Effect of insulin-like growth factor I (IGF-I) at high concentrations on blastocyst development of bovine embryos produced *in vitro*

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This study was carried out to determine the effects of oestrous cow serum containing insulin-like growth factor I (IGF-I) and supplementation with recombinant IGF-I on subsequent development of bovine embryos produced *in vitro*. When culture medium was supplemented with oestrous cow serum containing 34.8 ng endogenous IGF-I ml⁻¹, more embryos ($P < 0.01$) developed to blastocysts by day 9 and more blastocysts hatched on day 13 after insemination ($P < 0.01$) than in the control group. The effect of the addition of 10, 50 and 100 ng IGF-I ml⁻¹ to culture media containing oestrous cow serum and granulosa cells was also evaluated. Supplementation with 10 ng IGF-I ml⁻¹ did not improve embryo development at any stage. The addition of 50 and 100 ng IGF-I ml⁻¹ did not affect development during the first three cell divisions. However, on day 7 these groups yielded a higher embryo rate than did the control group. Similarly, the proportion of blastocysts on day 9 was enhanced. The addition of 100 ng IGF-I ml⁻¹ also increased the proportion of blastocysts. These data suggest that IGF-I at high concentrations accelerates the development to the blastocyst stage by shortening the transition from the morula to the blastocyst stage. The addition of 100 ng IGF-I ml⁻¹ increased the proportion of hatched blastocysts on day 13. The addition of oestrous cow serum and IGF-I to TCM 199 free of granulosa cells did not increase the proportion of embryos on day 7. However, the progress to blastocysts and hatched blastocysts on days 9 and 13 was significantly lower ($P < 0.05$). The addition of IGF-I to culture medium without oestrous cow serum but with granulosa cells resulted in significantly lower embryo development than in the control group or in the group supplemented with oestrous cow serum and IGF-I ($P < 0.01$). The results support the hypothesis that culture media containing high concentrations of IGF-I combined with oestrous cow serum and granulosa cells can improve the development of embryos produced *in vitro*.

Introduction

Successful methods have been developed for producing bovine embryos *in vitro*. Seven days after the fertilization of oocytes selected for production *in vitro*, up to 40% have developed into embryos (Brackett and Zuelke, 1993). However, the percentage of blastocysts (day 9) and hatched blastocysts (day 13) is lower than expected (Kreffft, 1993; Palma *et al.*, 1993) because the development of some embryos is arrested at earlier stages. Thus, it is necessary to improve the culture media to enhance morula and blastocyst development.

Growth factors have been shown to play a regulatory role in the functioning of the ovary (Adashi *et al.*, 1985; Carson *et al.*, 1989; Oliver *et al.*, 1989; Echterkamp *et al.*, 1994) and the uterus (Bringstock *et al.*, 1989; Boehm *et al.*, 1990; Chegini *et al.*, 1992; Persson *et al.*, 1992) resulting in a trophic effect on the endometrium and the embryo. Insulin-like growth factor I (IGF-I) is an important growth factor in blood and is of

particular importance in the interaction between the uterine endometrium and conceptus (Simmen *et al.*, 1993).

Several research groups reported that addition of growth factors to culture media stimulates the development of bovine embryos *in vitro* (Rinderknecht and Humbel, 1988; Coskun *et al.*, 1991; Larson *et al.*, 1992; Harper and Kaye, 1992; Keefer, 1992). Earlier studies showed that IGF-I induces mitosis of granulosa cells (Armstrong and Xia, 1993), the maturation of oocytes (Harper and Brackett, 1993; Lorenzo *et al.*, 1994) and bovine embryo development (Kaye *et al.*, 1992). Insulin-like growth factors (IGFs) have a high affinity for soluble binding proteins that can modulate receptor binding, and hence influence the biological activity of IGFs.

The purpose of this study was to examine the effect of supplementing the culture medium with different concentrations of IGF-I on the development of *in vitro* matured and fertilized (IVM/IVF) oocytes when oestrous cow serum was used. In addition, the development of embryos up to the stage of hatched blastocysts was compared with the development of embryos produced in protein-free medium, to assess whether

serum and IGF-I supplementation can stimulate preimplantation development of bovine embryos in *in vitro* culture. The effect of supplementation with IGF-I in TCM 199 free of serum and granulosa cells was also investigated to determine whether IGF-I acts synergistically with oestrous cow serum and directly on embryos or via granulosa cells. The development of embryos produced in different culture systems was evaluated by their ability to develop *in vitro* into blastocysts at day 9 and into hatched blastocysts at day 13 after fertilization.

Material and Methods

Collection of oocytes, maturation, fertilization and culture

Bovine ovaries were obtained from a local abattoir and were transported to the laboratory in phosphate-buffered saline (PBS, pH 7.35, 284 mOsmol l^{-1}) at 25–30°C. Within 4 h oocytes were aspirated from follicles 2–8 mm in diameter using a micro/macro aspirator (Labotect, Göttingen) and a 20 g needle. Only cumulus–oocyte complexes with multilayered, compact cumulus cells and dark, evenly granulated cytoplasm were allocated to the experimental groups.

