**NOTE** *Theriogenology*

# Improvement of Preimplantation Development of *In Vitro*-Fertilized Bovine Zygotes by Glucose Supplementation to a Chemically Defined Medium

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ABSTRACT. The influences of glucose supplementation on early development of bovine embryos in BSA-free synthetic oviduct fluid were examined. Among the groups supplemented with 1.5, 2.0, 4.0 or 5.6 mM glucose either at 0, 72 or 144 hr after fertilization, blastocysts yield significantly increased in the group supplemented with 4.0 mM glucose 144 hr after fertilization compared to the controls without glucose supplementation. The results suggest that appropriate amounts of glucose supplemented to the medium at the specific stage of embryo culture may be useful for the production of bovine blastocysts.
KEY WORDS: bovine, chemically defined medium, embryo, embryo culture, glucose

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In general, fetal bovine serum (FBS) is widely employed as a supplement in the media used for culturing *in vitro* fertilized bovine oocytes. Nevertheless, the use of FBS has disadvantages as it contains unknown factors, and the subsequent rate of development of the fertilized oocytes to the blastocyst stage is known to vary among serum lots. Thus, there is a need for a reliable *in vitro* embryo culture protocol that uses a chemically defined medium and yields a high rate of good quality embryos. Many reports showed that the rate of development to the blastocyst stage is lower in chemically defined, serum-free media than in standard culture media containing serum [2, 9, 14] or BSA [1, 15]. Although we have reported significantly improved embryo development to the blastocyst stage by the supplementation of chemically-defined medium with epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) [14], blastocyst development in this modified chemically-defined medium was still lower than that obtained in medium supplemented with serum.

On the other hand, most of embryo culture systems include glucose as the main energy source. The importance of glucose as an energy substrate for bovine zygotes has been demonstrated in several studies [4, 7], and its inhibitory effect on bovine embryo development at the early stages has also been described [1, 6, 8, 12]. Although the optimum timing and doses of the glucose addition to the culture media have been addressed in several reports [4, 5, 7, 11], there is limited knowledge on the effect of glucose supplementation on *in vitro* development of bovine embryos under chemically defined conditions [5]. Since the development of embryos to the blastocyst stage in protein-free media was slower than that in media supplemented with serum [2], it is necessary to determine the optimum timing and concentration of glucose supplementation when a chemically defined medium is used. Therefore, the objective of the present study was to determine the optimum timing and concentration of glucose supplementation to a protein free, chemically defined synthetic oviduct fluid (SOF) in order to maximize *in vitro* development of bovine embryos to the blastocyst stage.

The animal experiments in this study were approved by the Institutional Animal Experiment Committee of Kanagawa Prefectural Livestock Industry Technology Center. The experiments were performed in six separate laboratories, using the same experimental reagents, such as the culture media and frozen semen lot, and the results were aggregated. Preliminary experiments were conducted previously at each laboratory site using a protein-free SOF supplemented with 1 mg/m*l* polyvinyl alcohol (PVA) for embryo culture, and there was no significant difference in the rates of blastocyst development among these laboratories.

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Table 1. Effects of timing and concentration of glucose supplementation in SOFaa-E+I medium on development to the blastocyst stage of bovine embryos observed 192 hr after fertilization

| Timing of glucose supplementation [b] (hr) | No. of oocytes (replcates) | % of blastocyst at each concentration of glucose (mM) [a] | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 1.5 | 2.0 | 4.0 | 5.6 |
| 0 | 1205 (9) | 19.2 ± 2.5 [c] | 24.0 ± 3.1 [c] | 20.2 ± 3.5 [c] | 12.6 ± 3.6 [cd)A)] | 3.1 ± 1.7 [d)A)] |
| 72 | 549 (6) | 19.6 ± 4.1 | 19.7 ± 3.7 | 20.1 ± 3.7 | 24.7 ± 5.1 [AB)] | 16.9 ± 3.4 [B)] |
| 144 | 600 (6) | 16.9 ± 2.0 [c] | 22.9 ± 3.2 [cd] | 27.8 ± 3.7 [cd] | 31.3 ± 3.7 [d)B)] | 29.0 ± 2.3 [cd)C)] |

a) Data are expressed as the mean ± S.E.M., b) Time of glucose supplementation after *in vitro* fertilization, c–d) Values in the same row with different superscripts are significantly different ($P<0.05$), A–C) Values in the same column with different superscripts are significantly different ($P<0.05$).

