capacity of blastocysts to hatch *in vitro* is known to be a critical point in development and an indicator of good viability. Not only the hatching process, but also post-thawing capacity of frozen embryos developed *in vitro* should be included in future research to assess the developmental capacity, unless a transfer trial is performed.

In conclusion, the present results indicate that additions of glutamine, EAA and taurine in SOFM supplemented with 10% HS improve the in vitro development of IVM – and IVF – derived bovine oocytes. For the culture after IVF, the addition of glucose and renewal of the medium every 48 h are unnecessary in the present culture method.

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References

1) GREVE T, MADISON V, AVERY B, CALLESEN H, HYTTEL P. In vitro production of bovine embryos : A progress report and the consequences on the genetic upgrading of cattle populations. Anim. Reprod. Sci., 33 : 51 – 69. 1993.

2) GORDON I, Lu KH. Production of embryos in vitro and its impact on livestock production. Theriogenology, 33 : 77 – 87. 1990.

3) Lu KH, GORDON I, M, MCGOVERN H.

Pregnancy established in cattle by transfer of embryos drived from in vitro fertilization of oocytes matured in vitro. Vet. Rec., 121 : 259 −260. 1987.

4) FUKUI Y, ONO H. In vitro development to blastocysts of in vitro matured and fertilized bovine oocytes. Vet. Rec., 122 : 282. 1988.

5) GOTO K, KAJIHARA Y, KOSAKA S, NAKANISHI Y, OGAWA K. Pregnancies after co-culture of cumulus cells with bovine embryos derived from in-vitro matured follicular oocytes. J. Reprod. Fertil., 83 : 753. 758. 1988.

6) AOYAGI Y, FUKUI Y, IWAZUMI Y, URAKAWA M, ONO H. Effects of culture systems on development of in vitro-fertilized bovine ova into blastocysts. Theriogenology, 34 : 749−759. 1990.

7) FUKUDA Y, ICHIKAWA M, NAITO K, TOYOSA Y. Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells in vitro up to the blastocysts stage. Biol. Reprod., 41 : 114−119. 1990.

8) EYESTONE WH, FIRST NL. Co-culture of early cattle embryos to the blastocysts stage with oviductal tissue or in conditioned medium. J. Reprod. Fertil., 85 : 715−720. 1989.

9) FUKUI Y, MCGOWAN LT, JAMES RW, PUGH PA, TERVIT HR. Factors affecting the in-vitro development to blastocysts of bovine oocytes matured and fertilized in vitro. J. Reprod. Fertil., 92 : 125−131. 1991.

10) TAKAHASHI Y. FIRST NL. In vitro development of bovine one-cell embryos : Influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology, 37 ; 963−978. 1992.

11) ROSENKRANS CF, Jr, FIRST NL. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in Vitro. J. Anim. Sci., 72 : 434−437. 1994.

12) PINYOPUMMINTR T, BAVISTER BD. In vitro. matured/in vitro-fertilized bovine oocytes can develop into morula/blastocysts in chemically defined, protein-free culture media. Biol. Reprod., 45 : 736−742. 1991.

13) BAVISTER BD, ROSE-HELLEKANT TA, PINYOPUMMINTR T. Development of in vitro matured/in vitro fertilized bovine embryos into morulae and blastocysts in defined culture media. Theriogenology, 37 : 127−146. 1992.

14) KIM JH,. NIWA K, LIM JM, OKUDA K. Effects of phosphate, energy substrates, and amino acids on development of in vitro-matured, in vitro-fertilized bovine oocytes in a chemically defined, protein-free culture medium. Biol. Reprod., 48 : 1320−1325. 1993.

15) KIM JH, FUNAHASHI H, NIWA K, OKUDA K. Glucose requirement at different developmental stages of in vitro fertilized bovine embryos cultured in semi-defined medium. Theriogenology, 39 : 875−886. 1993.

16) PUGH PA, THOMPSON JG, MCGOWAN LT, MCMILLAN WH, TERVIT HR. Survival after transfer of fresh or frozen bovine embryos produced in vitro in a cell-and serum-free medium. Proc. Aust. Soc. Reprod. Biol., 25 : 86 abstr. 1993.

17) MOOR RM, TROUNSON AO. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. J. Reprod. Fertil. 49 : 101−109. 1977.

18) PARRISH JJ, PARRISH JL, LEIDFRIED-RUTLDGE ML, CRITSER FS, EYESTONE WH, FIRST NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology. 25 : 591 −600. 1986.

19) FUKUI Y. Effect of follicle cells on the acrosome reaction, fertilization, and developmental conpetence of bovine oocytes matured in vitro. Molec. Reprod. Dev., 26 : 40−46. 1990.