For *in vitro* maturation the oocytes were cultured in TCM 199 (Seromed, Berlin), supplemented with L-glutamine (100 mg l^{-1}), $NaHCO_3$ (3 g l^{-1}), Hepes (1400 mg l^{-1}), pyruvate (250 mg l^{-1}), L-lactic-calcium-salt (600 mg l^{-1}), gentamycin (55 mg l^{-1}), porcine FSH (pFSH, 10 μ g ml^{-1} , Schering, Kenilworth, NJ) and 10% (v/v) heat-treated (56°C, 30 min) oestrous cow serum. Concentrations of hormones and IGF-I were determined using a radioimmunoassay as described by Schams *et al.* (1991). Briefly, IGF-I was determined by radioimmunoassay after acid–ethanol extraction of 50 μ l serum. Separation of bound and free hormones was accomplished by a combination of second-antibody technique with 12% polyethylene glycol. Intra- and interassay coefficients of variation were < 10% and < 16%, respectively. The oestrous cow serum contained 34.8 ng IGF-I ml^{-1} , 5.7 pg oestradiol ml^{-1} , 0.7 ng progesterone ml^{-1} , 50.6 ng FSH ml^{-1} and 4.9 ng LH ml^{-1} . Thirty selected cumulus–oocyte complexes were each placed in a 400 μ l drop and cultured for 20 h at 39°C, in a humidified atmosphere of 5% CO_2 :95% air. Frozen semen from the same bull was used for all *in vitro* fertilizations. Each straw was thawed in a water-bath at 39°C for 10 s. Motile spermatozoa were separated by a modified swim-up technique in a Tyrode's lactate solution supplemented with BSA (6 mg ml^{-1} , Sigma, St Louis, MO), sodium pyruvate (0.1 μ g ml^{-1} , Sigma) and gentamicin (50 μ l ml^{-1} , Seromed). Semen was washed once in the same medium (3–5 ml) by centrifugation at 200 g for 10 min. The supernatant was discarded, and the sperm pellet was resuspended in 100–200 μ l of the same medium. Sperm concentration was determined and resuspended spermatozoa were added to a final concentration of 1.0×10^6 spermatozoa ml^{-1} .

After maturation the cumulus–oocyte complexes were transferred to 400 μ l drops of fert-Talp medium (Bavister and Yanagimachi, 1977) supplemented with 6 mg BSA ml^{-1} (Sigma) and 10 μ g heparin ml^{-1} (Sigma).

In vitro fertilization was performed for 20–24 h at 39°C in a humidified atmosphere of 5% CO_2 :95% air. After fertilization

the cumulus cells were removed from the oocytes by vortexing for 1 min in a 10 ml conical tube containing 400 μ l culture medium. Subsequently 30 denuded oocytes were placed in each 400 μ l drop of TCM 199 with $0.8\text{--}1.2 \times 10^6$ granulosa cells ml^{-1} , covered with equilibrated paraffin oil and cultured for 3–13 days in a humidified atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 at 39°C. The culture was examined on days 2, 3, 7, 9 and 13 after insemination to determine the cleavage rate (day 2), development rate of embryos (day 7) and blastocysts (days 9 and 13). In all experiments the effect of culture conditions on early embryo development was evaluated by counting the number of blastomeres on day 3. The embryos produced were freed from the zona pellucida by a brief exposure to PBS at pH 2.5 for 40–60 s. The blastomeres were dissociated by exposing them to 0.25% (w/v) trypsin in PBS for 10 to 20 min and counted. Embryo development was evaluated by determining the proportion of morulae and blastocysts on day 7, blastulation on day 9 and the formation of hatched blastocysts on day 13.

Experiment 1

The presumptive zygotes ($n = 1417$) were cultured in 400 μ l drops of either TCM 199 plus 10% oestrous cow serum containing 348 ng endogenous IGF-I ml^{-1} (34.8 ng ml^{-1} in culture droplets) ($n = 690$) or in TCM 199 without oestrous cow serum (control, $n = 719$) for 3–13 days. For the evaluation of the developmental capacity of embryos, 588 presumptive zygotes were cultured for 13 days with 10% oestrous cow serum and 617 without oestrous cow serum with seven replications. For measuring the effect of oestrous cow serum on the number of blastomeres on day 3 after insemination, 110 oocytes were cultured with 10% oestrous cow serum to evaluate 86 embryos and 102 oocytes were cultured without oestrous cow serum as a control group to evaluate 82 embryos on day 3 after insemination.

