

Effect of Growth Factors on Bovine Blastocyst Development in a Serum-Free Medium

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Shamsuddin, M.: Effect of growth factors on bovine blastocyst development in a serum-free medium. Acta vet. scand. 1994, 35, 141-147. – To investigate the effect of growth factors on pre-implantation development, bovine zygotes, produced by in vitro fertilization (IVF) of in vitro-matured (IVM) oocytes, were cultured in a serum-free medium to which the following growth factors were added one at a time: epidermal growth factor (EGF), acidic fibroblast growth factor (a-FGF), insulin-like growth factor-II (IGF-II), platelet-derived growth factor from human platelets (PDGF), and platelet-derived growth factor-AB, human, recombinant (PDGF-AB). All growth factors were added at a dose of either 10 or 50 ng/ml, except PDGF which was added at a dose of either 5 or 15 ng/ml. The control medium was TCM 199 supplemented with sodium pyruvate (0.25 mmol/l), BSA (10 mg/ml), insulin (5 µg/ml), transferrin (5 µg/ml), and sodium selenite (5 ng/ml). Embryos were cultured for 8 days (day of insemination = Day 0). The mean percentages of first cleavage on Day 2 varied from 67% to 86% and the differences between the 2 doses, or between the control and growth factor-treated groups were not significant ($p \geq 0.13$). The effects of the two doses on subsequent development up to the blastocyst stage did not differ either ($p \geq 0.12$). There was no stimulatory effect of any of the used exogenous growth factors on embryo development up to the morula or blastocyst stage on Day 7, or blastocyst stage on Day 8. Moreover, medium supplemented with PDGF had fewer blastocysts than the control ($p \leq 0.03$). The results indicate that growth factor supplementation may not necessarily increase the yield of blastocysts from bovine IVM-IVF oocytes in a serum-free medium.

embryo; IVM-IVF.

Introduction

There is convincing evidence that the transcription of embryonic genome takes place at the fourth cell cycle of bovine pre-implantation development (*Barnes & Eyestone 1990, Barnes & First 1991*). Embryos at this stage are more susceptible to adverse conditions, which may be prevailing during culture *in vitro*, and are often arrested in their development (*Barnes & Eyestone 1990*). However, with the refinement of techniques and avail-

ability of highly purified chemicals and water, it is now possible to culture bovine one-cell embryos up to the blastocyst stage in commercially available tissue culture medium (*Pinyopummintr & Bavister 1991, Shamsuddin et al. 1993a, 1993b*). Even protein-free medium supported the development of bovine IVM-IVF embryos up to the blastocyst stage; however, serum supplementation increased the rate of blastulation (*Pinyopummintr & Bavister 1991, Takagi et al. 1991*). The transcription

of bovine embryonic genome at the fourth cell cycle was suggested to be regulated by certain growth factors (Larson *et al.* 1992a, 1992b, Thibodeaux *et al.* 1993). However, the expression of growth factor receptors was detected at all stages of bovine development up to the blastocyst (Schultz *et al.* 1992, Watson *et al.* 1992) indicating that growth factors may be generally involved in embryonic development instead of being specific to the fourth cell cycle. Exposure to exogenous growth factors was reported as increasing the yield of blastocysts, although the efficacy of growth factor-supplemented medium was compared with that of a poor efficacious control medium; the embryo development in the control medium was completely blocked either at the 8-cell stage (Larson *et al.* 1992b) or at the morula stage (Thibodeaux *et al.* 1993). Recently, a serum-free culture medium has been formulated which consistently supported a high rate of blastocyst development; the speed and frequency of blastocyst development (30% of presumptive zygotes) were comparable to that of an oviduct cell co-culture system (Shamsuddin *et al.* 1993b). This serum-free medium, TCM 199 supplemented with bovine serum albumin (BSA), insulin, transferrin and selenium, has been used as a control in the present study to address the question whether growth factor supplementation can stimulate the bovine pre-implantation development up to blastocyst stage in culture.

