Stage-Specific Effect of Growth Hormone on Developmental Competence of Bovine Embryos Produced In-Vitro

Hisataka IWATA1, Mayuko OHOTA1, Shu HASHIMOTO2, Koji KIMURA3, Mamiko ISAJI4 and Masashi MIYAKE4


Abstract. Many efforts have been made to develop effective culture conditions for the production of bovine blastocysts. Growth hormone (GH) and glucose are known to affect in vitro embryo development. To improve in vitro culture conditions, the culture medium containing fetal calf serum (FCS) or bovine serum albumin (BSA) was supplemented with GH at various periods of development, and the effects of GH on the rate of development and the quality of the blastocysts were studied. Then, starting at the morula stage, the effect of glucose and GH on the rate of development was studied. In all experimental periods, FCS was more effective than BSA at improving the development rate and increasing the cell number of blastocysts. Adding GH to the culture medium between 18 and 48 h after fertilization (1–8 cell stage embryo) did not affect either the rate of blastulation or the cell number regardless of the serum protein (FCS or BSA). From 48 to 120 h after fertilization (5-cell to morula stage) GH increased the cell number of the blastocysts in the presence of BSA, but not in the presence of FCS. From 120 to 192 h after fertilization (morula to blastocyst stage), GH improved the developmental rate and cell number in the presence of FCS, although there was no significant difference when BSA was used instead of FCS as the serum protein. When cows were implanted with blastocysts developed in the presence of GH, their pregnancy rate did not differ from that of the control. Increasing the glucose concentration in the medium from 1.5 mM to 3 mM starting at the morula stage (120 h after fertilization) slightly decreased the rate of development, but on the other hand, decreasing the glucose concentration to 0 mM did not affect either the rate of development or the cell number. Also, then GH had no effect on the developmental rate or the cell number in the absence of glucose. In conclusion, when the medium was supplemented with serum, GH improved embryo development from the morula stage, but an increased concentration of glucose decreased embryo development. Furthermore, GH did not improve the pregnancy rate of blastocysts developed in vitro.

Key words: Growth hormone, Glucose, Bovine embryo

T he development of bovine embryos in vitro is well known to be highly affected by culture conditions. Therefore, much effort has been made to develop effective culture conditions for embryo development. Recent observations have shown that the injection of bovine growth hormone (GH) into heifers and cows increased the concentration of GH in serum or follicular fluid from 5–7 ng/ml to 31–53 ng/ml and improved oocyte maturation [1]. Furthermore, adding 100 ng/ml GH to the culture conditions improved oocyte maturation [2, 3] and early embryo development [4, 5]. In these reports,
GH was added to the medium throughout the culture period, so it is unclear whether it has equal activity throughout the culture period. To efficiently produce high quality embryos, it is important to know when development is most strongly affected by GH and to assess the quality of embryos treated with GH in vitro. FCS has been shown to improve embryo development and to increase the cell number of blastocysts [6–9]. However, GH has been reported to improve embryo development in chemically defined medium, but not in medium containing serum or in a co-culture system [4]. Therefore in this study, FCS and BSA were each used as the serum protein. GH was shown to increase the glucose uptake of mouse embryo [10]. Glucose is a major energy source for mammal cells, although a high glucose concentration during early embryo development is reported to be detrimental [11]. While some reports have shown that glucose metabolism dramatically increases from the morula stage to the blastocyst stage [12, 13], few reports have studied the effect of increased glucose concentration from the morula stage on embryo development [10].

In this study GH was added at various periods of development (18–48 h, 48–120 h and 120–192 h after fertilization) to in vitro culture medium containing either fetal calf serum (FCS) or bovine serum albumin (BSA) to determine its effect on embryo development. Embryos treated with GH from the morula stage were transferred to cows to determine the effect of GH on the pregnancy rate. The effect of adding glucose and GH from the morula stage on embryo development was also examined.

**Materials and Methods**

*In vitro maturation, fertilization and culture*

Bovine ovaries were collected from carcasses at a slaughterhouse. They were preserved in physiological saline at approximately 35 C and transported to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (3 to 5 mm in diameter) using a 20-G needle connected to a 10-ml syringe. COCs surrounded by compact and thick cumulus cells were randomly selected and cultured in a 100-µl droplet of maturation medium (15 oocytes/drop) under paraffin oil at 39 C in an atmosphere of 5% CO2 in air with maximum humidity. The media used for maturation (IVM), fertilization (IVF), and culture (IVC) were based on synthetic oviductal fluid (SOF) [11] and all of media were supplemented with 5 mM taurine, 1 mM L-glutamine (Nacalai Tesque, Kyoto, Japan) and 100 U/ml penicillin and 100 µg/ml streptomycin. Both nonessential amino acids (NEAA) and essential amino acids (EAA) were added to the media used for IVM and IVC at the concentrations present in Eagle’s minimum essential medium (MEM) and basal Eagle’s medium, respectively. The medium for IVM was supplemented with 1 g/l glucose, 10% fetal calf serum (FCS: Gibco BRL, Rockville, MD), 1 µg/ml β-estradiol (Sigma Chemical Co., St. Louis, MO, USA) and 1 µg/ml follicular stimulating hormone.