Experiment 2

In this experiment 1308 oocytes were selected for IVM/IVF. After fertilization, the 680 presumptive zygotes were cultured in TCM 199 plus 10% oestrous cow serum containing, as in Expt 1, 34.8 ng endogenous IGF-I ml^{-1} supplemented with 10 ng human recombinant IGF-I ml^{-1} (Boehringer Mannheim, Mannheim) ($n = 680$), or without the IGF-I supplement (control, $n = 628$). Thus the total concentration of IGF-I was 44.8 ng ml^{-1} and 34.8 ng ml^{-1} , respectively. The number of blastomeres on day 3 was assessed by examining 155 oocytes from the group cultured in media supplemented with IGF-I and 223 oocytes from the control group. The remaining oocytes (525 from the supplemented group and 405 from the control group) were cultured for 13 days, and the rate of development was assessed. The treatments were replicated seven times.

Experiment 3

Recombinant IGF-I was added at concentrations of 50 and 100 ng ml^{-1} to TCM 199 containing 10% oestrous cow serum (34.8 ng endogenous IGF-I ml^{-1} in culture droplets), giving

Table 1. Effect of 10% oestrous cow serum on embryo development rate

Serum (%)	Oocytes (n)	Cleavage (day 2)	Morulae and blastocysts (day 7)	Blastocysts (day 9)	Hatched blastocysts (day 13)
0	588	81.6% (480)	34.2% (201)	17% (100)*	7.1% (42)*
10	617	80.4% (496)	38.4% (237)	29.0% (179)*	21.5% (133)*

Values in parentheses are numbers of oocytes reaching that stage.

*Values within columns differ ($P < 0.01$).

concentrations of 84.8 and 134.8 ng IGF-I ml⁻¹, respectively. As in Expt 1, these two groups (50 ng ml⁻¹, $n = 550$ and 100 ng ml⁻¹, $n = 638$) were compared with embryos developed in TCM 199 plus 10% oestrous cow serum but without recombinant IGF-I ($n = 608$ oocytes). For determination of early developmental capacity, groups of oocytes (control, $n = 180$; 50 ng ml⁻¹, $n = 156$; and 100 ng ml⁻¹, $n = 183$) were used to produce the embryos (142, 111 and 143, respectively) to determine the number of blastomeres on day 3. The remaining oocytes (control, $n = 428$; 50 ng ml⁻¹, $n = 394$ and 100 ng ml⁻¹, $n = 455$) were used to evaluate the development of embryos up to day 13. The treatments were replicated seven times.

Experiment 4

After fertilization of the selected oocytes (1533) the presumptive zygotes were divided at random into four groups according to the supplementation of TCM199: 10% oestrous cow serum plus 100 ng IGF-I ml⁻¹ without granulosa cells (GC) (IGF-I + oestrous cow serum, $n = 411$), 100 ng IGF-I ml⁻¹ plus 50×10^3 granulosa cells but without oestrous cow serum (IGF-I + GC, $n = 388$), IGF-I without oestrous cow serum or GC (IGF-I, $n = 397$) and 10% oestrous cow serum plus GC but without IGF-I (control, $n = 337$). The treatments were replicated five times.

Statistical analyses

The percentage of embryos developing into cleaved embryos (day 2), morulae and blastocysts (day 7), blastocysts (day 9) and hatched blastocysts (day 13) was expressed as the proportion of all oocytes cultured for maturation. Differences were tested for statistical significance by Chi-square test with Yates' correction. When the frequency of developed embryos was expected to be below 5, Fischer's Exact Probability Test was used in addition. The cell number on day 3 was subjected to analysis of variance. $P < 0.05$ was considered to be significant.

Results

Experiment 1: effect of oestrous cow serum

The mean percentages of embryos developing to different stages when cultured in TCM 199 supplemented with 10%

oestrous cow serum is shown in Table 1. There was no significant difference in the rate of embryo development on days 2 and 7. However, oestrous cow serum had a significant effect on the number of blastocysts and hatched blastocysts produced by day 9 and day 13, respectively ($P < 0.01$; Table 1). There were no significant differences in the proportions of 2–4, 5–8 and > 8-cell embryos produced when the media were supplemented with 10% oestrous cow serum (16.3%, 49.5% and 34.1%, respectively, compared with 18.3%, 47.6% and 34.1%, respectively, for the control group).

Experiment 2: effect of medium supplemented with 10 ng recombinant IGF-I ml⁻¹

There was a significant difference in cleavage rate between the group treated with additional 10 ng IGF-I ml⁻¹ and the control group ($P < 0.05$; Table 2). However, there was no significant difference between the groups on day 7 (morula and blastocyst formation) day 9 (blastocyst formation) or day 13 (hatching of blastocysts). The number of blastomeres on day 3 was not different between the group supplemented with IGF-I (19.3%, 46.3% and 34.4%) and the control group (17.3%, 48.2% and 34.5% for 2–4, 5–8 and > 8-cell embryos, respectively, $P > 0.05$).