Material and methods

One-cell bovine embryos were produced by *in vitro* fertilization (IVF) of *in vitro*-matured (IVM) oocytes. Briefly, cumulus-oocyte complexes (COC) were collected from small ovarian follicles (<7 mm) at slaughter and cultured for maturation in tissue culture medium 199 (TCM; Earle's salts with glutamine; Bio-

chrom, Berlin, Germany; buffered with 26.2 mmol/l sodium bicarbonate; pH 7.2) supplemented with oestrous cow serum (Shamsuddin *et al.* 1993c). After 24 to 25 h of culture, the expanded COC were inseminated with spermatozoa selected by a modified swim-up through a hyaluronic acid (HA; 1 mg/ml) preparation (Shamsuddin & Rodriguez-Martinez 1993). Ten to 15 COC were co-incubated with about 50×10^3 spermatozoa in 50- μ l droplets of Fert-TALP (Parrish *et al.* 1988). The Fert-TALP was supplemented with BSA (6 mg/ml), heparin (5.0 μ g/ml), D-penicillamine (20 μ mol/l), hypotaurine (10 μ mol/l) and epinephrine (1 μ mol/l). The semen used was derived from a bull with proven high fertility. After 20 to 22 h of sperm-COC co-incubation, the presumptive zygotes (not differentiated from unfertilized oocytes) were denuded by repeatedly passing them through a small glass pipette. Ten to 20 zygotes were cultured in 50- μ l droplets under paraffin oil of either control medium alone or of medium with one of the growth factors. The control medium was TCM supplemented with BSA (10 mg/ml), insulin (5 μ g/ml), transferrin (5 μ g/ml), sodium selenite (5 ng/ml), sodium pyruvate (0.25 mmol/l), sodium penicillin-G (50 IU/ml) and streptomycin sulphate (50 μ g/ml). The growth factors were added one at a time to the control medium from frozen-thawed stock solutions. The final media were equilibrated for at least 2 h before use. Epidermal growth factor (EGF; # E-1264) and acidic fibroblast growth factor (a-FGF; # F-5267) were reconstituted in TCM supplemented with BSA (10 mg/ml) at 20 ng/ μ l and 1 ng/ml, respectively. Insulin-like growth factor-II (IGF-II; # I-2139) was reconstituted (1 μ g/10 μ l) in 10 mmol/l acetic acid. Platelet-derived growth factor from human platelet (PDGF; # P-8147) was reconstituted (1 ng/ μ l) in 1 M/l acetic acid. Platelet-derived growth factor-AB, human, recombinant

Table 1. Growth factor dose-effect on first cleavage of bovine zygotes and their subsequent development to morula (M) and blastocyst (BL) stages.

Growth factor	Dose ^a ng/ml	n	Day 2	Day 7		Day 8
			% cleaved	% M	% BL	% BL
EGF	10	127	72.6± 1.9	19.9±3.8	9.1±4.0	24.1±6.4
	50	90	76.9± 4.6	21.1±5.7	2.4±2.4	14.2±4.3
a-FGF	10	106	85.6± 4.9	29.0±5.0	8.5±1.4	24.3±5.0
	50	117	85.9± 4.3	14.3±5.0	10.3±3.7	19.0±3.1
IGF-II	10	106	78.4± 3.2	22.7±4.5	7.6±1.0	16.0±3.2
	50	107	69.2± 3.0	22.4±4.1	7.6±5.0	18.7±1.0
PDGF	5	88	83.0±11.8	20.5±4.3	2.2±2.2	5.9±3.0
	15	95	66.7± 5.5	22.1±4.5	3.0±1.6	9.0±2.9
PDGF-AB	10	98	75.5±10.0	19.1±5.1	2.1±1.1	13.6±2.1
	50	109	78.9± 2.1	22.9±0.4	6.3±2.1	15.3±3.9

n = number of zygotes cultured; day of insemination = Day 0; values are mean±SEM of 3 observations; a = the difference between the effects of two doses did not differ significantly in any growth factor ($p \geq 0.12$); EGF = epidermal growth factor; a-FGF = fibroblast growth factor acidic; IGF-II = insulin-like growth factor-II; PDGF = platelet-derived growth factor; PDGF-AB = platelet-derived growth factor AB, human, recombinant.

