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# Effects of Protein Source and Energy Substrates on the *In Vitro* Development of Bovine Embryos in a Two-step Culture System

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## Abstract

**In this study, we examined the effects of a two-step culture system, which involves the use of different culture media for early cleavage and later stage embryos, on the *in vitro* development of bovine embryos. We also investigated the effect of glucose, phosphate and citrate on the *in vitro* early developmental period of bovine embryos in a two-step culture system. Moreover, the supplementation of different protein sources (BSA-V, BSA-FAF and FBS) during IVC did not affect the frequency of blastocyst development. Using two-step culture, embryos were cultured in protein-free media for an initial 5 days. This was then followed by the same culture media or an FBS supplemented media. The developmental rates of blastocysts in the FBS containing group were significantly higher than in the replaced with no serum containing group. Embryos cultured in mSOF supplemented with 1.5 mM glucose plus 1.2 mM phosphate were significantly inhibited. The inhibition of developmental competence by glucose plus phosphate was consistent with the existence of 0.5 mM sodium citrate. This study indicates that a two-step culture system, which applies different conditions for early cleavage embryos, i.e., serum-free media, vs. later stage embryos, with serum containing media, may be effective for *in vitro* production systems. In addition, the developmental competence of bovine embryos was depressed in the presence of glucose plus phosphate as compared to either alone or the absence of both. Therefore, the avoidance of this negative effect should allow more optimal conditions to be developed for *in vitro* production.**

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## Introduction

The production of bovine embryos by *in vitro* fertilization (IVF) and culture has been greatly improved so that today transferable embryos are routinely obtained from immature oocytes [12]. Although a variety of culture systems are employed for *in vitro* embryo production, the developmental rate of blastocysts is, still too low and further research upon the dependence of metabolic change during the developmental period is needed.

Co-culture systems that include oviduct epithelial cells [29], uterine fibroblast cells [28] or trophoblastic vesicles [8] are routinely used. However, these culture systems lack adequate definition, which is required to guarantee quality control and repeatability [25]. To eliminate excessive variability, and to better understand pre-implantational development, simplified culture systems have been employed [21, 30].

Serum and BSA are the most common components of media for mammalian embryo culture. Serum, which contains hormones, growth factors, vitamins, peptides, and an array of defined and non-defined molecules, is generally included as the fixed nitrogen source for the pre-implantation embryo [10]. However, serum has been found to have a biphasic influence on development of bovine embryos, being deleterious to the first cleavage division but stimulatory for blastocyst development. Moreover, different batches of commercially available BSA might inhibit or stimulate embryonic development [4]. A two-step culture approach that applies different conditions for early cleavage and later stage pre-implantation embryos may be a more effective culture system [1, 20].

Glucose used to be routinely used in embryo culture media. However, it was found to be inhibitory and appears to have been partly or largely responsible for the impeding development. Moreover, the use of glucose in culture media has obstructed the ability to support development of cleavage stage embryos from numerous species, such as the mouse [2], hamster [23], bovine [11] and the human [3].

Phosphate stimulates the activity of the glycolytic pathway, and as a result causes a decrease in ATP production via mitochondrial respiration (TCA cycle and oxidase phosphorylation) [24]. Kim *et al*. [11] reported that without phosphate, glucose alone showed no detrimental effect on early embryonic development. Gray *et al*. [7] suggested that citrate, which is an energy substrate in the TCA cycle, has a beneficial effect on cleavage and blastocyst formation.

*In vivo*, embryos progress from the oviduct to the uterus, usually at about the eight-cell stage [17]. The secretions of these two compartments differ considerably in composition. Moreover, concentrations of key constituents may change in a dynamic way. However, these facts have not generally been incorporated into designing culture media for pre-implantation embryo development, which is almost always comprised of a single formulation for all stages [20]. Many have investigated the in vitro culture of bovine embryos, but the relation between metabolic changes and developmental stages remain unclear. The objectives of this study were to examine the effects of different protein sources supplemented in culture media on the *in vitro* development of bovine embryos, and to examine the effects of glucose, phosphate and citrate upon the developmental frequency of bovine embryos.