Experiment 3: effect of supplementation with 50 and 100 ng recombinant IGF-I ml⁻¹

There was no significant difference in the cleavage rate on day 2 (Table 3) or in the proportion of blastomeres (2–4, 5–8 and > 8-cell embryos) on day 3 between the groups supplemented with 0, 50 or 100 ng IGF-I ml⁻¹ (19.1%, 47.5% and 33.4% compared with 19.8%, 45.9% and 34.3%, and 16.9%, 47.5% and 35.6%, respectively, $P > 0.05$).

On day 7 and day 9 the rate of morula and blastocyst formation was higher when 50 or 100 ng IGF-I was added to the culture medium than when embryos were cultured in the control medium without IGF-I (Table 3). Similarly when the presumptive zygotes were cultured in medium containing 100 ng IGF-I ml⁻¹, the number of hatched blastocysts on day 13 was significantly higher ($P > 0.01$) than in the control group.

The percentage of oocytes reaching different stages of embryo development by days 7 and 9 are shown (Figs 1 and 2). On day 7 the proportion of oocytes reaching the morula and blastocyst stages was not different between embryos cultured

Table 2. Effect of additional insulin-like growth factor I (10 ng IGF-I ml⁻¹) on bovine embryo development rate

IGF-I added (ng ml ⁻¹)	Oocytes (n)	Cleavage (day 2)	Morulae and blastocysts (day 7)	Blastocysts (day 9)	Hatched blastocysts (day 13)
0	405	77.0% (312)*	37.7% (153)	28.9% (117)	21.0% (85)
10	525	81.7% (429)*	37.1% (195)	28.4% (149)	20.8% (109)

Values in parentheses are the number of oocytes that reach that stage.

*Values within columns are significantly different ($P < 0.05$).

Table 3. The effect of additional insulin-like growth factor I (50 and 100 ng IGF-I ml⁻¹) on the development rate of embryos

IGF-I (ng ml ⁻¹)	Oocytes (n)	Cleavage (day 2)	Morulae and blastocysts (day 7)	Blastocysts (day 9)	Hatched blastocysts (day 13)
0	428	80.1% (343)	32.0% (137) ^c	28.0% (120) ^{a,c}	21.2% (91) ^c
50	394	80.7% (318)	36.5% (144)	34.5% (136) ^b	25.1% (99) ^a
100	455	79.3% (361)	40.9% (186) ^d	38.2% (174) ^c	32.1% (146) ^{b,d}

Values in parentheses are the number of oocytes that reach that stage.

Values within columns with different superscripts differ significantly ^{a,b} $P < 0.05$, ^{c,d} $P < 0.01$.

with 50 ng IGF-I ml⁻¹ and those cultured without IGF-I (62.6%). The addition of 100 ng IGF-I ml⁻¹ resulted in a higher proportion of blastocysts (68.0%, $P < 0.01$) compared with the control group and there were significantly more early ($P < 0.01$) and expanded blastocysts ($P < 0.05$) in this group.

The early blastocyst arrest seen on day 9 decreased when 100 ng IGF-I ml⁻¹ (6.4%; $P < 0.05$) was added to the culture media compared with the control group (13.1%). Supplementation with 50 ng IGF-I ml⁻¹ had no significant effect (11.8%). Supplementation with 100 ng IGF-I ml⁻¹ resulted in a lower proportion of blastocysts (6.4%) compared with the control group (13.1%) and with the group supplemented with 50 ng IGF-I ml⁻¹ (12.5%); however, this difference was not significant ($P < 0.06$). In the group cultured in medium with 100 ng IGF-I ml⁻¹ more blastocysts had hatched by day 9 (67.2%) than in the control group (54.7%; $P < 0.05$), indicating a more rapid development of blastocysts (Fig. 2).

The proportion of blastocysts that progressed to hatched blastocysts from day 9 to day 13 was 75.8% (91/120), 72.8% (99/136) and 83.4% (146/175) for 0, 50 and 100 ng IGF-I ml⁻¹, respectively, and did not differ significantly between groups.

Experiment 4: effect of supplementation with 100 ng IGF-I ml⁻¹ together with granulosa cells and oestrous cow serum

There was no difference in the cleavage rate on day 2 between the four treatments groups (Table 4). On day 7 the

group cultured with IGF-I and oestrous cow serum but without granulosa cells did not differ from the control group. However, the groups cultured with IGF-I but without oestrous cow serum, and without granulosa cells or oestrous cow serum had significantly fewer morulae and blastocysts on day 7 and fewer blastocysts on days 9 and 13 ($P < 0.01$).

On days 9 and 13 the proportions of blastocysts and hatched blastocysts after culture in oestrous cow serum plus IGF-I and in IGF-I alone were significantly lower than in the control group ($P < 0.05$). There was no significant difference in the embryonic development rate between the groups cultured with IGF-I and granulosa cells and IGF-I alone.