(PDGF-AB; # P-6684) was purchased in solution (10 ng/μl). All stock solutions were preserved at -20°C until use, when further dilutions, if needed, were made with culture medium. All growth factors were added at a dose of either 10 or 50 ng/ml, except PDGF which was added at a dose of either 5 or 15 ng/ml. Unless otherwise stated, the chemicals were purchased from Sigma, St. Louis, MO, USA. All zygotes from the sperm-COC co-incubation were pooled and divided equally to different treatments; at least 2 treatments were run in parallel. The experiment includes 3 replicates for every dose of a growth factor and 6 replicates for control. The effects of different treatments were evaluated by the proportions of embryos that cleaved on Day 2, by the proportions of morulae and blastocysts on Day 7, and by the proportions of blastocysts on Day 8 (day of insemination = Day 0). All cultures and equilibrations were performed at 39°C and 5% CO₂ in humidified air.

The effects of the growth factor supplementation on embryo development were determined by the Kruskal-Wallis test (Proc NPAR1WAY; SAS Institute Inc. 1987), with comparisons being conducted first between the 2 doses of each growth factor and then between the control and individual growth factor-treated groups.

Results

In total, 1043 presumptive zygotes were cultured after supplementing the medium with one of 5 growth factors at either of 2 doses (Table 1). An additional 206 zygotes were cultured in the control medium (Table 2). The mean percentages of embryos that cleaved at least once varied from 67% to 86%. The effects of the 2 doses of growth factors on embryo development up to the blastocyst stage did not differ significantly, except a fewer blastocysts in 50 ng/ml than in 10 ng/ml of

Table 2. Effects of growth factor supplementation on first cleavage and subsequent development of bovine embryos up to blastocyst stage (abbreviations as in Table 1).

Treatment	n	Day 2	Day 7		Day 8
		% cleaved	%M	%BI	%BI
EGF	217	74.7±2.4	20.5±3.1	5.7±2.6	19.2±4.1
a-FGF	223	85.7±2.9	21.6±4.6	9.4±1.8	21.7±2.9
IGF-II	213	73.8±2.8	22.6±2.7	7.6±2.3	17.3±1.6
PDGF	183	74.8±6.9	21.4±2.8	2.6±1.2 ^a	7.5±2.0 ^a
PDGF-AB	207	77.2±4.6	21.0±2.4	4.2±1.4	14.4±2.0
Control	206	81.3±2.5	20.7±3.4	10.5±4.0	22.2±3.7

Values are mean±SEM of 6 observations; ^aValues are significantly lower than the corresponding values of the control ($p \leq 0.03$).

EGF ($p \geq 0.13$). Therefore, data on embryo development under the 2 doses of each growth factor were pooled and compared with data on the control medium.

Supplementation of culture medium with EGF, a-FGF or IGF-II did not have any significant positive or negative effect on the yield of morulae and blastocysts on Day 7, and blastocysts on Day 8 (Table 2; $p \geq 0.26$). However, the percentage of blastocysts in PDGF-supplemented medium was significantly lower than that in control medium both on Day 7 and on Day 8 ($p \leq 0.03$). Medium supplemented with PDGF-AB also tended to have fewer blastocysts than the control medium ($p \leq 0.09$).