## Materials and Methods

### *In vitro* maturation (IVM)

Oocyte collection and IVM were performed as described by Lee and Fukui [12]. Briefly, bovine ovaries were collected immediately postmortem at a local abattoir and transported to the laboratory in saline (25-30℃) containing antibiotics (100IU/㎖ penicillin, 100㎍/㎖ streptomycin). Follicular fluid, with oocytes, was aspirated from small antral follicles (2-7 ㎜) using an 18-g needle connected to a 10 ㎖ syringe. By using a stereomicroscope, only cumulus-intact oocytes with evenly granulated cytoplasm were selected from the follicular fluid. The cumulus-oocyte complexes (COCs) were washed twice in TCM199 supplemented with 3 ㎎/㎖ BSA, 2 mM sodium bicarbonate and 10 mM hepes. A group of 10-12 randomly selected oocytes were then allocated to each drop of maturation medium (TCM199 supplemented with 10% FBS, 25 mM sodium bicarbonate, 1 mM glutamine, 2.5 ㎍/㎖ FSH (Antrin, Denka Pharm, Tokyo., Japan) and 1 ㎍/㎖ estradiol (Sigma Co, MO., USA).

For IVM, oocytes were cultured for 24h in 50 ㎕ drops of medium overlaid with 10㎖ of paraffin oil in sterile Petri dishes (60×15 ㎜, Corning Costar Co., USA). Embryos were incubated in 5% CO2 in air with saturated humidity at 39℃.

### *In vitro* fertilization (IVF)

Frozen semen was thawed in a 37℃ water bath for 30 sec, then subjected to swim-up separation in Tyrode's medium for 50 min to increase the proportion of motile sperm. The final sperm concentration used in IVF was 2.0×10⁶/㎖. The capacitation of sperm was enhanced by including 8 ㎍/㎖ heparin sulfate in the IVF medium. Incubations for IVF were performed in 5% CO2 in air with saturated humidity for 30h at 39℃.

### *In vitro* culture (IVC)

mSOFM was used as the medium for this study (Table 1). The oocytes in each IVF drop were stripped off cumulus cells by pipetting and then washed 2 times in mSOFM. IVC incubations were conducted at 5% CO2, 7% O2 and 90% N2 under saturated humidity at 39℃. The proportions of embryos reaching blastocysts were examined on day 8 (192 h). At this time, blastocyst cell numbers were evaluated by Hoechst33342 staining. Briefly, embryos were removed from culture on day 8 pi and transferred to a slide in 2-3 ㎕ of medium, and 15 ㎕ of Hoechst 33342 stain prepared with sodium citrate (2.3%) and ethyl alcohol was added. The slide was then incubated on a warming plate for 5 min, the extra stain was discarded, and Permount was added along with a coverslip. Total cells in each blastocyst were counted under a fluorescence microscope.

### Statistical analysis

Embryo culture in Exp.1 was done in unchanged media for 10 days, and in Exps. 2, 3, and 4 the media was changed 120-132 hrs after IVF with different media.

All embryos were evaluated for the morphological stage of development reached. The data were analyzed by logistic regression model(PROC Logistic Procedure, Statistic Analysis System(SAS), Version 6.04).

## Results

### Exp 1. Effects of protein source on the frequency of development to blastocyst

No significant differences in blastocyst development were observed between the treatments groups (Table 2), and no differences were observed in the percentages of embryos reaching the hatching blastocyst stage as a percentage of the total number of embryos for FBS (10.5%), BSA-V (8.9%), and BSA-FAF (10.9%).

### Exp 2. Effects of serum on the frequency of development to the blastocyst stage

Development to blastocyst by replacing with the same media was significantly ($p<0.05$) lower than that achieved by replacing with serum containing media (Table 3). No significant differences in the percentages of embryos reaching the hatching blastocyst stage as a percentage of the total number of embryos were observed for FBS (11.0%), BSA-V (8.6%), and BSA-FAF (14.3%).

### Exp 3. Effects of glucose and/or phosphate in the two-step culture system

The effects of glucose and/or phosphate were examined in

**Table 1.** Compostion of modified syntheic oviduct fluid medium used for the *in vitro* culture of bovine embryos

| Component | Units | Early stage | Later stage | Wahing |
|---|---|---|---|---|
| NaCl | mM | 107.70 | 107.70 | 107.70 |
| KCl | mM | 7.16 | 7.16 | 7.16 |
| NaHCO3 | mM | 25.07 | 25.07 | 4.01 |
| KH2PO | mM | 1.19* | 1.19 | 1.19* |
| Na-lactate (60% syrup) | mM | 3.30 | 3.30 | 3.30 |
| Na-pyruvate | mM | 0.33 | 0.33 | 0.33 |
| caCl2 | mM | 1.71 | 1.71 | 1.71 |
| MgCl2 | mM | 0.49 | 0.49 | 0.49 |
| HEPESa | mM | — | — | 10.50 |
| Glucose | mM | 1.50* | 1.50 | 1.50* |
| EAAb | % | 2 | 2 | 2 |
| NEAAc | % | 1 | 1 | 1 |
| BSAd | ㎎/㎖ | 8 | — | 6 |
| FBSe | % | — | 10 | — |

* Supplementation depended upon experimental design.
a N-[2-hydroxyethy1]piperazine-N ′-2-ethanesulfonic acid.
b Essential amino acids.
c Non-essential amino acids.
d Bovine serum albumin (fatty acid free, fraction V)
e Fetal bovine serum.

