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

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This study was carried out to identify the possible effects of epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) on cumulus expansion and nuclear maturation in bovine oocytes matured *in vitro* in serum-free media. Bovine follicular oocytes were matured *in vitro* and were then classified as cumulus cell-enclosed oocytes or denuded oocytes. They were then cultured for 24 h in TCM-199 (tissue culture medium-199) with EGF (50 ng ml⁻¹), IGF-I (100 ng ml⁻¹) and EGF plus IGF-I (50 and 100 ng ml⁻¹, respectively). At the end of the culture period, the morphology of oocytes was evaluated for cumulus expansion and nuclear stage of maturation. In Expt 1, percentages of oocytes reaching maximum cumulus expansion were: 12.5% (control), 46.5% (EGF), 15.2% (IGF-I) and 52.8% (EGF plus IGF-I). In Expt 2, the respective rates of nuclear maturation were: 35.6%, 52.1%, 45.5% and 61.4% for cumulus cell-enclosed oocytes, and 35.3%, 46.6%, 45.4% and 42.5% for denuded oocytes; the same growth factor treatments were used in both cases. There was no significant effect of IGF-I on cumulus expansion. Maximum rates of cumulus expansion and nuclear maturation were obtained in the presence of both growth factors. These results lead to the following conclusions: (i) EGF, either alone or together with IGF-I, stimulates cumulus expansion; and (ii) both growth factors, acting alone or together, enhance nuclear maturation in oocytes surrounded by compact cumulus cells.

Introduction

Bovine oocytes mature *in vivo* at the time of the pre-ovulatory gonadotrophin surge, but can also mature *in vitro* in a suitable medium (Edwards, 1965). The maturation depends on the interaction of many factors and enables oocyte development to reach metaphase II (Tonetta and DiZerega, 1989). A more detailed study of conditions that are necessary for maturation *in vitro* is required.

Gonadotrophins are the primary regulators of nuclear maturation in mammalian oocytes *in vivo*. However, more recent observations imply that gonadotrophin is only one of a complex sequence of factors, such as growth factors, that appear to regulate ovarian function. Epidermal growth factor (EGF) stimulates ovarian granulosa cell proliferation *in vitro* in various species (Gospodarovicz and Bialecki, 1979; May *et al.*, 1987), modulates granulosa cell differentiation (Hsueh *et al.*, 1981) and stimulates the growth of small follicles in women (Westergaard *et al.*, 1990). It appears that EGF, like LH, promotes oocyte maturation by disrupting its communication with cumulus cells (Dekel and Sherizly, 1985) or that it creates a positive maturation signal (Downs, 1989). Insulin-like growth factor I (IGF-I) is a potent mitogen for granulosa cells

(Hernández *et al.*, 1988), even in the absence of FSH (Veldhuis *et al.*, 1986), and acts as a biological amplifier of the action of FSH in the ovary (Hsu and Hammond, 1987). With respect to the maturation of oocytes *in vitro*, as yet undefined follicular factors influence oocyte maturation (Tonetta and DiZerega, 1989). Herlerr *et al.* (1992) showed that addition of IGF-I to maturation medium improves the quality of bovine embryos. Identification of these 'follicular factors' would contribute to the knowledge of intrafollicular regulation of oocyte maturation.

The aim of the present study was to establish whether EGF and IGF-I, alone and in combination, added to serum-free maturation media without addition of exogenous hormones enhance the cumulus expansion and the maturation rate of bovine oocytes *in vitro*.

Materials and Methods

All reagents and media were purchased by Sigma Chemical Co. (St Louis, MO) except IGF-I (Boehringer Mannheim GmbH, Mannheim). Ovaries were obtained from an abattoir and were transported to the laboratory in PBS (Dulbecco) at 39°C within 1 h. Contents of small visible antral follicles (2–8 mm in diameter) were aspirated with an 18-gauge needle attached to a 10 ml disposable syringe, and placed in conical tubes kept at

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Table 1. Effect of growth factors on cumulus expansion of bovine oocytes matured *in vitro*

Treatment	Number of oocytes ^a	Cumulus expansion				LS Means* ($\pm$ SEM)
		0 (%)	+(%)	++ (%)	+++ (%)	
Control	80	67.5	12.1	7.6	12.5	0.65 $\pm$ 0.14 ^b
EGF	92	22.8	18.4	12.3	46.5	1.86 $\pm$ 0.13 ^c
IGF-I	87	34.4	30.3	20.7	15.2	1.12 $\pm$ 0.14 ^b
EGF + IGF-I	91	15.3	12.1	18.6	52.8	2.11 $\pm$ 0.13 ^c

^aData pooled from five independent experiments.

Cumulus expansion – 0: no detectable response equals 0; +: minimum observable response equals 1; ++: moderate response equals 2, and +++ indicates the maximum degree of cumulus expansion equals 3.

*LS Means ($\pm$ SEM) with different superscripts are significantly different, ^b versus ^c $P < 0.05$.

39°C. After 30 min, the sedimented oocytes were transferred to a 35 mm plastic Petri dish containing 2 ml of Hepes-buffered washing medium (TCM 199 + 2% fetal calf serum (FCS)) and given five washes.

Selected oocytes (with homogeneous and evenly granulated cytoplasm) were divided into cumulus cell-enclosed (with intact and unexpanded cumulus) and denuded oocytes (without layers of cumulus cells) according to a previous criteria (Lorenzo *et al.*, 1992). These were placed into drops (50 μ l) of maturation medium under oil, and cultured in 35 mm Petri dishes at 39°C in air with 5% CO₂, 95% humidity for 24 h. The oocytes (five per drop) were matured in TCM-199 with the addition of L-glutamine and sodium bicarbonate. Growth factors were added to the maturation media; there were four different treatments: control, EGF (50 ng ml⁻¹), IGF-I (100 ng ml⁻¹) and EGF plus IGF-I (50 and 100 ng ml⁻¹, respectively). Except in the case of the isolation medium no serum source was used in the maturation media.

