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Effects of Growth Hormone and Growth Factors on the Improvement of Culture Conditions of *In vitro* Produced Bovine Embryos

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Abstract: The effect of growth hormone (GH), activin, insulin and epidermal growth factor (EGF) was examined on nucleus maturation, cleavage after fertilization and development of bovine oocytes to blastocysts *in vitro*. COCs were cultured in the presence of medium alone mSOFaa [Modified oviduct synthetic fluid with amino acids] (control), activin (10ng/ml), EGF (10ng/ml), GH (100ng/ml) and insulin 5μg/ml. There was an increase ($P < 0.05$ and $P < 0.01$) in the percentage of oocytes that reached metaphase II when EGF and GH were added to maturation medium. EGF had a higher percentage and insulin a lower (97 and 87, respectively). All the additives tested did not increased cleavage rate. The tendency was the same in terms of developmental to blastocyst and hatching rates. As a trend EGF and GH showed higher overall effects compared to others. When mean total cell number was observed, all the growth additives, except insulin, increased ($P < 0.05$ and $P < 0.01$, respectively) blastocyst cell number compared to the control. Among the factors tested, GH and EGF had higher cell number, with mean cell counts of 173.5 ± 1.1 and 151.6 ± 2.0, respectively. Addition of GH and growth factors in culture media can improve the quality of embryos that are capable of high pregnancy rates after transfer to the recipients yielding healthy offspring.

Key words: Activin-A, EGF, GH, insulin, *in vitro* fertilized, blastocysts

Introduction

Biotechnologies for mammals such as gene transfer, somatic cell cloning in particular with embryonic stem cells, involve the manipulation of oocytes and embryos *in vitro*. A common objective of these applications is to harvest *in vitro* produced (IVP) cattle embryos from these biotechnologies which are capable of high pregnancy rates after transfer to the recipients and yielding healthy offspring free of calving difficulties. Yet more extensive use of these embryos in recent years indicates that *in vitro* technologies may produce IVP blastocysts that are developmentally compromised compared to their *in vivo* counterparts. This may be attributed to the heterogeneous population of oocytes obtained from this follicle population and also by deficiencies in culture media used for oocyte maturation, fertilization and embryo culture (Leibfried-Rutledge, 1994).

Hormones like LH, FSH and estrogens are frequently used as additives in culture media to enhance the quality of the maturation process (Zuelke and Bracketf, 1990). However, folliculogenesis and oocyte maturation is regulated not only by the gonadotropins and steroids but also by other hormones such as growth hormone (GH) and a variety of locally produced paracrine acting cytokines. Among these regulators there are several growth factors such as insuline-like growth factors (IGFs), epidermal growth factor (EGF), transforming growth factor α (TGFα), TGFβ and activin (Bevers *et al.*, 1997).

Culture of bovine cumulus oocytes complexes (COCs) in the presence of growth hormone accelerates the process of nuclear maturation and increases cumulus expansion, fertilization rate of cleaved embryos and of blastocysts due to the improved distribution of cortical granules (Izadyar *et al.*, 1998).

Insulin has been shown to increase the rate of glucose transport in the blastocysts, blastocysts metabolism *in vitro* and the rate of morphological development of mouse embryos during preimplantation period *in vitro* (Gardner and Kaye, 1991). There is also an evidence of insulin acting synergistically with amino acids in improving development of rat embryos *in vitro*.

Epidermal growth factor is a mitogenic factor with the ability to stimulate the proliferation and differentiation of ovarian granulosa cells. It has been demonstrated that epidermal growth factor stimulates *in vitro* maturation of bovine oocytes in the presence of gonadotropins (Kobayashi *et al.*, 1994; Lorenzo *et al.*, 1994) and even in the absence of them (Park *et al.*, 1997). Park *et al.* (1999) demonstrated that EGF stimulates the production of plasminogen activators by cumulus cells, which may play a role in a cytoplasmic maturation of oocytes, which results in normal fertilization and development of oocytes.

In early preimplantation embryos, it has been noticed that activin A (Recombinant human activin A) stimulates the development and early preimplantation of embryos to the blastocyst stage when they are cultured in a chemically defined medium (Yoshioka and Komamae, 1996). The same authors demonstrated that when activin A was added to the bovine embryos, there was an increase in the number of one-cell embryos reaching morula or blastocyst stage.

