In Vitro Culture of Bovine One-Cell Embryos Derived from In Vitro Fertilization Using a Semi-Chemically Defined Medium

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ABSTRACT. The effects of alteration of metabolic substrates and supplementation of amino acids on in vitro development of bovine embryos were examined. One-cell embryos derived from in vitro fertilization were cultured using a semi-chemically defined medium, Brinster's Medium, containing 3 mg/ml bovine serum albumin (BMOC-3) as a basal medium. BMOC-3 did not support embryonic development to the morula stage, but blastocysts were obtained by omitting glucose and lowering the lactate level from 20.12 to 3.0 mM and the pyruvate level from 0.5 to 0.3 mM (mBMOC-3). Supplementation of amino acids (glutamine plus the other 12 essential amino acids, as well as further addition of 7 non-essential amino acids) improved the embryonic developmental rate to the blastocyst stage with an increase in cell number equal to that for co-cultured embryos with bovine oviductal cells in TCM 199 with 10% fetal calf serum. These results clarified that the one-cell bovine embryo can develop to the blastocyst stage in a semi-chemically defined medium, and that amino acids support embryonic development in vitro.——KEY WORDS: amino acid, bovine embryo development, lactate, semi-chemically defined medium.


Bovine embryos have been successfully cultured from the eight-cell to the blastocyst stage, while the culture of one-cell embryos to the blastocyst stage has proved to be much more difficult [29]. This is due to a developmental block that occurs in vitro at the eight-cell stage [4, 7, 28]. Progress in embryo co-culture systems has made it possible to culture one-cell bovine embryos up to the blastocyst stage using various somatic cells in co-culture [4, 7, 9, 12] or in conditioned medium [8]. In a co-culture system, somatic cells may provide unknown embryo growth promoting factor(s) and/or delete embryo toxic factor(s) from a basic medium [8] making it difficult to define the exact requirements for development of the embryo independent of other cell types. Several workers reported that early bovine embryos developed into blastocysts without somatic cell support when they were cultured in synthetic oviduct fluid (SOF) [27] and SOF added to human serum instead of bovine serum albumin (BSA) [16], under a gas atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Furthermore, one-cell bovine embryos can develop to the blastocyst stage in CR1 [24], hamster embryo culture medium (HECM) [19] and modified SOF (mSOF) supplemented with amino acids [25], under a gas atmosphere of 5% CO₂ in air. These media contain relatively low concentration of lactate (3.3-10.0 mM) and no glucose.

Consequently, the present experiments were designed to evaluate the development of one-cell bovine embryos in other semi-chemically defined media with altered metabolic substrates and amino acid supplementation.

MATERIALS AND METHODS

In vitro fertilization of ovarian oocytes: Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (2 to 5 mm in diameter) on bovine ovaries obtained from a slaughterhouse. They were washed three times with a maturation medium; 25 mM HEPES-buffered TCM 199 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 0.5 mM sodium pyruvate, 0.02 AU/ml FSH (Antrin, Denka-Seiyaku Co., Kawasaki, Japan), 1 μg/ml estradiol-17β (Sigma Chemical Co., St. Louis, MO) and antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin). Only oocytes with an intact, unexpanded cumulus and evenly granulated cytoplasm [15] were then cultured for 22 to 24 hr with the maturation medium. Maturation cultures as well as all other cultures were maintained at 39°C in humidified air with 5% CO₂.

In vitro fertilization was performed using frozen semen from one bull by the method previously described by Niwa and Ohgoda [17]. The frozen-thawed semen was diluted with modified Brackett and Oliphant isotonic medium (BO medium) [2] without BSA but supplemented with 10 mM caffeine (Caffeine-Sodium Benzoate, Sigma). The sperm were then washed three times by centrifugation at 500 × g for 5 min. The COCs were coincubated for 6 hr with spermatooza (5 × 10⁴ cells/ml) in 100 μl droplets of BO medium supplemented with 5 mM caffeine, 10 μg/ml heparin (Sigma) and 10 mg/ml fatty-acids free BSA (Sigma).

