Insulin and Insulin-Like Growth Factor-I (IGF-I) Stimulate the Development of Bovine Embryos Fertilized *In Vitro*

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ABSTRACT. To study the effects of insulin and insulin-like growth factor-I (IGF-I) on early development of bovine embryos, one-cell embryos were cultured in a chemically defined, protein-free medium: modified synthetic oviduct fluid. The addition of 10 $\mu\text{g/ml}$ insulin or 20 ng/ml IGF-I increased the percentage of morulae at Day 5 (5 days after insemination). The cell number of blastocysts which developed after 2 days culture of morulae obtained at Day 5 was increased by supplementation with insulin or IGF-I. The combination of insulin and IGF-I had neither additive nor synergistic effect on the embryonic development. These results clearly show that insulin and IGF-I improve the *in vitro* development of bovine embryos. — **KEY WORDS:** bovine embryo development, insulin, insulin-like growth factor-I.

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Recent studies have demonstrated that bovine embryos derived from oocytes matured and fertilized *in vitro* can develop to blastocysts in chemically defined, protein-free media [6, 20, 24, 30]. However, embryonic development using these cell-free culture systems is still deficient. A somatic cell-free culture system may lack some embryotrophic factors.

Insulin has been shown to enhance development of mouse [9–11, 18, 27], rat [15, 34], porcine [21, 26] and bovine embryos *in vitro* [22, 28]. In the mouse, IGF-I stimulates the development of the preimplantation embryo [12, 13]. Recently, it has been suggested that bovine embryos in the oviduct are exposed to IGF-I which is synthesized from the oviduct [25]. Therefore, IGF-I is expected to have a similar beneficial effect on the development of bovine embryos. However, previous reports have shown that IGF-I supplementation dose not improve bovine embryo development *in vitro* [6, 14]. Both insulin and IGF-I can cross-react with each other's receptor and elicit the same effects [8, 17]. In mouse embryos, IGF-I may act to stimulate protein synthesis by cross-reaction with the insulin receptor [12, 13, 18]. It is expected that some interactions exist between the effects of insulin and IGF-I on the development of bovine embryos as in mouse embryos.

The aim of the present study was to investigate the effects of insulin and IGF-I on the development of preimplantation bovine embryos using a chemically defined medium. To determine the effect of insulin and IGF-I on the development beyond the developmental arrest at the 8-cell stage (8- to 16-cell block) [5], the embryonic development to the morula stage was examined. Furthermore, the effects of insulin and IGF-I on the development of morulae to blastocysts were studied.

Cumulus-oocyte complexes (COCs) were collected by aspiration from small antral follicles (2–6 mm in diameter) of bovine ovaries obtained from a slaughterhouse. They were washed three times with HEPES-buffered Tyrode's medium [1] (TALP-HEPES) supplemented with 3 mg/ml bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, U.S.A.), 0.2 mM sodium pyruvate and 50 $\mu\text{g/}$

ml gentamicin (Sigma). *In vitro* maturation was performed in 25 mM HEPES-buffered TCM-199 (Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco), 0.2 mM sodium pyruvate, 0.02 U/ml FSH, 1 $\mu\text{g/ml}$ estradiol-17 β and 50 $\mu\text{g/}$ ml gentamicin. COCs were cultured in 50 μl drops of the maturation medium (10 COCs per drop) for 22 hr at 39°C under 5% CO₂ in humidified air. *In vitro* fertilization was performed using frozen semen from one bull. The motile sperm were separated using Percoll solution. Percoll (Pharmacia BioProcess, Uppsala, Sweden) was diluted to 45 and 90% (v/v) with modified Brackett and Oliphant isotonic medium [4] (BO) omitting BSA. Two ml of 90% Percoll solution was overlaid with 2 ml of 45% Percoll solution (Percoll gradient layer). Frozen-thawed semen was put on top of the Percoll gradient layer and centrifuged at 700 $\times$ g for 20 min. The top layers were removed and the sperm pellet was washed using BO medium by centrifugation at 500 $\times$ g for 5 min. The 10–15 COCs were coincubated with spermatozoa (5 $\times$ 10⁶ cells/ml) in a 100 μl drop of BO medium supplemented with 3 mg/ml fatty acid-free BSA (Sigma) and 2.5 mM theophylline (Sigma) for 20 hr at 39°C under 5% CO₂ in humidified air as described previously [31]. After insemination, the cumulus cells were removed from oocytes by vortex agitation in 100 μl TALP-HEPES for 1 min. Cumulus-free oocytes were washed three times and cultured in each medium at 39°C under 5% CO₂, 5% O₂ and 90% N₂. Synthetic oviduct fluid [32] (SOF) with slight modifications [30] was used for the basal culture medium (mSOF). Glucose and BSA were deleted from SOF, but 1 mg/ml polyvinyl alcohol (Sigma) and 20 amino acids were added. A premixed solution of basal medium Eagles (BME) essential amino acids (ICN Biomedicals Inc., Costa Mesa, CA, U.S.A.), minimum essential medium (MEM) nonessential amino acids (ICN) and 1 mM glutamine (Sigma) were added as amino acids.