**Table 2.** Effect of protein source on the *in vitro* development of 2-cell bovine embryos.

| Protein source | No. of embryos cultured* | No. (%) of blastocysts | Mean blastocyst cell no. ± s.e.(n) |
|---|---|---|---|
| FBS | 241 | 62 (25.7) | 80.1±5.2 (32) |
| BSA-Va | 239 | 55 (23.0) | 87.4±6.3 (28) |
| BSA-FAFb | 245 | 66 (26.9) | 89.0±6.2 (33) |

* Two-cell embryos were selected at 30 hours after IVF (8 replicates).
a Bovine serum albumin-fraction V.
b Bovine serum albumin-fraction V, fatty acid free.

**Table 3.** Effect of replacement with the same media or 10% fetal bobine serum containing media on the in vitro development of 2-cell bovine embryos

| Period of culture | | No. of embryos cultured* | No. of blastocysts | Mean blastocyst cell no. ± s.e.(n) |
|---|---|---|---|---|
| Culture to Day 5 | After culture to Day 10 | | | |
| FBS | FBS | 220 | 52 (23.6)ab | 82.4±4.1 (22) |
| BSA-FAF | BSA-FAF | 213 | 47 (22.1)a | 96.6±6.5 (20) |
| BSA-FAF | FBS | 226 | 78 (34.5)b | 95.3±8.0 (25) |

* Two-cell embryos were selected at 30 hours after IVF (7 replicates).
a Bovine serum albumin-fraction V, fatty acid free.
a,b Different superscripts in the same column differ significantly ($p<0.05$).

a two-step culture system. Development to blastocyst in medium containing both glucose and phosphate was significantly ($p<0.05$) lower than the in glucose alone (Table 4). No significant differences were found in the percentages of embryos reaching the hatching blastocyst stage as a percentage of the total number of embryos for glucose and phosphate (16.7%), glucose alone (24.4%), phosphate alone (25.2%), and none (24.4%).

**Exp 4. Effects of glucose and/or phosphate in sodium citrate**

Effects of glucose and/or phosphate in sodium citrate containing medium were also examined. Development to blastocyst and the cell numbers of embryos cultured in different media were not significantly different (Table 5). However, significant differences were found in embryo hatchings as a percentage of total embryos in the media containing glucose alone (27.9%), none (25.7%), and both glucose and phosphate (14.4%).

## Discussion

A two-step culture protocol was used in the present study to allow for the different nutritional requirements of cleavage stages and of the differentiation stage (morulae and blastocysts) for development *in vitro*. If oviductal- and uterine-stage embryos have differing nutrient requirements, which seems likely in view of their metabolic differences, then the use of a single culture formulation to support complete pre-implantation development will result in a comprise medium that is sub-optimal for both developmental phases. This may partly account for the low frequencies of blastocyst development when the same formulation is used throughout embryo culture [1, 20].

A major biological role of serum albumin is that it be taken up by the embryo and broken down to provide energy substrates and the amino acids for metabolic and anabolic processes and for the chelation of heavy metal ions or other toxins [18]. Serum sources also contain amino acids that play an important role as energy sources, osmoregulators and pH stabilizers [4]. In the present study, protein sources did not have different effects blastocyst development, which is similar to that found by Pinyopummintr and Bavister [19]. Serum has been shown to be inhibitory to early development *in vitro*, and to actually inhibiting the first cleavage division of IVF cow embryos [19], and stimulating blastulation [4]. Moreover, in the present study serum supplementation exhibited a biphasic effect, and showed that in the BSA-FAF group, serum supplementation in the late developmental stage was better than replacement with serum free media. These responses may be analogous to those obtained with porcine embryos [4]. However, in the BSA-V group, replacement with serum containing media or serum free media produced no difference. Components such as vitamins, fatty acids, growth factors, which are present in serum, may be essential to development during the later stages. Moreover, fatty acid-free preparations of BSA could have some or all contaminants, possibly introduced during the preparation of BSA-V, removed by extraction procedures, and these contaminants may mimic the effect of serum.