Discussion

The effect of several mitogenic growth factors on the bovine pre-implantation development was studied using a serum-free culture medium as control (Shamsuddin *et al.* 1993b). The efficacy of the control medium has been consistently higher than that of controls used in several recent studies where growth factors were tested (Larson *et al.* 1992a, 1992b, Flood *et al.* 1993, Thibodeaux *et al.* 1993). Under the

prevailing conditions, the results of the present study show that addition of commercially available growth factors failed to stimulate the bovine embryo development in culture. In accordance with the results of the present study, EGF did not stimulate the chronological development of bovine embryos up to the blastocyst stage (Takagi *et al.* 1991, Keefer 1992, Flood *et al.* 1993). However, EGF-treated blastocysts tended to hatch at a higher rate (Keefer 1992, Flood *et al.* 1993). In mouse embryos, EGF stimulated the mitotic and hatching rates when embryos were cultured singly in micro-drops (Paria & Dey 1990). This effect was undetectable when embryos were cultured in a group (Wood & Kaye 1989, Paria & Dey 1990). It was therefore speculated that when cultured in a group, growth factors produced by one embryo may stimulate development in the fellow embryos; thereby limiting the effect of the exogenous growth factors (Paria & Dey 1990). Nevertheless, in monovulatory animals a paracrine growth factor action between embryos in vivo may be unlikely.

Acidic-FGF did not show any effect on the cultured bovine embryos in the present study. Accordingly, supplementation of culture me-

dium with basic-FGF did not lead to any significant change in the frequency of blastocyst development in the bovine (Larson *et al.* 1992b, Flood *et al.* 1993). The a-FGF is homologous to the b-FGF regarding the sequence and number of amino acids (Thomas *et al.* 1987) and both were equally mitogenic to epithelial cell lines (Simmen *et al.* 1993). Larson *et al.* (1992b) reported a synergistic effect of b-FGF and transforming growth factor- β on the development of embryos from the 8-cell stage to the 16-cell stage; however, the effect was not detectable when development up to the blastocyst stage was considered.

In the present study, insulin-like growth factor-II did not influence the bovine blastocyst development, which is consistent with the results of Flood *et al.* (1993). Receptors for insulin, IGF-I and IGF-II have been reported from pre-implantation embryos from bovine and other species (Kaye *et al.* 1992, Schultz *et al.* 1992, Watson *et al.* 1992). However, the embryotrophic effects of IGFs in culture are controversial (Paria & Dey 1990, Harvey & Kaye 1992). Although growth factors of the insulin family share many common characteristics, the sources of their origin seem to differ; insulin and IGF-I are maternally derived while the most important source of IGF-II seems to be the paternal allele (see Kaye *et al.* 1992, Schofield & Engstrom 1992, for review). It is likely that IGF-II serves an autoregulatory function in embryos that receive a normal functioning ligand allele from the father, and thereby the effect of exogenously supplied IGF-II was limited. Using a mutant mouse model, Baker *et al.* (1993) have demonstrated a slower embryonic and postnatal growth rate in IGF-II deficient mice.

Natural PDGF from human platelets has significantly reduced the development of blastocysts in culture compared to the control, as found in the present study. This is consistent

with the result of Larson *et al.* (1992a). Using the same commercial PDGF as used in the present study, Flood *et al.* (1993) reported a yield of about 12% blastocysts; the values did not differ from the control. A stimulatory effect of PDGF was found on the fourth cell cycle of cultured bovine embryos (Larson *et al.* 1992a, Thibodeaux *et al.* 1993); however, the two studies showed contrasting effects of PDGF on the yield of blastocysts. In the present study, the percentage of morulae did not differ between control and PDGF-treated groups, indicating that the transcription of embryonic genome at the fourth cell cycle of bovine development may not be dependent upon PDGF.

It is not clearly understood why PDGF reduces blastocyst development. Two questions can be raised: 1) since natural PDGF was dissolved in a highly acidic solution, there is a risk that the pH of the culture medium was lowered by the stock solution, and thereby the activities of other essential compounds were reduced; 2) natural PDGF might have carried contaminants which were detrimental to embryos. In order to address those questions, a highly purified, recombinant PDGF containing a heterodimer similar to that in the natural ones (PDGF-AB) was included in the present study. The data on PDGF-AB also indicated a tendency for a reduced yield of blastocysts, compared to the control. The PDGF, a serum mitogen with major pleiotropic effects, is involved in autocrine or paracrine stimulation of cell growth, provided that specific receptors are present in the responsive cells. The presence of α and β -type receptors for PDGF and of PDGF-A has been determined immunocytochemically in the trophoblast of elongated, preimplantation bovine embryos (Rodriguez-Martinez *et al.* 1989, Persson *et al.* 1991). Although the expression of PDGF receptors has also been shown in all