In Experiment 1, to determine the effects of insulin and IGF-I on the development beyond the 8- to 16-cell block of bovine embryos, one-cell embryos were divided into 4 groups (2 $\times$ 2 factorial design), and cultured for 4 days in

(1) mSOF; (2) mSOF + 10 $\mu\text{g/ml}$ insulin (Sigma); (3) mSOF + 20 ng/ml IGF-I (Sigma) or (4) mSOF + 10 $\mu\text{g/ml}$ insulin + 20 ng/ml IGF-I. Embryos were fixed at Day 5 (122–124 hr after insemination; Day 0=day of insemination), and the total cell number, including metaphase plates but excluding pyknotic nuclei, was counted using the air-drying method previously described by Takahashi and First [30]. Embryos which had 16 or more cells were defined as morulae.

In Experiment 2, the effects of insulin and IGF-I on the embryonic development from morula to blastocyst were examined. To obtain morulae at Day 5, one-cell embryos were cultured for 4 days in mSOF alone. Since bovine embryos have both receptor genes for insulin and IGF-I at all preimplantation stages [33], insulin and IGF-I were not added to the culture medium to avoid their effects on embryos before the morula stage. Thereafter, compacted morulae at Day 5 were selected and were divided into 4 groups (2×2 factorial design) as in Experiment 1, and were cultured for 48 hr from Day 5 to Day 7. All blastocysts obtained at Day 7 (170–172 hr after insemination) were fixed and the total cell number was counted.

Data were analyzed by 2-way analysis of variance (ANOVA). When ANOVA revealed a significant interaction in treatment effects, the data were compared using Duncan's multiple range-test.

In Experiment 1, as a result of 2-way ANOVA (Table 1), it was found that supplementation with insulin and IGF-I did not affect the initial cleavage rate and the mean cell

number of morulae. The 2-way ANOVA showed that there was a significant interaction between the effects of insulin and IGF-I on the percentage of embryos that reached the morula stage ($p < 0.01$). Insulin and IGF-I significantly increased ($p < 0.01$) the percentage of morulae.

In Experiment 2, the 2-way ANOVA revealed that the addition of insulin or IGF-I had no effect on the percentages of blastocysts at Day 7, but there was a significant interaction between the effects of insulin and IGF-I on the cell number of blastocysts as shown in Table 2. Insulin and IGF-I significantly increased ($p < 0.01$) the cell number of blastocysts.

In bovine embryos, the maternal-zygote transition begins at the 4- to 8-cell stage and embryos are most sensitive to *in vitro* culture conditions during transcriptional shifts from the maternal to the embryonic genome [2, 3]. Developmental arrest (8- to 16-cell block) follows in the subsequent cell cycle [5]. It has been reported that the synergistic beneficial effect of insulin and amino acids on the development of bovine embryos into the morula stage may be caused by facilitating the transport of amino acids by insulin [22]. Insulin and IGF-I may be related to the quantitative and qualitative changes in protein synthesis or to the onset of new RNA synthesis between 8- and 16-cell stage which may arise by transcriptional shifts [3, 7]. The rate of protein synthesis of bovine embryos increases dramatically between the 16-cell and the blastocyst stages of development [7]. In the mouse, insulin and IGF-I