*In vitro fertilization (IVF)* was performed as described previously [15]. Eighteen hours after fertilization, zygotes were denuded by vortexing (30 sec) and cultured in a 100-µl droplet of modified-SOF (m-SOF) medium containing 1% FCS and 1.5 mM glucose. Forty-eight hours after fertilization, the number of cleaved embryos (>4-cell) was counted and these cleaved embryos were transported in a 100-µl droplet of m-SOF medium containing 5% FCS and 1.5 mM glucose. The embryos were cultured at 39 C in an atmosphere of 5% CO2, 5% O2 and 90% N2 with maximum humidity. Each experiment was repeated 4 times. The rate of blastulation was assessed at days 6, 7 and 8 after fertilization and the cell number of all the blastocysts was counted at day 7 after fertilization. For the counting of the total cell numbers, blastocysts were fixed in acetoalcohol (ethanol: acetic acid: water; 3: 1: 1) and air-dried on slides for 1 min, then subjected to Giemsa staining.

**Experimental design**

In experiments 1–3, the effect of GH supplemented at various culture periods (18–48 h, 48–120 h and 120–192 h post fertilization) on embryo development were studied. Under our culture conditions, a normal embryo develops to about the 5–8 cell stage at 48 h after fertilization, and then at 120 h after fertilization, the embryo develops to the early morula stage. These morulas contain 25.3 ± 6 cells on average. In experiments 1–3, FCS or BSA (fatty acid free, Nacalai Tesque, Kyoto, Japan) was used as the serum protein to clarify the effect of GH.

In experiment 1, 18 hours after fertilization, 30 one-cell stage zygotes were randomly selected for
each experimental group. The medium used for the experiment was m-SOF containing 1% FCS or 7 mg/ml BSA. Some of each medium was supplemented with 100 ng/ml recombinant bovine GH (ICN Biomedical, Inc. Costa Mesa CA, USA). Forty-eight hours after fertilization, the embryos were washed and cultured in IVC medium containing 5% FCS for 6 days.

In experiment 2, 48 hours after fertilization, 20 normal cleaved zygotes (>4-cell) were randomly selected and transferred to experimental groups. The medium used for the experiment was m-SOF containing 5% FCS or 7 mg/ml BSA, and 0 or 100 ng/ml GH. One hundred and twenty hours after fertilization, the embryos were washed and cultured in IVC medium containing 5% FCS for 3 days (192 h after fertilization). In total of 330 embryos were selected from 617 cleaved embryos (>4 cells) developed from 1005 oocytes.

In experiment 3, 120 hours after fertilization, 12 normal morula stage embryos were randomly selected from 5-day-old embryos for each experimental group and cultured for 3 days (192 h after fertilization). The medium used for the experiment was m-SOF containing 5% FCS or 7 mg/ml BSA, and 0 or 100 ng/ml GH. In total, 192 morulas were selected from 348 morulas developed from 1140 oocytes.

In experiment 4, the amount of glucose in the culture medium was increased to 3 mM from 1.5 mM or decreased to 0 mM from 1.5 mM. The effect of GH (0 or 100 ng/ml) on the developmental competence of the embryos was then studied. Normal morula stage embryos (120 h after fertilization) were collected and randomly transferred to the experimental groups as described in experiment 3. In total, 240 morulas were selected from 451 morulas developed from 1560 oocytes.

In experiment 5, 7-day-old blastocysts that had developed from the morula stage in the presence or absence of GH were freshly transferred to cows. Recipients were selected and the embryos were transferred as described previously [16]. The pregnancy rate was assessed at 60 days after the transfer by palpation. For each experiment group, 30 blastocysts were transferred to 30 cows. Two cows in the GH (+) group died, and no data could be determined for them.

**Statistical analysis**

To analyze the rate of blastulation, the frequencies of the data were compared using Fisher’s protected least significant difference (PLSD) test following an analysis of variance (ANOVA). All data were subjected to an arcsine transformation before statistical analysis. A p value less than 0.05 was considered to be significant. The mean cell numbers of blastocysts were compared using the Scheffe test following ANOVA. A p value less than 0.05 was considered to be significant.