20) TERVIT HR, WHITTINGHAM DG, ROWSON LEA. Successful culture in vitro of sheep and cattle ova. J. Reprod. Fertil., 30 : 493−497. 1972.

21) WALKER SK, QUINN P, ASHMAN RJ,

SMITH DH, SEAMARK RF. Protein supplementation for the culture of one-cell embryos of sheep. Proc. Aust. Soc. Reprod. Biol., 18 : 9 abstr. 1986.

22) THOMPSON JG, SIMPSON AC, PUGH PA, TERVIT HR. Requirement for glucose during in vitro culture of sheep preimplantation embryos. Molec. Reprod. Dev., 31 : 253－257. 1992.

23) BATT PA, GARDNER DK, CAMMERON AWH. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. Reprod. Fertil. Dev. 3 : 601－607. 1991.

24) MATSUYAMA K, MIYAKOSHI H, FUKUI Y. Effect of glucose levels during the in vitro culture in synthetic oviduct fluid medium on in vitro devlopment of bovine oocytes matured and fertilized in vitro. Theriogenology, 40 : 595－605. 1993.

25) PETRRS RM, JOHNSON BH, REED ML, ARCHIBONG AE. Glucose, glutamine and inorganic phosphate in early development of the pig embryos in vitro. J. Reprod. Fertil., 89 : 269－275. 1990.

26) GARDNER DK, LANE M, SPITZER A, BATT PA. Amino acids and increased embryo density stimulate development of sheep zygotes in vitro. Proc. Aust. Soc. Reprod. Biol., 24 : 90 abstr. 1992.

27) ZIELKE HR, ZIELKE CL, OZAND PT. Glutamine : a major energy source for cultured mammalan cells. Fed. Proc., 43 : 121－125. 1984.

28) CHATOT CL, TASCA RJ, ZIOMEK CA. Glutamine uptake and utilization by preimplantation mouse embryos in CZB medium. J. Reprod. Fertil., 89 : 335－346. 1990.

29) RIEGER D. Relationships between energy metabolism and development of early mammalian embryos. Theriogenoiogy, 37 : 75－93, 1992.

30) GARDNER DK, LANE M. Amino acids and ammonium regulate mouse embryo development in culture. Biol. Reprod., 48 : 377－385. 1993.

31) LANE M, GARDNER DK. Amino acids increase mouse embryo viability. Theriogenology, 41 : 233 abstr. 1994.

32) TROUNSON AO, PUSHETT D, MACLELLAN LJ, LEWIS I, GARDNER DK. Current status of IVFM／IVF and embryo culture in human and farm animals, Theriogenology, 41 : 57－66. 1994.

33) LANE M, GARDNER DK. In situ removal of embryo-toxic ammonium generated by the metabolism and breakdown of amino acids in culture media. Proc. Aust. Soc. Reprod. Biol., 25 : 32 abstr. 1993.

34) HARVEY MB, KAYE PL. Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts in vitro. Development, 110 : 963－967. 1990.

35) KAYE PL, PEMBLE LB, HOBBS JG. Protein metabolism in preimplantation mouse embryos. Prog. Clin. Biol. Res., 217B : 103－106. 1986.

36) HARVEY MB, KAYE PL. Insulin stimulates protein synthesis in compacted mouse embryos. Endocrinology, 122 : 1182－1184. 1988.

37) ZHANG X, ARMSTRONG DT. Presence of amino acids and insulin in a chemically defined medium improves development of 8－cell rat embryos in vitro and subsequent implantation in vivo. Biol. Reprod., 42 : 662－668. 1990.

38) DUMOULIN JCM, EVERS JLH, BRAS M, PIETERS MHEC, GERAEDTS JPM. Positive effect of taurine on preimplantation development of mouse embryos in vitro. J. Reprod. Fertil., 94 : 373－380. 1992.

39) BAVISTER BD, BARRETT DK, MCKIENAN SH. Hypotaurine stimulates in vitro development of early one-cell hamster embryos to the morula／blastocyst stage. Biol. Reprod., 44

(suppl 1) : 155 abstr. 1990.

40) PETERS RM, REED ML. Addition of taurine or hypotaurine to culture medium improves development of one-and two-cell pig embryos in vitro. Theriogenology., 35 : 253 abstr. 1991.

41) REED ML, ILLERA MJ, PETERS RM, In vitro culture of pig embryos. Theriogenology, 37 : 95−109. 1992.