Table 1. Effects of insulin and insulin-like growth factor-I (IGF-I) on the development of one-cell bovine embryos into the morula stage^{a)}

Supplementation		No. of replicates (No. of oocytes)	% ova (mean $\pm$ s.e.m.)		No. of cells in morulae (mean $\pm$ s.e.m.)
Insulin	IGF-I		cleaved at Day 2	developed to morulae at Day 5	
–	–	4 (125)	80.0 $\pm$ 0.8	34.6 $\pm$ 0.7 ^{b)}	34.7 $\pm$ 0.6
+	–	4 (118)	81.3 $\pm$ 0.8	44.7 $\pm$ 0.9 ^{c)}	38.2 $\pm$ 1.4
–	+	4 (120)	82.4 $\pm$ 1.2	45.4 $\pm$ 0.7 ^{c)}	39.1 $\pm$ 1.4
+	+	4 (123)	82.1 $\pm$ 1.2	43.5 $\pm$ 1.7 ^{c)}	38.6 $\pm$ 1.6

a) One-cell embryos were cultured for 4 days (Day 1 to Day 5) in mSOF with (+) or without (–) insulin (10 $\mu\text{g/ml}$) and IGF-I (20 ng/ml). Day 0=day of *in vitro* insemination.

b-c) Values with different superscripts are significantly different ($p < 0.01$).

Table 2. Effects of insulin and insulin-like growth factor-I (IGF-I) on the development of bovine morulae into the blastocyst stage^{a)}

Supplementation		No. of replicates (No. of morulae)	% ova (mean $\pm$ s.e.m.)		No. of cells in blastocysts (mean $\pm$ s.e.m.)
Insulin	IGF-I		developed to blastocysts at Day 7		
–	–	4 (44)	70.1 $\pm$ 8.2		145.1 $\pm$ 1.7 ^{b)}
+	–	4 (48)	71.7 $\pm$ 9.5		166.3 $\pm$ 2.4 ^{c)}
–	+	4 (40)	75.1 $\pm$ 9.2		163.9 $\pm$ 2.1 ^{c)}
+	+	4 (42)	69.7 $\pm$ 2.9		161.8 $\pm$ 3.6 ^{c)}

a) Morulae were cultured for 2 days (Day 5 to Day 7) in mSOF with (+) or without (–) insulin (10 $\mu\text{g/ml}$) and IGF-I (20 ng/ml). Day 0=day of *in vitro* insemination.

b-c) Values with different superscripts are significantly different ($p < 0.01$).

stimulate the transport of amino acids and the synthesis of proteins [10, 19]. Furthermore, the synthesis of some specific proteins in preimplantation mouse embryos are affected by insulin and IGF-I [29]. It is suggested that insulin and IGF-I might support cell proliferation of bovine embryos by promoting the transport of amino acids and the synthesis of proteins as in mouse embryos.

In mouse embryos, IGF-I may act to stimulate protein synthesis by way of a cross-reaction with the insulin receptor [12, 13, 18]. Transcripts for ligand and receptor genes of insulin, IGF-I and IGF-II were detected at all stages of preimplantation bovine embryos [33]. The combination of insulin and IGF-I had neither additive nor synergistic effect on the development to morulae (Experiment 1) and the cell number of blastocysts (Experiment 2). These results suggest that insulin and IGF-I can cross-react with each other's receptors similarly as in mouse embryos. Therefore, previous researchers could not find any beneficial effect of supplementation with IGF-I on the development of bovine embryos, because their basic media were supplemented with insulin or serum which contained IGF-I [6, 14].

In bovine embryos, the immunocytochemical detection of insulin and IGF-I receptors was reported at the blastocyst stage [16]. However, the binding of insulin and IGF-I and the localization of insulin and IGF-I receptors are not known before the blastocyst stage. Further experiments are needed to investigate the binding of insulin and IGF-I and the cellular localization of insulin and IGF-I receptors in bovine embryos. It is necessary to clarify the mechanism mediating the effects of insulin and IGF-I on the development of preimplantation bovine embryos.

In conclusion, the present study demonstrates that insulin and IGF-I promote the development of preimplantation bovine embryos. Furthermore, it is suggested that the beneficial effects of insulin and IGF-I may be mediated by a cross-reaction with each other's receptors.

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