In this study experiments were conducted to examine the effectiveness of the growth hormone (GH), activin-A, insulin and EGF on cumulus expansion, nuclear maturation, cleavage rates and development to blastocyst of *in vitro* produced bovine embryos and to determine which one of them is the most effective.

Materials and Methods

Collection and culture of cumulus oocytes complexes: Bovine ovaries were collected from Japanese Black cows at a local abattoir, and were transported to the laboratory in Ringer's solution supplemented with penicillin-G (100 IU/ml) and streptomycin sulphate (0.2μg/ml) at 30-32°C in a thermo flask within 3 h. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 2-8 mm follicles and selected based on the presence of a multilayered compact cumulus investment. Selected COCs were rinsed in a maturation medium, TCM199 (Earle's salt; Gibco, Grand Island, NY, U.S.A.) + 10% heat-treated fetal cow serum (FCS; Life Technologies, Auckland, New Zealand) + 0.001 mg/ml follicle stimulating hormone (FSH; Denka Pharmaceutical Co., Kawasaki, Japan) + 50 μg/ml gentamycin sulphate (Sigma, St. Louis, MO, USA). Then, in groups of 35, randomly allocated in each well of a 4-well culture dish (Nunc A/S, Roskilde, Denmark) containing 500μl of the same media and cultured for 24 h. Except where otherwise indicated, culture was performed at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

Assessment of nuclear maturation: To investigate the effects of Activin-A, EGF, Insulin, and growth hormone (GH) on nuclear maturation, cumulus oocytes complexes (COCs) were cultured for 24 h in five different groups: 1) maturation media (MM) alone (control); 2) 10 ng/ml recombinant human activin-A (R&D Systems, Inc. Minneapolis, MN, USA); 3) 10 ng/ml epidermal growth factor (EGF; Sigma); 4) 5 μg/ml insulin (Sigma); and 5) 100 ng/ml growth hormone (GH; Sigma) added to MM.

After culture, the nuclear status of the oocytes was determined by aceto-orcein (Orcein acetate) staining. Cumulus complex

oocytes (COCs) were denuded by treating them with 0.5% hyaluronidase (Sigma) for 2 min followed by repeated pipeting with a narrow-bore micropipette in a modified phosphate-buffered saline (mPBS) solution. COCs were fixed overnight in acetic acid: ethanol (1 : 3), stained in 1% orcein and observed under a phase contrast micrscope and observed under a phase-contrast microscope (Olympus, Japan) for examination of their nuclear status. Oocytes that reached metaphase II stage were recorded as matured.

Sperm preparation, IVF and IVP: Twenty four hours after culture in MM, COCs were fertilized *in vitro*. Fertilization was carried out at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Frozen and thawed spermatozoa used for IVF were centrifuged twice over 2 µM caffeine in modified synthetic oviduct fluid with amino acids (mSOFaa), for 5 min each at 500 g at 25°C. The sperm sample was collected by removing the supernatant except for the last 150 µl containing the sperm pellet. The pellet was then suspended in Caffeine-mSOFaa supplemented with 1% (w/v) Bovine serum albumin (BSA: Fatty acid free; Sigma) and 2 µg/ml heparin (Shimizu Pharmaceutical Co., Japan) to yield a final sperm concentration of 5×10^6 spermatozoa/ml. One hundred micro liters of sperm suspension was covered with mineral oil (Sigma) and pre-incubated for 1 h. Cultured oocytes were transferred into sperm micro drop (20-25 oocytes per micro drop) for fertilization of 18 h.
Eighteen hours after coincubation, 25-30 presumptive zygotes were cultured in 500 µl of the culture medium at 38.5°C in a humidified atmosphere of 5% CO_2 in air in five different treatment groups: 1) mSOFaa + 10% (v/v) heat-treated fetal cow serum (Control); 2) mSOFaa + 10 ng/ml rh Activin-A; 3) mSOFaa + 10 ng/ml (EGF); 4) mSOFaa + 5 µg/ml insulin and 5) mSOFaa + 100 ng/ml GH. On the second day (48 h of culture) 250 µl of fresh medium was added. Blastocysts were scored morphologically on day 7 of culture and used for vitrification. Best blastocysts were used for vitrification and the other embryos were cultured for further assessment.