42) LIM JM, NIWA K, KIM JH, OKUDA K. Effects of glutamine and taurine in a chemically defined medium on the development of bovine oocytes fertilized in vitro. J. Mamm. Ova Res., 10 : 98−99 (in Japanese). 1993.

43) LEIDFRIED ML, BAVISTER BD. Fertlizability of in vitro matured oocytes from golden hamters. J. Exp. Zool., 226 : 481−485. 1983.

44) MILLER GF, GLIEDT DL, LESTER TD, PIERSON JN, RAKES JM, RORIE RW. Addition of bovine oviductal epithelial cells (BOEC) and / or penicillamine, hypotaurine and epinephrine (PHE) to bovine in vitro fertilization (IVF) medium increases the subsequent embryo cleavage rate. Theriogenology, 37 : 259 abstr. 1992.

45) KAYE PL. Metabolic aspects of the physiology of the preimplantation embryo. In:Rossant J, Pedrsen RA (eds), Experimental Approches to Mammalian Embryonic Development, Cambridge University Press, 267−292. 1986.

46) HUXTABLE RJ. Physiological actions of taurine. Physiol. Rev. 72 : 101−163. 1992.

47) TAKAHASHI M, NAGAI T. HAMAMO S, KUWAYAMA M, OKUMURA N, OKANO A. Effect of thiol compounds on in vitro development and intracellular glutathione conntent of bovine embryos. Biol. Reprod., 49 : 228−232. 1993.

48) FLIID MR, WIEBOKD JL. Glucose metabolism by implantation pig embryos. J. Reprod. Fertil., 84 : 7−12. 1988.

49) THOMPSON JG, SIMPSON AC, PUGH PA, WRIGHT RWJr, TERVIT HR. Glucose utilization by sheep embryos derived in vivo and in vitro. Reprod. Fertil. Dev., 3 : 571−576. 1991.

50) GARDNER DK, LANE M, BATT PA. Uptake and metabolism of pyruvate and glucose by individual sheep preattachment embryos developed in vivo. Molec. Reprod. Dev., 36 : 313−319. 1993.

51) O'FALLON JV, WRIGHT RWJR. Quantitative determination of the pentose phosphate pathway in preimplantation mouse embryos. Biol. Reprod., 34 : 58−64. 1986.

52) TIFFIN G, RIEGER D, BETTERIDGE KJ, YADAV BR, KING WA. Glucose and glutamine metabolism in preattachment cattle embryos in relation to sex and stage of development. J. Reprod. Fertil., 93 : 125−132. 1991.

53) RENARD JP, PHILIPON A, MENEZO Y. In vitro uptake of glucose by bovine blastocysts. J. Reprod. Fetrtil., 58 : 161−164. 1980.

54) SESHAGIRI PB, BAVISTER BD. Glucose inhibits development of hamster 8−cell embryos in vitro. Biol. Reprod., 40 : 599−606. 1989.

55) GARDNER DK, LANE M, SPITZER A, BATT PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells : Amino acids, vitamins, and culturing embryos in groups stimulate development. Biol. Reprod., 50 : 390−400. 1994.

ウシ体外成熟・体外受精卵の体外発生に及ぼす種々の栄養素および培養液交換の影響

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和　文　摘　要

ウシ体外成熟・体外受精卵の体外発生に及ぼす栄養素として，グルタミン／アミノ酸，インシュリン，タウリン／ハイポタウリン，グルコースの添加効果および培養中の培養液交換の影響を検討した。培養液には10%ヒト血清（0.44mMグルコース含有）を添加した合成卵管液を用い，媒精後10日間培養した。

グルタミン（１mM）および必須アミノ酸液（１%）を添加した場合，非添加区に比べて拡張胚盤胞（24.5%）およびふ化胚盤胞（12.1%）への発生率は有意に増加した（実験１）。グルタミンおよび必須アミノ酸を添加した血清含有培養液へのインシュリン（10μg／ml）添加効果は認められなかった（実験２）。タウリン（10mM）の添加により分割率は有意に増加したが，タウリン，ハイポタウリン（５mM）および両者の添加は胚への発生率を改善しなかった（実験３）。媒精時または媒精後５日目のグルコース（1.5mM）の添加による胚の発生率の向上も認められなかった（実験４）。更にグルコース（1.5mM）の有無に関わらず，培養期間中２日毎の培養液の交換は胚の発生率に有意な効果を示さなかった。

本結果により，ウシ体外成熟・体外受精卵の体外発生において10%ヒト血清を含む合成卵管液にグルタミンおよび必須アミノ酸の添加は有効であったが，その他の栄養素の添加効果は認められなかった。また，２日間の培養液交換は不要であることが示唆された。

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