Discussion

The results of the present study suggest that the *in vitro* development of bovine embryos improves in a culture medium containing high concentration of IGF-I in addition to oestrous cow serum and granulosa cells.

In the present study, the concentrations of IGF-I in serum are similar to those reported by Echternkamp *et al.* (1990) with exogenous IGF-I present at the usual concentration for cell culture (10 ng ml⁻¹) and at higher concentrations.

The addition of 10 ng IGF-I ml⁻¹ to TCM 199 containing 10% oestrous cow serum had a positive effect on the cleavage rate ($P < 0.05$). However, the difference in the cleavage rate

Fig. 1. Effect of insulin-like growth factor I (IGF-I) on the development of bovine embryos on day 7 after insemination. The percentage of morulae, early blastocysts, blastocysts, expanded blastocysts and total blastocysts present 7 days after culture of presumptive zygotes (day 1) in TCM 199 plus 10% oestrous cow serum, containing 38.4 ng endogenous IGF-I ml^{-1} supplemented with 0 ($\square$), 50 ($\blacksquare$) and 100 ($\boxplus$) ng exogenous human recombinant IGF-I ml^{-1} . Total numbers of embryos examined at the IGF-I final concentrations of 34.8, 84.8 and 134.8 ng IGF-I ml^{-1} were 107, 107, 137, respectively. Statistical analysis is a comparison by means of Chi-square analysis: $^{a,b}P < 0.05$ and $^{c,d}P < 0.01$.

may have been only a temporary effect, as no positive effect on the number of blastomeres (on day 3) was detectable 1 day later. Similarly, the supplementation of culture media with 50 or 100 ng IGF-I ml^{-1} did not affect early embryo development. These results corroborate other studies conducted on bovine embryos (Barnes and First, 1991), but conflict with reports on mice. Kaye *et al.* (1992) showed that the supplementation of IGF-I stimulates the *in vitro* development of two-cell mouse embryos 48 h after *in vitro* fertilization. These divergent observations may be due to developmental characteristics of murine and bovine embryos. Blastulation in mouse embryos, for instance, occurs within 72 h after IVF, whereas this occurs in cow embryos 5 to 7 days after IVF and thus growth factor receptors are expressed later than in murine embryos (Schultz *et al.*, 1992).

The results presented here also corroborate studies conducted on bovine embryos which demonstrated that IGF-I has no effect on IVM/IVF/IVC embryos during the transition period from maternal to embryonic control of development (Barnes and First, 1991). In accordance with this, the control medium containing neither IGF-I nor serum was not inferior in stimulating early development to media supplemented with oestrous cow serum and different concentrations of IGF-I.

Whereas 10 ng IGF-I ml^{-1} did not improve bovine embryo development, the supplementation of 50 and 100 ng ml^{-1}

Fig. 2. Effect of insulin-like growth factor I (IGF-I) on the development of blastocysts on day 9 after insemination. Percentage of early blastocysts, blastocysts, expanded blastocysts and hatched blastocysts 8 days after culture of presumptive zygotes (day 1) in TCM 199 plus 10% oestrous cow serum, containing 38.4 ng endogenous IGF-I ml^{-1} supplemented with 0 ($\square$), 50 ($\blacksquare$) and 100 ($\boxplus$) ng human recombinant IGF-I ml^{-1} . Total numbers of embryos examined at the IGF-I final concentrations of 34.8, 84.8 and 134.8 ng IGF-I were 137, 144 and 186, respectively. Statistical analysis is a comparison by means of Chi-square analysis: $^{a,b}P < 0.05$.

increased the yield of embryos on day 7. These data are consistent with the results from the studies in mice by Harvey and Kaye (1992) but conflict with results of studies reporting that no positive effect on bovine embryo development was observed on day 7 after fertilization when IGF-I was added at 50 ng ml^{-1} (Herrler *et al.*, 1993). These authors may have failed to demonstrate clearly the positive effect of IGF-I because they used a lower concentration of LH in the maturation medium, which could have caused lower developmental rates (Herrler *et al.*, 1993).

The results of the present study also suggest that there is a dose-response relationship between IGF-I and embryonic development, since the medium containing 50 ng IGF-I ml^{-1} yielded a lower proportion of hatched blastocysts on day 9 than the medium containing 100 ng IGF-I ml^{-1} .

The result with 100 ng IGF-I ml^{-1} is similar to that reported by Matsui *et al.* (1994) but is inconsistent with the report of Lee and Fukui (1995), who used a culture medium without supplementation with oestrous cow serum or another protein source.

In Expt 3 embryonic development in TCM 199 plus granulosa cells was compared with that in TCM 199 plus granulosa cells supplemented with 10% oestrous cow serum containing 34.8 ng endogenous IGF-I ml^{-1} and significant differences were found until day 7.