In vitro culture of one-cell embryos: Six hours after insemination, the COCs were washed three times with each culture medium and incubated in 100 μl droplets covered with paraffin oil under the same conditions as for maturation and fertilization. Eighteen hours after insemination, one-cell embryos were stripped of cumulus cells by

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vortexing, washed three times, and cultured in each medium for 7 days. Brinster's Medium (BMOC-3) [3] was used as the basal culture medium. The medium consisted of 94.88 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂·2H₂O, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄·7H₂O, 25.07 mM NaHCO₃, 5.56 mM glucose, 20.12 mM sodium lactate, 0.50 mM sodium pyruvate, 100 U/ml penicillin, 50 µg/ml streptomycin, 20 µg/ml phenol red and 5 mg/ml BSA. In Experiment 1, BMOC-3 was altered with metabolic substrates to be glucose-free BMOC-3 or glucose-free BMOC-3 containing 3.00 mM sodium lactate and 0.30 mM sodium pyruvate (mBMOC-3). In Experiment 2, a premixed solution of basal medium Eagles (BME) essential amino acids (EAA; Flow Laboratories, Irvine, Scotland), minimum essential medium (MEM) non-essential amino acids (NEAA; Flow) and 1.0 mM glutamine were added to mBMOC-3. One-cell embryos were also co-cultured with bovine oviducal cells (BOC) in TC medium supplemented with 10% FCS, 0.5 mM sodium pyruvate and antibiotics (TC199+10%FCS).  

Isolation and culture of bovine oviductal cells: Bovine oviducts were obtained at a slaughterhouse, trimmed free of connective tissue and rinsed in Dulbecco's phosphate-buffered saline supplemented with 10% heat-inactivated calf serum (Gibco). After blotting on sterile gauze to remove excess fluid and blood, oviducts were cut longitudinally with fine dissecting scissors. Mucosal tissue was scratched with a curette, suspended in TC199+10%FCS and pipetted gently. Oviductal cells were then washed three times by centrifugation at 100 × g for 5 min. The pellet was resuspended in TC199+10%FCS to a ratio of 1:100 (v/v); 3 ml of suspension was placed in a 35 mm plastic petri dish, cultured for 48 hr and then used as co-culture. Media were replenished every 48 hr by replacing 50% of the total volume with fresh TC199+10%FCS.  

Evaluation of embryonic development and counting total cell number: Initial cleavage and development to the morula and blastocyst stages were determined under a stereomicroscope 2 days (approximately 42 hr), 5 days (122 to 124 hr) and 8 days (190 to 194 hr) after in vitro fertilization. In Experiment 2, all of the blastocysts obtained were air-dried [25]. Their total cell numbers, including metaphase plates but excluding pyknotic nuclei were counted under a bright-field microscope.  

Statistical analysis: In Experiment 1, data pooled from two replicates were analyzed by χ²-test with Yates' correction. In Experiment 2, statistical comparisons for percentage data and total cell numbers were done by ANOVA followed by Duncan's multiple range test.  

RESULTS  

Effects of lactate and glucose levels on development of embryos to the blastocyst stage (Experiment 1): None embryos developed to the morula stage in BMOC-3 with original components. By omitting glucose, 5.2% of embryos developed to the morula stage, but they did not reach the blastocyst stage. In the absence of glucose, when the lactate and pyruvate levels were lowered to 3.0 mM and 0.3 mM, respectively, both the initial cleavage rate (75.4%) and the percentage of morulae (16.4%) were significantly higher than the other groups (P<0.05) and 8.2% of embryos developed to the blastocyst stage (Table 1).  

Effects of amino acids on development of embryos to the blastocyst stage (Experiment 2): Results of in vitro culture of embryos in mBMOC-3 supplemented with amino acids are shown in Table 2. The addition of glutamine alone to mBMOC-3 did not improve the development of embryos. The percentage of blastocysts significantly increased (P<0.05) by further addition of EAA (14.6%) or both EAA and NEAA (16.6%), these results were similar to those obtained by co-culture (13.9%). The mean number of cells for blastocysts cultured in mBMOC-3 with glutamine + EAA + NEAA (104.1 cells) and those co-cultured in TC199+10%FCS with BOC (109.2 cells) were greater than those cultured in mBMOC-3 (78.7 cells, P<0.05).  

DISCUSSION  

BMOC-3 did not support the development of bovine one-cell embryos to the morula stage, but blastocysts were obtained by omitting glucose and lowering the lactate and pyruvate levels from the original level of 20.12 to 3.0 mM and from 0.5 to 0.3 mM, respectively (Table 1). Takahashi and First [25, 26] reported a similar finding, that elevation of lactate levels from: 3.3 to 33.0 mM markedly inhibited the development of bovine one-cell embryos when
embryos were cultured in glucose-free SOF. The concentration of lactate in sheep and rabbit oviductal fluid was reported to be 2 to 4 mM [11, 14, 20], which is lower than in media commonly used for culture of mammalian embryos (20 to 30 mM). The concentration of pyruvate in rabbit and mouse oviducal fluid was also reported to be 0.14 to 0.37 mM [14]. A proper lactate/pyruvate ratio has been indicated to be essential for balancing the oxidation-reduction potential of embryos [8, 25]. A higher lactate level (20.12 mM) might induce imbalance of oxidation-reduction potential and energy deficiency in embryos. The present results indicate that a high lactate level (20-30 mM) which has been commonly used for culture of mammalian embryos is one of the causes of the 8- to 16-cell block as suggested by Takahashi and First [26].