**Results**

In experiments 1, 2 and 3, using BSA instead of FCS significantly decreased both the rate of development and the cell number of blastocysts (Table 1–3). During the early developmental period (18 to 48 h after fertilization), when GH was supplemented to the medium, no differences were observed in either the rate of development or the cell number, regardless of the serum protein (Table 1). Later in the development period (48 to 120 h after fertilization), adding GH to the medium containing FCS had no effect on either the rate of development or the cell number. However, when BSA was used instead of FCS, GH improved embryo development significantly (GH–: 26% vs GH+: 35%; Table 2). In the period between the morula stage and the blastocyst stage, GH significantly improved both the rate of blastulation and the cell number in the medium containing FCS but not in the medium containing BSA (Table 3). In experiment 4, when the glucose concentration was increased from 1.5 mM to 3 mM starting at the morula stage, the rate of blastulation was significantly decreased (3 mM: 65 vs 1.5 mM: 74%; Table 4). However, decreasing the glucose concentration from 1.5 mM to 0 mM did not affect either the rate of blastulation or the cell number. Furthermore, adding GH to the medium containing 3 mM glucose slightly increased the cell number of blastocysts (103.5 ± 17.9 vs 92.8 ± 16.8). However, in the glucose-free medium, GH did not affect either the rate of blastulation or the cell number. In experiment 5, no difference was observed in the pregnancy rates obtained from embryos developed with and without GH (GH+: 42.9% vs GH–: 43.3%).
Discussion

In this study, using BSA instead of FCS decreased both the rate of development and the cell number, regardless of when BSA was added to the culture medium. These results indicate that serum is more efficient as a protein source than BSA for the mass production of bovine embryos. In this

Table 1. Effect of GH treatment during 18 to 48 h after fertilization on developmental competence of embryos

<table>
<thead>
<tr>
<th>FCS or BSA</th>
<th>GH ng/ml</th>
<th>No. of embryos</th>
<th>No. of trials</th>
<th>No. (%) of blastocysts Day 6*</th>
<th>Day 7*</th>
<th>Day 8*</th>
<th>Cell No. ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS 0</td>
<td>120</td>
<td>4</td>
<td>10(8)</td>
<td>119* ± 23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>120</td>
<td>4</td>
<td>9(7)</td>
<td>127a ± 27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA 0</td>
<td>120</td>
<td>4</td>
<td>7(6)</td>
<td>81b ± 13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>120</td>
<td>4</td>
<td>7(6)</td>
<td>81b ± 21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Days after insemination.

Table 2. Effect of GH treatment during 48–120 h after fertilization on developmental competence of embryos

<table>
<thead>
<tr>
<th>FCS or BSA</th>
<th>GH ng/ml</th>
<th>No. of embryos</th>
<th>No. of trials</th>
<th>No. (%) of blastocysts Day 6*</th>
<th>Day 7*</th>
<th>Day 8*</th>
<th>Cell No. ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS 0</td>
<td>80</td>
<td>4</td>
<td>12(15)*</td>
<td>106* ± 27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>80</td>
<td>4</td>
<td>16(20)*</td>
<td>102± 23</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BSA 0</td>
<td>80</td>
<td>4</td>
<td>3(4)</td>
<td>73b ± 13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>80</td>
<td>4</td>
<td>4(5)</td>
<td>87ab ± 21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Days after insemination.

Table 3. Effect of GH treatment during 120–192 h after fertilization on developmental competence of embryos

<table>
<thead>
<tr>
<th>FCS or BSA</th>
<th>GH ng/ml</th>
<th>No. of embryos</th>
<th>No. of trials</th>
<th>No. (%) of blastocysts Day 6*</th>
<th>Day 7*</th>
<th>Day 8*</th>
<th>Cell No. ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS 0</td>
<td>48</td>
<td>4</td>
<td>10(21)</td>
<td>86* ± 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>48</td>
<td>4</td>
<td>11(23)*</td>
<td>99b ± 18</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BSA 0</td>
<td>48</td>
<td>4</td>
<td>5(10)</td>
<td>85b ± 18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>48</td>
<td>4</td>
<td>7(15)</td>
<td>96b ± 12</td>
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</tbody>
</table>

* Days after insemination.