Bovine ovaries were collected at a slaughterhouse and transported to the laboratory in saline. Cumulus-oocyte complexes (COCs) were collected from follicles with a diameter of approximately 3–5 mm using a 10-m*l* syringe with an 18-gauge needle. COCs with more than one layer of cumulus cells closely adhered to the zona pellucida, and an oocyte with homogeneous cytoplasm was selected. The selected COCs were washed three times with a maturation medium consisting of TCM-199 (12340-030, Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 0.02 AU (Armour Units) porcine follicle stimulating hormone (Antrin R10, Kyoritsu Seiyaku, Tokyo, Japan), 5% FBS (SH30070, HyClone Laboratories, Logan, UT, U.S.A.), 1 $\mu$g/m*l* estradiol-17β (E1132, Sigma-Aldrich, St. Louis, MO, U.S.A.) and 0.2 mM sodium pyruvate (P2256, Sigma-Aldrich). Fifteen to twenty oocytes were placed in a 100-$\mu l$ drop of the maturation medium, which was then covered with liquid paraffin (26137-85, Nacalai Tesque, Kyoto, Japan) and incubated in a $CO_2$ incubator (38.5°C, 5% $CO_2$ in humidified air) for 20–22 hr. *In vitro* fertilization (IVF) was performed according to our previous report [14]. In this study, semen samples collected from a single Japanese Black bull and frozen on the same day were used. They were thawed in water at 37°C and washed twice by centrifugation (460 × *g*, 5 min) with 6 m*l* IVF medium (IVF 100; Research Institute for the Functional Peptides, Yamagata, Japan). Then, the sperm pellet was resuspended to a concentration of $1 \times 10^7$ spermatozoa/m*l*, and the suspension was used to make fertilization drops (100-$\mu l$) with a final concentration of $5.0 \times 10^6$ spermatozoa/m*l*. The COCs were washed three times with 350-$\mu l$ droplets of IVF 100, transferred into the fertilization drops and incubated for 6 hr at 38.5°C under a humidified atmosphere of 5% $CO_2$ in air. Fifteen oocytes were placed in each fertilization drop. After IVF, the cumulus cells were removed from the oocytes by pipetting through a narrow-bore glass pipette. Cumulus-free presumptive zygotes were placed in a culture medium which was glucose-free modified synthetic oviduct fluid [15] supplemented with 2% (v/v) Basal Medium Eagle essential amino acids (B6766, Sigma-Aldrich), 1% (v/v) Minimum Essential Medium amino acids (11140-050, Gibco) (SOFaa), 1 mg/m*l* PVA (P8136, Sigma-Aldrich), 100 *n*g/m*l* EGF (E4157, Sigma-Aldrich) and 50 *n*g/m*l* IGF-I (I3769, Sigma-Aldrich) (SOFaa-E+I). Twenty presumptive zygotes were incubated in each 100-$\mu l$ drop covered with liquid paraffin at 38.5°C in 5% $CO_2$, 5% $O_2$ and 90% $N_2$ until 192 hr post-fertilization. Glucose was added to the SOFaa-E+I medium at different concentrations (0, 1.5, 2.0, 4.0 and 5.6 mM) at different time points (0, 72 and 144 hr) after fertilization, and the developmental rates of embryos were investigated. During glucose addition, the half amount of the culture media was aspirated from each drop with a micropipette under a stereo microscope, and then, preincubated fresh culture media containing the double concentration of glucose were introduced. The percentages of embryos developing to blastocysts were subjected to arcsine transformation before statistical analysis. A two-way analysis of variance (2-way ANOVA) was performed using SPSS (SPSS 16.0 J, User's Guide, SPSS, Tokyo, Japan) followed by Tukey's HSD test. Data are presented as mean ± S.E.M., and a $P$ value of less than 0.05 was considered statistically significant.