In Expts 1 and 4, the proportion of hatched blastocysts on day 9 and of hatched blastocysts on day 13 was considerably

Table 4. The effect of granulosa cells (GC), oestrous cow serum (ECS) and insulin-like growth factor I (100 ng IGF-I ml⁻¹) on the development rate of bovine embryos

Group	Oocytes (n)	Cleavage (day 2)	Morulae and blastocysts (day 7)	Blastocysts (day 9)	Hatched blastocysts (day 13)
ECS + GC (control)	337	79.5% (268)	35.9% (121) ^c	32.5% (108) ^{a,c}	30.3% (102) ^{a,c}
ECS + IGF-I	411	79.6% (327)	30.2% (124) ^c	25.5% (105) ^{b,c}	23.8% (98) ^b
GC + IGF-I	388	79.6% (309)	19.6% (76) ^d	12.1% (47) ^d	7.2% (28) ^d
IGF-I	397	79.6% (316)	16.1% (64) ^d	8.6% (34) ^d	5.0% (20) ^d

Values within columns with different superscripts differ significantly: ^{a,b}*P* < 0.05; ^{c,d}*P* < 0.01.

lower with the culture medium containing granulosa cells and 100 ng IGF-I ml⁻¹. TCM 199 with granulosa cells was able to stimulate development up to day 7, but then the development of the embryos decreased. The granulosa cells in TCM 199 without oestrous cow serum did not build a monolayer as did the control group supplemented with serum. Whether the TCM 199 without serum directly affected the development of embryos or acted via the granulosa cells was not evaluated in this study. In addition, embryonic development was significantly lower when the culture medium was supplemented only with IGF-I. These results support the contention that there is an interaction between oestrous cow serum, IGF-I and granulosa cells in the *in vitro* development of IVM/IVF embryos (Thibodeaux *et al.*, 1995). Furthermore the results reported here suggest that IGF-I acts on the development of bovine embryos indirectly, via granulosa cells and in agreement with previous studies that showed that supplementation with growth factors could be beneficial to embryonic development in the absence of serum but could not replace it (Palma *et al.*, 1995).

This supposition is also corroborated by the finding that IGF-I receptors are located in the plasma membrane of granulosa cells (Adashi *et al.*, 1988) and that IGF-I stimulates the proliferation and differentiation of these cells (Spicer *et al.*, 1993). In addition, the IGF-I binding capacities of granulosa cells are enhanced by FSH and LH (Veldhuis and Rogers, 1987; Gong *et al.*, 1993) and oestrogens.

The fact that a high culture density improves embryo development (Wiley *et al.*, 1986; Paria and Dey, 1990; Palma *et al.*, 1992; Ferry *et al.*, 1994; Keefer *et al.*, 1994) provides support for a cooperative interaction between preimplantation embryos due to the growth factors released by them. Interestingly, the addition of 100 ng IGF-I ml⁻¹ did not only result in a higher proportion of hatched blastocysts, but also accelerated the formation of early and expanded blastocysts on day 7. It has been reported that mouse embryos develop faster in high culture densities (Paria and Dey, 1990; Lane and Gardner, 1991) and that the transition from morula to blastocyst stage occurs 24 h earlier in ovine and bovine embryos produced *in vitro* than in those collected after superovulation (Walker *et al.*, 1992; Greve *et al.*, 1993), which may be due to the higher temperature in the incubator (Greve *et al.*, 1993).

The results presented here indicate that high concentrations of exogenous IGF-I added to the culture system in the presence of granulosa cells, serum and endogenous IGF-I accelerate embryo development. In the experiments reported here, the

in vitro development of embryos cultured with 100 ng IGF-I ml⁻¹ was not reduced on day 13 of culture as well as after freezing (Willemsen *et al.*, 1995). As embryonic development was only examined *in vitro* up to day 13 this provides only limited evidence and has to be substantiated by testing the *in vivo* viability after transfer. The mechanism by which IGF-I exerts a mitogenic and differentiating effect on *in vitro* cultured embryos is not known. In *in vitro* culture IGF-I can act directly on the embryo via receptors on morulae and blastocysts (Mattson *et al.*, 1988; Heyner *et al.*, 1989). In conclusion IGF-I plays an important role in the IVM/IVF/IVC development of bovine embryos and it improves the development of embryos produced *in vitro* when added to culture media containing oestrous cow serum and granulosa cells. Under these culture conditions, treatment with IGF-I at high concentrations does not have a negative effect but may enhance the development of IVM/IVF embryos cultured *in vitro* for a relatively long time, for example 13 days.