Table 4. Effect of GH treatment during 120–192 h after fertilization on developmental competence of embryos

<table>
<thead>
<tr>
<th>Glucose mM</th>
<th>GH ng/ml</th>
<th>No. of embryos</th>
<th>No. of trials</th>
<th>No. (%) of blastocysts Day 6*</th>
<th>Day 7*</th>
<th>Day 8*</th>
<th>Cell No. ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>48</td>
<td>4</td>
<td>12(25)</td>
<td>101</td>
<td>± 23</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>48</td>
<td>4</td>
<td>10(21)</td>
<td>103</td>
<td>± 30</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>0</td>
<td>48</td>
<td>4</td>
<td>12(25)</td>
<td>98</td>
<td>± 17</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>48</td>
<td>4</td>
<td>7(15)</td>
<td>93</td>
<td>± 17</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>48</td>
<td>4</td>
<td>8(17)</td>
<td>104</td>
<td>± 18</td>
<td></td>
</tr>
</tbody>
</table>

* Days after insemination.

Discussion

In this study, using BSA instead of FCS decreased both the rate of development and the cell number, regardless of when BSA was added to the culture medium. These results indicate that serum is more efficient as a protein source than BSA for the mass production of bovine embryos. In this
experiment, a positive effect of GH was observed in experiments 2 and 3 but not in experiment 1. It is reported that mRNA of the GH receptor (GHR) was found to be expressed from the GV stage of the oocyte to the blastocyst stage [4] and was 5.9 times higher in 6-day-old embryos than in 2-day-old embryos. Furthermore, the GHR protein was first visualized 3 days after fertilization [5]. This result, together with the present results, indicates that GH is not effective for embryo development during the early developmental stage of the embryo. From 48 to 120 h post-fertilization (from the 5-cell stage to the morula stage), GH improved embryo development in the medium containing BSA but not in the medium containing FCS. This discrepancy may be due to the fact that serum conceals the effect of GH because serum has many developmental factors [17]. On the other hand, GH significantly improved the developmental competence of morula-stage embryos in the medium containing FCS but not in the medium containing BSA. However, there was a slight increase in the cell number, though the difference was not significant (Table 3). This may be due to the small number of data points or to the poor ability of BSA to support embryo development. These results suggest that when embryos are cultured in serum-containing medium, the effective period for GH supplementation starts at 120 h post-fertilization (the morula stage).

Glucose is a major energy source for mammalian cells. During the early developmental stage of bovine embryos, almost all glucose is metabolized through the pentose phosphate pathway. During this stage, the level of glucose metabolism in the cells is normally very low, and increasing the glucose concentration at this time inhibits embryo development [11]. Starting at the 8–16 cell stage, glucose metabolism rapidly increases as a result of the development of the glycolytic pathway [13]. Although adding 1.5 mM glucose after 48 h post-fertilization has been shown to have a beneficial effect on embryo development [18], adding 5.5 mM glucose from the morula stage had only a small effect on the rate of blastulation and quality [14]. In this study, when the glucose concentration was increased from 1.5 to 3 mM at the morula stage, the rate of blastulation at day 7 after fertilization was lower than that obtained with 1.5 mM glucose. On the other hand, starting at the morula stage, reducing the glucose concentration did not affect the rate of blastulation. This result suggests that an increased glucose concentration, instead of increasing glucose metabolism, is detrimental to 5-day-old embryos (morula). Although the role of GH on embryo development is not clear, GH was reported to increase the glucose uptake of the mouse embryo [19]. This effect of GH on glucose transport is due to direct recruitment of the transporters GLUT 1 and GLUT 4 to the plasma membrane [20]. In this study GH was found to have a positive effect on the cell number of blastocysts in medium supplemented with 1.5 and 3 mM glucose. On the other hand, reducing the glucose concentration from 1.5 mM to 0 mM had no effect on either the development rate or the cell number. These results suggest that the positive effect of GH on the development of the bovine embryo is related to the glucose contained in the medium.

Administration of bovine somatotropin at the time of insemination was found to enhance embryonic development and to increase the pregnancy rate [21]. However, little is known about how in vitro development of embryos in the presence of GH affects the pregnancy rate. Our results show that GH increased embryo development and the cell number, but it did not improve the pregnancy rate. In general, there are many differences between embryos developed in-vivo and embryos developed in-vitro, and this difference results from suboptimal culture condition in vitro (22). In this study, embryos were cultured with GH for 2 days (from 5 days after fertilization to 7 days). Our results suggest that unknown factors derived from the in vitro culture condition conceal the effect of GH treatment on the rate of blastulation, or that 2 days of GH treatment is not of long enough duration to improve embryo development in vitro.

In conclusion, when serum was used as the protein source, GH improved embryo development from the morula stage, but an increased concentration of glucose decreased embryo development. In addition, GH did not improve the pregnancy rate of blastocysts developed in vitro.
References