earlier stages of bovine development up to blastocyst in a serum-containing medium with oviduct cells (Watson *et al.* 1992), a better understanding of PDGF-receptor behaviour under more defined conditions is necessary to answer whether the effects of this polypeptide are blocked by other chemicals or vice versa. The lack of positive effect of growth factors in culture does not necessarily mean that growth factors are not involved in the bovine pre-implantation development *in vivo*. The concentration, half-life, physical and chemical properties of stock solutions and culture medium, storage period and temperature, transportation and sources all can affect the potency of a biological product. In the present study, each growth factor was used in 2 doses. The outcome of the 2 doses did not differ significantly, except for a tendency of the higher dose of EGF (50 ng/ml) to reduce blastocyst yield. Supplementation with other doses or several growth factors together may show stimulatory effect on embryo development but this remains to be determined.

Acknowledgements

The author thanks Professor Heriberto Rodriguez-Martinez and Dr. Birgitta Larsson for critically revising the manuscript and for translating the summary into the Swedish language, and Ms. Aili Colbing and Ms Gunilla Lekselius for technical assistance. Frozen semen was generously supplied by Elitsem, Eskilstuna, Sweden. The project received financial support from the Council of Ministers of the Nordic Countries and the Swedish Council for Forestry and Agricultural Research.

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Sammanfattning

Effekten av tillväxtfaktorer på notkreatursembryons utveckling till blastocyster i ett serum-fritt medium.

För att undersöka effekten av tillväxtfaktorer på embryoutvecklingen före implantationen användes notkreatursembryon i en-cellstadiet, framtagna genom *in vitro* befruktning (IVF) av *in vitro* mognade (IVM) oocyter. Embryonerna odlades i ett serumfritt medium till vilket en av följande tillväxtfaktorer tillsattes: epidermal growth factor (EGF), acidic fibroblast growth factor (a-FGF), insulin-like growth factor-II (IGF-II), platelet-derived growth factor från humana trombocyter (PDGF) och human rekombinant platelet-derived growth factor-AB (PDGF-AB). Alla tillväxtfaktorer tillsattes i doser av 10 eller 50 ng/ml, utom PDGF som tillsattes i antingen 5 eller 15 ng/ml. Som kontrollmedium användes TCM 199 till vilket tillsatts natriumpyruvate (0.25 mmol/l), BSA (10 mg/ml), insulin (5 µg/ml), transferrin (5 µg/ml) och natrium-selenit (5 ng/ml). Embryonerna odlades vid 39°C och 5% CO₂ i fuktmatad luft under 8 dagar (dag 0 = dagen för befruktningen). Den genomsnittliga andelen oocyter som genomgått första delningen på dag 2 varierade från 67% till 86%. Skillnaderna mellan de 2 doserna eller mellan kontrollgrupp och behandlingsgrupper var inte signifikant ($p \geq 0.13$). Det var inte heller någon skillnad i effekt av de två doserna på utvecklingen till blastocyststadiet ($p \geq 0.12$). Ingen av de exogent tillförda tillväxtfaktorerna hade någon stimulerande effekt på embryoutvecklingen till morula eller blastocyststadiet på dag 7 eller till blastocyststadiet på dag 8. Färre blastocyster utvecklades i medium med tillsats av PDGF än i kontrollmediet ($p \leq 0.03$). Resultaten indikerar att tillsattande av tillväxtfaktorer till ett serum-fritt medium inte stimulerar utvecklingen av IVM-IVF oocyter till blastocyster.

(Received September 21, 1993; accepted December 22, 1993).

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