Blastocyst cell number determination: Hatched blastocysts from each group were prepared for cell counts. Briefly, embryos were fixed with 1% (v/v) formaldehyde (Sigma) for 10 min at room temperature and then placed in a drop of mounting medium on a slide. The mounting medium consisted of 25% (v/v) glycerol in PBS containing 2.5 mg/ml sodium azide and 2.5 µg/ml Hoechst 33342 (Sigma). A coverslip was placed on top of the embryos and the edge of the slide was sealed with fingernail polish. The total cell numbers were counted under Nikon fluorescent microscope (Nikon Optiphot, Tokyo, Japan).

Statistical analysis: The differences between growth factors, GH and control on nuclear maturation, cleavage, development to blastocysts and mean cell number in blastocysts were assessed by ANOVA, and means were compared using a least significant difference test. All percentage data were converted using arcsine transformation prior to ANOVA. A probability level of $P < 0.05$ and $P < 0.01$ was considered statistically significant.

Results

Effect of growth factors and GH on nuclear maturation: A total of 1673 oocytes were used for either checking the nuclear status of oocyte after *in vitro* culture in MM (n = 560) or for IVF (n = 1113). There was an increase ($P < 0.05$) in the percentage of oocytes that reached metaphase II when Activin, EGF and GH were added to the maturation media over the control (Table 1). The addition of insulin did not show any significant difference over control. Between growth factors tested and GH, there was no significant difference in the percentage of oocytes that reached metaphase II, although EGF and GH had higher percentage and insulin had lower.

Effect of growth factors and GH on *in vitro* embryo production: Addition of EGF, activin, insulin and GH in the culture media did not have significant effect on the cleavage, although they had higher percentages than control (Table 1). This may be explained as a result of the bull's effect and quality of the sperm. As a tendency, EGF and GH had higher mean cleavage rate (90 and 89%, respectively) than activin and insulin (84 and 83%, respectively). The mean cleavage rate for control was 77%. When the development to blastocyst was analyzed, activin, GH and EGF had higher ($P < 0.05$ and $P < 0.01$, respectively) percentage of oocytes developed to blastocyst than control. Insulin did not have any significant effects from control. The mean blastocyst production rate for control was 25%. The hatching rates of blastocysts did not differ significantly, but expressed the tendency for betterment showed by the corresponding additives used in this experiment (Table 1).
To evaluate further the quality of the blastocysts produced under growth hormone and growth factor culture conditions, the cell number was determined. The mean cell count per blastocyst was distinctly affected by the culture media (Table 1). Embryos cultured in medium alone (MM) had a mean cell count of 103.2 ± 3.4. All the growth additives, except insulin, increased ($P < 0.05$ and $P < 0.01$, respectively) blastocyst cell number compared to the control. Among the factors tested, GH and EGF had higher cell number, with mean cell counts of 173.5 ± 1.1 and 151.6 ± 2.0, respectively.

Discussion

This study clearly demonstrates that cumulus expansion, *in vitro* maturation, cleavage, blastocyst development and hatching ability of *in vitro* produced bovine embryos can be altered by the addition of growth factors and hormones. We were able to demonstrate that culture factors can improve nuclear maturation and favor the cleavage and development of embryos to hatched stage and increase total cell number per embryo. Many studies have been done to determine the effects of GH and growth factors tested here individually. There are few reports on the comparison between them.
Autocrine secretion of growth factors and growth hormone by embryos and expression of specific receptors at particular stages strongly implicate growth factors as mediators in early embryonic events (Izadyar *et al.*, 2000; O'Neil, 1998; Teruel *et al.*, 2000). O'Neil (1997) reported that when embryos were cultured at a concentration of one embryo μl^{-1} of medium, there is an enhancement in blastulation and cell number per embryo. Reducing embryo concentration to one embryo per 10-100 µl result in a loss of autocrine embryotrophic effect. In our case the concentration of the media was reduced as we cultured 35 oocytes/embryo in 500µl medium, assuming that there was no autocrine effect.

Table 1: Maturation of oocytes, cleavage, development to blastocysts and cell number of bovine embryos cultured in mSOFaa supplemented with GH or growth factor

Treatment	No. of oocytes stained	No. (%) Matured	No. of oocytes used for fertilization	No. (%) Cleaved	No. (%) developed to blastocysts	No. (%) hatched blastocysts	Mean cell number in blastocysts
1 Control	120	84 (70)^{a}	251	193 (77)	64 (25)^{cp}	12 (5)	103.2± 3.4^{er}
2 Activin	100	92 (92)^{b}	202	170 (84)	85 (42)^{d}	20 (10)	136.0± 3.6^{f}
3 EGF	120	116 (97)^{b}	216	194 (90)	115 (53)^{q}	55 (25)	151.6± 2.0^{f}
4 Insulin	100	87 (87)	198	164 (83)	80 (40)	18 (9)	118.3± 2.1
5 GH	120	113 (94)^{b}	246	219 (89)	121 (49)^{d}	47 (19)	173.5± 1.1^{s}