Optimal glucose concentration depends upon the culture medium; i.e., 1.0-1.5 mM in SOF [6] and TLP [11], and 5.56 mM in TCM199. The beneficial effects of co-culture included a reduced glucose concentration, an increase in the levels of L-lactate and pyruvate [5], and a reduced oxygen concentration [27]. The inhibitory effect of glucose has also been reported in the hamster [23], mouse [2] and bovine [16]. Edwards et al. [5] reported that the mammalian preim-

**Table 4.** Effect of glucose and/or phosphate in mSOF medium on development of 2-cell bovine embryos

| Treatment | | No. of embryos cultured* | No. of blastocysts | Mean blastocyst cell no. ± s.e. (n) |
|---|---|---|---|---|
| Glucose (1.5mM) | Phosphate (1.2mM) | | | |
| + | + | 131 | 42 (32.1)a | 89.1±11.6 (21) |
| + | − | 135 | 59 (43.7)b | 102.9±6.4 (25) |
| − | + | 135 | 51 (37.8)ab | 95.6±7.9 (20) |
| − | − | 131 | 51 (38.9)ab | 96.3±12.5 (22) |

* Two-cell embryos were selected at 30 hours after IVF (7 replicates).
a,b Different superscripts in the same column differ significantly ($p<0.05$).

**Table 5.** In vitro developmental rates of 2-cell bovine embryos cultured in citrate containing mSOF medium with or without glucose and/or phosphate

| Treatment | | No. of embryos cultured* | No. of blastocysts | Mean blastocyst cell no. ± s.e. (n) |
|---|---|---|---|---|
| Glucose (1.5mM) | Phosphate (1.2mM) | | | |
| + | + | 104 | 32 (30.8) | 82.2±10.1 (15) |
| + | − | 104 | 41 (39.4) | 98.7±5.4 (17) |
| − | + | 103 | 42 (40.8) | 90.5±8.0 (13) |
| − | − | 105 | 44 (41.9) | 103.5±8.3 (18) |

* Two-cell embryos were selected at 30 hours after IVF (7 replicates).

plantation embryo does not utilize glucose readily prior to compaction, but rather uses pyruvate, L-lactate or amino acids as energy sources. In addition, it was found that energy production by oxidative phosphorylation or glycolysis is necessary for cleavage and maintaining the developmental capacity [26]. In an *in vitro* culture of mouse embryos, preferred energy production was changes tricarboxylic acid cycle to glycolysis. Embryos consume pyruvate preferentially during the early developmental stages, before glucose becomes the predominant energy substrate in the blastocyst [13]. In this study, glucose alone had no effect on the development of bovine embryos, but glucose together with phosphate inhibited embryo development to the blastocyst stage. This result resembles that obtained by Barnett and Bavister [1], and Moore and Bondioli [16]. In glucose/phosphate containing media, phosphate stimulates cellular glycolysis by activating three key glycolytic enzymes (hexokinase, phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase) and this enhanced glycolysis results in the inhibition of mitochondrial respiration [24]. In the early developmental stages, glycolysis poorly supports embryo development, presumably due to greatly reduced energy generation (eight vs. two ATPs) [1], energy generation by the Kreb's cycle is a benefit during the early embryonic developmental periods than glycolysis [16]. But, after compaction the embryo is more likely to use glycolysis [15], further research upon metabolic changes during the developmental period is needed to clarify the roles of glucose and/or phosphate.

Citrate is an allosteric activator of acetyl-CoA carboxylase and thus plays a key role in the control of fatty acid synthesis, which stimulates blastocyst formation and the growth of rabbit embryos [7]. A recent study about the effect of citrate on *in vitro* development, showed that citrate has no effect on developmental competence, and together with glucose and phosphate inhibits blastocyst formation [22]. A study by Keskintepe et al. [9] indicated that 0-0.9mM citrate without amino acids had no effect on *in vitro* culture, but with non-essential amino acids stimulated blastocyst formation. According to Liu and Foote [14] nonessential amino acids (NEAA) have a stimulatory effect upon all developmental stages, and essential amino acids (EAA) enhance blastocyst formation and the hatching of blastocysts, but EAA were found to be toxic during the early developmental stages. In the present study, citrate was found to have no effect on developmental capacity when NEAA and EAA were supplemented in SOF media. Studies are needed to determine if the embryos would benefit from the addition of citrate to culture medium containing NEAA alone.

In conclusion, the use of a two-step culture system, including the use of a serum-free media at the cleavage stages, followed by the inclusion of serum for the differentiated stages (i.e., the morula and blastocyst), appears to be a valid strategy for optimizing blastocyst production. Moreover, in the early developmental stages, the stimulation of glycolysis would result in insufficient ATP production and the inhibition of embryo development. The avoidance of this negative effect could provide an optimal culture system.

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