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References

- Adashi EY, Resnick CE, D'Ercole AJ, Svoboda ME, van Wyk JJ (1985) Insulin-like growth factors as interovarian regulators of granulosa cell growth and function. *Endocrine Reviews* **6** 400–420
- Adashi EY, Resnick CE, Hernandez ER, Svoboda ME and Van Wyk JJ (1988) Characterization and regulation of a specific cell membrane receptor for somatomedin C/insulin like growth factor I in culture rat granulosa cells. *Endocrinology* **122** 194–201
- Armstrong DT and Xia P (1993) Differential mitogenic actions of insulin-like growth factor-I and FSH on bovine cumulus cells and granulosa cells. *Theriogenology* **39** 181 (Abstract)
- Barnes FL and First NL (1991) Embryonic transcription in *in vitro* cultured bovine embryos. *Molecular Reproduction and Development* **29** 117–123
- Bavister BD and Yanagimachi R (1977) The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa *in vitro*. *Biology of Reproduction* **16** 228–237
- Boehm KD, Daimon M, Gorodeski IG, Sheean LA, Utian WH, Ilan J (1990) Expression of the insulin-like and platelet-derived growth factor genes in human uterine tissues. *Molecular Reproduction and Development* **27** 93–101
- Brackets B and Zuelke KA (1993) Analysis of factors involved in the *in vitro* production of bovine embryos. *Theriogenology* **39** 43–64
- Bringstock DR, Heap RB, Brown KD (1989) Polypeptide growth factors in uterine tissues and secretions. *Journal of Reproduction and Fertility* **85** 747–758
- Carson RS, Zhang Z, Hutchinson LA, Herington AC, Findlay JK (1989) Growth factors in ovarian function. *Journal of Reproduction and Fertility* **85** 735–746