Column with different superscripts are statistically different $^{a-b, c-d}$ and $^{e-f}$ ($P < 0.05$) while $^{p-q}$ and $^{r-s}$ ($P < 0.01$)
1) mSOFaa + 10% FCS (Control); 2) mSOFaa + 10 ng/mL rh Activin ; 3) mSOFaa + 10 ng/mL EGF;
4) mSOFaa + 5 µg/mL insulin and 5) mSOFaa + 100 ng/mL GH

Growth factors and growth hormone receptors interaction during the regulation of early embryo has been the most clearly demonstrated. Of all the growth factors, activin-A has the least information that is currently available on expression and distribution in the blastocysts.

Several studies have analyzed the effect of EGF on the development of embryos *in vitro* (Merriman *et al.*, 1998; Morita *et al.*, 1994; Paria *et al.*, 1990). Furthermore, EGF significantly enhanced trophoblast outgrowth and the percentage of hatching mouse blastocyst itself at the specific concentration (Kim *et al.*, 1999). In our study EGF has revealed higher maturation and developmental capacities than GH and other growth factors used. This may be due to its high mitogenic action (Lee *et al.*, 1995)). Related to this may be the finding that EGF stimulates plasminogen activators secretion, which has effects on various reproductive processes including implantation (Park *et al.*, 1999). Insulin has shown to have broad effects on preimplantation embryos. It has been shown to promote glucose uptake by cells and is also mitogen. The effect of insulin on protein synthesis was reported to depend on the developmental stages. At the 8-cell stage insulin increased synthesis of some proteins and decreased others (Shi *et al.*, 1994); however at later stages of development the effect was mostly stimulatory (Harvey *et al.*, 1988). It has also been demonstrated that insulin inhibited endogenous protein degradation in blastocysts increasing the uncatabolized protein pool (Dunglison *et al.*, 1993). The uncatabolized proteins may be toxic to the embryos. This may be the reason for very poor survival rate, hatching rate and even the mean cell number compared with other growth factors and GH in our study. Recently, Herrler *et al.* (1998) demonstrated that the addition of insulin to the culture medium decreased the apoptosis and increased cell proliferation of preimplantation rabbit embryos.

In this experiment, embryos cultured in the presence of insulin increased to a relatively higher percentage of blastocyst formation and blastomere numbers than control, agreeing with the previous author.

Cytokines play a functional role in the process of cellular proliferation. In early preimplantation embryos, it has been noticed that cytokines are produced as paracrine or autocrine factors. In this study activin-A increased the nuclear maturation, cleavage, blastocyst development, hatching and cell number of blastocyts, whereas, cumulus expansion was not altered by the addition of Activin-A to the medium. Yoshioka *et al.* (1996) previously showed a role of activin A on bovine embryo development.

The presence of functional GH receptors at an early stage raises the possibility that GH may be involved in early embryonic growth and development. The functionality of GH in bovine embryos is clearly demonstrated by the actions of GH on cleavage and blastocyst formation as well as hatchability of the blastocyst. Our results revealed that addition of GH to the maturation and culture media enhanced the nuclear maturation and subsequent development of embryos, suggesting that these embryos have a better quality than control ones. It has been shown that culture of oocytes and embryos in the presence of GH resulted in an improved embryo development in terms of more blastocyst and increased blastocyst formation (Dong *et al.*, 2001; Fukaya *et al.*, 1998; Izadyar *et al.*, 2000). Moreover, mouse blastocysts that have been cultured with GH are reported to have more blastomeres and have a higher chance for implantation after transfer to the recipient uteri (Fukaya *et al.*, 1998). In this study GH had higher number of blastomeres suggesting that if the embryos will be transferred, they will have more chance for implantation than other growth factors tested here.

In summary, the present findings suggest that the addition of the growth factors and GH in the culture media has a favorable effect on maturation, cleavage, blastocyst formation, hatching and cell number of blastocysts *in vitro*. We found that EGF had better overall results than other additives tested. GH had a higher number of blastomeres than other additives. Insulin had lower results in all aspects compared to other additives.

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