It has been demonstrated that glucose is not only unnecessary but at 5.56 mM, it also has detrimental effects on early embryonic development to the blastocyst stage in the presence of lower level of lactate (3.3 mM) [25, 26]. Although, detrimental effect of 5.56 mM glucose was not confirmed, the present results support the previous finding that one-cell bovine embryos can develop into the blastocyst stage without glucose.

Glutamine has been shown to improve the development of mouse [5] and pig [18] embryos overcoming their respective blocks. In cattle, glutamine can be metabolized by blastocysts and may be utilisable as an energy source [22]. In our present experiment, however, the beneficial effect of glutamine supplementation was not observed (Table 2). Rieger [21] suggested that metabolism of the carbon chain of glutamine is an opportunistic rather than obligatory source of energy production in the embryo, simply serving to consume excess amounts of glutamine and α-ketoglutarate arising from synthetic transformation reductions.

Amino acids have been reported to be essential for in vitro development of early embryos in hamsters [1], rats [30] and cattle [23, 25]. In the present experiment, supplementation of amino acids (glutamine + EAA or glutamine + EAA + NEAA) beneficially affected embryonic development to the blastocyst stage (Table 2). Rosenkranz and First [23] demonstrated that EAA and NEAA increased the blastocyst percentage by 10.6 and 8.1%, respectively. Cell number in the blastocysts obtained after culturing embryos in mBMCOC-3 with glutamine + EAA + NEAA was significantly higher than that of the control (mBMOC-3), and was similar to that obtained by co-culture. These findings are comparable to a previous report in which the number of cells in the blastocysts cultured in mSOF supplemented with the amino acids increased to 111.5 cells at 7 days after in vitro fertilization, compared with the control of 71.5 cells [25]. The total cell number of blastocysts 8 days after in vitro fertilization, especially of those cultured in the present mBMCOC-3 supplemented with amino acids, correspond to those of recent studies in HECM [19] and in co-culture systems [10, 13] in which cell numbers in the blastocysts are reported to be 70 to 150 cells 8 to 10 days after in vitro fertilization. Supplement of amino acids to a culture medium may increase the pool size of endogenous amino acids and de novo protein synthesis [25, 30]. However, the synergistic effect of glutamine, EAA and NEAA was not obvious, since the present experiment was not completely designed, and there was no significant difference between glutamine + EAA and glutamine + EAA + NEAA. Further studies are needed to elucidate the influence of individual amino acids on the embryonic development.

In conclusion, the present study clearly demonstrates that one-cell bovine embryos can develop successfully to the blastocyst stage in a semi-chemically defined medium by lowering the lactate and pyruvate levels, omitting glucose and supplement of amino acids. Frequency of embryonic development up to the blastocyst stage in this medium was equivalent to that for the co-culture system with oviductal cells. Further analysis will be required for the establishment of suitable conditions for in vitro

### Table 2. Effect of amino acid supplementation to mBMCOC-3 on the in vitro development of one-cell bovine embryos derived from in vitro fertilization (Experiment 2)

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. of trials</th>
<th>% of cleaved at Day 2</th>
<th>% of morulae at Day 5</th>
<th>% of blastocysts at Day 8</th>
<th>Cell number in blastocysts (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAA</td>
<td>Gln</td>
<td>NEAA</td>
<td>(embryos)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mBMCOC-3</td>
<td>5 (161)</td>
<td>74.0±2.1</td>
<td>15.0±1.5</td>
<td>8.1±0.7</td>
<td>78.7±5.9</td>
</tr>
<tr>
<td>+</td>
<td>5 (101)</td>
<td>74.9±1.6</td>
<td>17.1±1.6</td>
<td>8.0±1.0</td>
<td>91.0±6.1</td>
</tr>
<tr>
<td>+</td>
<td>4 (102)</td>
<td>73.9±1.8</td>
<td>22.0±1.7</td>
<td>14.6±1.6</td>
<td>95.2±5.3</td>
</tr>
<tr>
<td>+</td>
<td>3 (109)</td>
<td>76.5±3.9</td>
<td>22.1±1.4</td>
<td>16.6±0.4</td>
<td>104.1±7.0</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

Day 0=Day of in vitro insemination.

a) Containing 3.0 mM Na-lactate, 0.3 mM Na-pyruvate and 3 mg/ml BSA but without glucose.
b) Glut: 1 mM glutamine, EAA: Essential amino acids of BME, NEAA: Non-essential amino acids of MEM.
c) d) Values with different superscripts within the same column differ significantly (P<0.05).
e) One blastocyst obtained in vitro culture was lost.
development of early bovine embryos.

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