The results of embryo development are shown in Table 1. There was a significant interaction between the concentration and the timing of glucose supplementation in the developmental rate of blastocysts ($P<0.05$). The percentage of blastocysts cultured in SOFaa-E+I medium supplemented with 4.0 mM glucose 144 hr after fertilization was significantly increased when compared with controls without glucose supplementation. Moreover, the blastocysts yield in the group supplemented with 4.0 mM glucose 144 hr after fertilization was significantly increased when compared with that in the group supplemented with 4.0 mM glucose 0 hr after fertilization. These results indicated that 4.0 mM glucose supplemented at 144 hr after fertilization is useful for embryo culture in this chemically defined medium. On the other hand, in previous studies using media containing serum, the optimum dose (1.5 or 5.56 mM) and timing (72, 96 or 120 hr after fertilization) of glucose supplementation varied among reports [4, 5, 7, 8]. The type of medium and serum could influence the effect of glucose supplementation on the embryo growth.

When compared among the groups supplemented with various amounts of glucose 0 hr after fertilization, the developmental rate of blastocysts decreased with higher concentration of glucose (4.0 or 5.6 mM), and percentages of blastocysts yield in the group supplemented with 5.6 mM glucose decreased significantly when compared with those with 0, 1.5 or 2.0 mM glucose.

In bovine embryos, Javed and Wright [3] described that glucose does not affect cell growth before the morula stage, and Matsumoto *et al*. [7] reported that glucose supplementation immediately after fertilization at the concentration of 3 mM or less to modified SOF medium containing 5%

superovulated cow serum did not affect blastocyst formation. However, there are some reports showing that addition of high concentration of glucose immediately after *in vitro* fertilization was harmful for early embryonic development [1, 6, 9, 13]. These findings are consistent with our results. Although the reason why high concentrations of glucose obstruct embryonic development is not clear, Rieger [10] showed that glucose increases the intracellular level of oxygen radicals harmful for mammalian embryo development.

Although glucose obstructs the development of the embryos when supplemented in the culture in high concentrations during the early stages, it has important roles on energy metabolism after the compacted morula stage and during blastulation [4, 8, 16]. Prior to the present study, we observed the timing of formation of blastocoels using time-lapse photography as a preliminary test, and blastocoels of embryos cultured in SOFaa-E+I medium could be identified from 132.8 ± 2.7 hr (Mean ± S.E.M.) after fertilization (unpublished data). In the present study, the rate of embryos developing to the stage of blastocyst was highest in the group supplemented with 4.0 mM glucose 144 hr after fertilization, nearly at the timing of formation of the blastocoel. These results suggest that appropriate amounts of glucose at the later stage of the culture are effective to enhance embryo development. On the other hand, Holm *et al*. [2] reported that bovine embryos developed to early blastocysts at 129.0 ± 2.9 hr after fertilization in serum-free SOFaa medium and at 107.5 ± 3.1 hr after fertilization in SOFaa medium supplemented with serum and suggested that serum in the culture medium accelerated the growth of embryos. It has been reported that in a culture medium containing serum, development of embryos was improved significantly when glucose was supplemented 72 hr [11] after *in vitro* fertilization, which is earlier than the effective timing of glucose supplementation for blastocyst development (144 hr after fertilization) in this study. These findings suggest that the presence of serum in the culture medium might affect the timing of effective glucose supplementation for the embryo development due to its accelerating effect on the embryo growth.

In conclusion, glucose may be useful for blastocyst production, if it is used in appropriate timing and concentration in chemically defined culture media.

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