- Chegini N, Rossi MJ, Masterson BJ (1992) Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and EGF and PDGF β -receptors in human endometrial tissue: localization and *in vitro* action *Endocrinology* **130** 2373–2385
- Coskun SA, Sanbuissho A, Lin YC, Rikihisa Y (1991) Fertilizability and subsequent developmental ability of bovine oocytes matured in medium containing epidermal growth factor (EGF) *Theriogenology* **36** 485–494
- Echternkamp SE, Spicer LJ, Gregory KE, Canning SF and Hammond JM (1990) Concentrations of insulin-like growth factor-I in blood and ovarian follicular fluid of cattle selected for twins *Biology of Reproduction* **43** 8–14
- Echternkamp SE, Howard HJ, Roberts AJ, Grizzle J, and Wise T (1994) Relationships among concentrations of steroids, Insulin-like Growth Factor-I, and Insulin-like factor binding proteins in ovarian follicular fluid of beef cattle *Biology of Reproduction* **51** 971–981
- Ferry L, Mermillod P, Massip A and Dessy F (1994) Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage *Theriogenology* **42** 445–453
- Gong JG, Mc Bride D, Bramley TA, Webb R (1993) Effects of recombinant bovine somatotrophin, insulin-like growth factor-I and insulin on the proliferation of bovine granulosa cells *in vitro* *Journal of Endocrinology* **139** 7–15
- Greve T, Avery B and Callesen H (1993) Viability of *in vivo* and *in vitro* produced bovine embryos *Reproduction in Domestic Animals* **28** 164–169
- Harper KM and Brackett G (1993) Bovine blastocysts development after *in vitro* maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins *Biology of Reproduction* **48** 409–416
- Harper KM, Kaye P (1992) Enhanced bovine oocyte quality after *in vitro* maturation (IVM) with insulin-like growth factor-I (IGF-I) and gonadotropins *Biology of Reproduction and Development Supplement* **1** 67 (Abstract)
- Harvey MB and Kaye PL (1992) Insulin-like growth factor-I stimulates growth of mouse preimplantation embryos *in vitro* *Molecular Reproduction and Development* **31** 195–199
- Herliker A, Lucas-Hahn A, Niemann H (1993) Effects of insulin-like growth factor-I on *in vitro* production of bovine embryos *Theriogenology* **37** 1213–1214
- Heyner S, Rao IV, Jarret L and Smith RM (1989) Preimplantation mouse embryos internalise maternal insulin via receptor-mediated endocytosis: pattern of uptake and functional correlations *Developmental Biology* **137** 45–58
- Kaye PL, Bell KL, Beebe LF, Duglison LFS, Gardner HG, Harvey MB (1992) Insulin-like growth factors (IGFs) in preimplantation development *Reproduction Fertility and Development* **4** 373–386
- Keefer CL (1992) Development of bovine embryos produced *in vitro* cultured individually in a simple medium: effect of EGF and TGF β 1 *Theriogenology* **37** 236
- Keefer CL, Stice SL, Paprocki AM and Golueke P (1994) *In vitro* culture of embryos. Cooperative interaction among embryos and role of growth factors *Theriogenology* **41** 1322–1331
- Kreff H (1993) Untersuchungen zur Wirkung von Wachstumsfaktoren bei der Entwicklung von Rinderembryonen *in vitro* PhD Thesis pp 1–135 Ludwig-Maximilian University Munich
- Lane M and Gardner DK (1991) Effect of incubation volume and embryo density on the development and viability of mouse embryos *in vitro* *Proceedings of the Symposium on Preimplantation Embryo Development* Newton Massachusetts 33 (Abstract)
- Larson RC, Ignatz GG, Currie WB (1992) Platelet derived growth factor (PDGF) stimulates development of bovine embryos during fourth cell cycle *Development* **155** 821–826
- Lee ES and Fukui Y (1995) Effect of various growth factors in a defined cultured medium on *in vitro* development of bovine embryos matured and fertilized *in vitro* *Theriogenology* **0077** 71–83
- Lorenzo PL, Illera MJ, Illera JC and Illera M (1994) Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation *in vitro* by the addition of epidermal growth factor and insulin-like growth factor I *Journal of Reproduction and Fertility* **101** 697–701
- Mattson BA, Rosenblum IY, Smith RM and Heyner S (1988) Autoradiographic evidence for insulin and insulin-like growth factor binding to early mouse embryos *Diabetes* **37** 585–589
- Matsui M, Takahashi Y, Hishinuma M, Kanagawa H (1994) Effect of insulin and insulin-like growth factor on the development of bovine embryos *Journal of Mammalian Ova Research* **11** 132–133
- Oliver JE, Aitman TJ, Powell JF, Wilson CA, Clayton RN (1989) Insulin-like growth factor-I gene expression in the rat ovary is confined to the granulosa cells of developing follicles *Endocrinology* **124** 2671–2676
- Palma GA and Brem G (1995) Effect of growth factors IGF-I, TDGF- α , EGF and PDGF on development of *in vitro* produced bovine blastocysts *Theriogenology* **43** 291 (Abstract)
- Palma GA, Clement-Sengewald A, Berg U and Brem G (1992) Role of the embryo number in the development of *in vitro* produced bovine embryos *Theriogenology* **37** 271 (Abstract)
- Palma GA, Krefft H, Clement-Sengewald A, Zhu J and Brem G (1993) Results of large *in vitro* bovine embryo production *Proceedings of the 10th Scientific Meeting of European Embryo Transfer Association* France 250 (Abstract)
- Palma GA, Wolf E and Brem G (1995) Effect of growth factors IGF-I, EGF, TDGF- α and PDGF in a protein-free medium on the development of IVM/IVF bovine embryos. In *Reproduction and Animal Breeding Advances and Strategy* pp 425–426 Eds G Enne, GF Greppi and A Lauria. Elsevier, Amsterdam
- Paria BC and Dey SK (1990) Preimplantation embryo development *in vitro*: cooperative interactions among embryos and role of growth factors *Developmental Biology* **87** 4756–4760
- Persson E, Rodriguez-Martinez H, Dantzer V (1992) Immunocytochemical localization of growth factors during porcine placentation *Proceedings of the 12th International Congress on Animal Reproduction* (The Hague) **3** 1267–1269
- Rinderknecht E, Humbel RE (1988) The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin *Journal of Biological Chemistry* **363** 6233–6239
- Schams D, Graf F, Meyer J, Graule B, Mauthner M and Wolny C (1991) Changes of hormones, metabolites and milk after treatment with somatotrope (recombinant methionyl bst) in Deutsches Fleck- and German Black and White cows *Journal of Animal Science* **69** 1583–1592
- Schultz GA, Hogan A, Watson A, Smith RM and Heyner S (1992) Insulin-like growth factors and glucose transporters temporal patterns of gene expression in early murine and bovine embryos *Reproduction Fertility and Development* **4** 361–371
- Simmen RCM, Ko Y, Simmen FA (1993) Insulin-like growth factors and blastocyst development *Theriogenology* **39** 163–175
- Spicer LJ, Alpisar E and Echternkamp SE (1993) Effects of insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production and (or) insulin-like growth factor I production *in vitro*. *Journal of Animal Science* **71** 1232–1241
- Thibodeaux JK, Myers MW, Prough SG and White KL (1995) Effect of serum extender containing growth factors on development of IVM and IVF bovine embryos. *Theriogenology* **44** 423–432
- Veldhuis JD and Rogers RJ (1987) Mechanisms subserving the steroidogenic synergism between follicle-stimulating hormone and insulin-like growth factor I (somatomedin C) *Journal of Biological Chemistry* **262** 7658–7664
- Walker SK, TM Heard and Seamark RF (1992) *In vitro* culture of sheep embryos without co-culture success and perspectives *Theriogenology* **37** 111–116
- Wiley L, Yamami MS and Van Muydon D (1986) Effect of potassium, type of protein supplement and embryo density on mouse preimplantation development *in vitro* *Fertility and Sterility* **45** 111–119
- Willemssen D, Palma GA and Wolf E (1995) Vitrification of *in vitro*-produced bovine embryos after culture in medium supplemented with insulin-like growth factor-I (IGF-I) *Theriogenology* **43** 352 (Abstract)