In Expt 1, cumulus–oocyte complexes were evaluated at the end of the culture period to assess the effect of growth factors on cumulus cell expansion. The criteria used for describing cumulus expansion was a subjective scoring system in which: 0 indicated no detectable response; + indicated the minimum observable response and +++ indicated the maximum degree of expansion, where all layers of cumulus cells expanded, even those closest to the oocyte.

At the end of the culture period in Expt 2, the oocytes were fixed to ascertain the influence of growth factors on nuclear maturation *in vitro* and the effect of the presence or absence of cumulus cells around the oocyte. Cumulus cell-enclosed and denuded oocytes were used. Cumulus cells of the latter group were removed with hyaluronidase (200 U ml⁻¹) and mechanically stripped. The oocytes were then pipetted onto a slide. A coverslip spotted with a paraffin wax:vaseline (10:1) mixture at each corner was placed directly over the centre of the drop containing the oocytes. Fixation of oocytes was carried out by placing the slides in acetic acid:ethanol (1:3, v,v) for 24 h and staining with a drop of aceto-orcein (2% orcein in 60% acetic acid) for 2 min, followed by washing under tap water. Nuclear maturation was evaluated and classified as germinal vesicle (GV), metaphase I (M I), including metaphase I, anaphase I and telophase I, metaphase II (M II) and degenerate.

Statistical analysis

The degree of cumulus expansion in Expt 1, and metaphase II stage in both groups of oocytes in Expt 2, were used as endpoint parameters for assessing the effect of growth factor on expansion and maturation of bovine oocytes *in vitro*. Statistical analysis of endpoints was carried out by using the Biomedical Data Program (BMDP, Dixon *et al.*, 1990). Expts 1 and 2 were repeated (groups of oocytes were processed on different occasions) five and seven times, respectively. Cumulus expansion at the end of the culture period was assigned a numerical value corresponding to the degree of expansion achieved, where minimum response (+) equals 1, moderate response (++) equals 2, and maximum response equals 3. These numerical scores were subjected to analysis of variance using the 7d procedure of BMDP. The data ($n = 350$ cumulus cell-enclosed oocytes) were analysed by replication ($n = 5$) and treatments ($n = 4$). Comparisons between least square means (LS $\pm$ SEM) were made by *t* test. Mean values for Expt 2 ($n = 914$) were compared by a variance analysis (7d procedure of BMDP), and differences between individual means were analysed by pairwise *t*-test and Bonferroni post-test to determine whether values were statistically different. A P value < 0.05 was accepted as denoting significant differences between groups.

Results

A total of 1264 bovine oocytes were used and evaluated in this study. In Expt 1 ($n = 350$), the respective data of cumulus cell-enclosed oocytes that attained cumulus expansion (% expansion +++ and LS means $\pm$ SEM) in the four different treatments, were: 12.5% and 0.65 $\pm$ 0.14 (control), 46.5% and 1.86 $\pm$ 0.13 (EGF), 15.2% and 1.12 $\pm$ 0.14 (IGF-I) and 52.8% and 2.11 $\pm$ 0.13 (EGF + IGF-I) (Table 1). Treatment with EGF resulted in a significantly higher incidence ($P < 0.05$) of cumulus expansion compared with control and IGF-I treatment. When EGF was used together with IGF-I, cumulus expansion was greater than for treatment with IGF-I alone ($P < 0.05$). No significant effect was observed after treatment with IGF-I compared with control treatment.

Table 2. Nuclear stages of bovine cumulus-cell enclosed oocytes on maturation *in vitro* with growth factors

Treatment	Number of oocytes ^a	Degenerate	Germinal vesicle	Metaphase I	Metaphase II
Control	96	16.0 ± 3.1	24.9 ± 6.0	22.7 ± 3.5	35.6 ± 3.3 ^b
EGF	128	9.3 ± 1.2	6.7 ± 1.0	31.9 ± 2.9	52.1 ± 4.7 ^c
IGF-I	105	13.3 ± 2.1	7.7 ± 3.3	29.3 ± 3.7	49.5 ± 3.1 ^c
EGF + IGF-I	141	5.9 ± 2.1	3.0 ± 1.4	29.8 ± 4.3	61.4 ± 3.6 ^d

Values are percentages ± SEM.

^aData pooled from seven independent experiments.

Values within column with different superscripts are significantly different.

^b versus ^c $P < 0.05$; ^b versus ^d $P < 0.01$; ^c versus ^d $P < 0.05$.

Table 3. Nuclear stages of bovine denuded oocytes on maturation *in vitro* with growth factors

Treatment	Number of oocytes ^a	Degenerate	Germinal vesicle	Metaphase I	Metaphase II
Control	69	21.9 ± 3.4	15.8 ± 4.1	28.1 ± 3.1	35.3 ± 6.1 ^b
EGF	117	15.7 ± 1.9	10.0 ± 2.7	28.8 ± 1.2	46.6 ± 4.0 ^b
IGF-I	114	14.8 ± 1.8	15.5 ± 1.3	25.3 ± 2.5	44.4 ± 4.9 ^b
EGF + IGF-I	144	12.8 ± 1.2	6.2 ± 1.1	40.5 ± 4.9	42.5 ± 2.7 ^b

Values are percentages ± SEM.

^aData pooled from seven independent experiments.

Values within column with the same superscript are not significantly different.

In Expt 2 ($n = 914$), maturation rates obtained for the four treatments with cumulus cell-enclosed oocytes ($n = 470$), were: 35.6% (control), 52.1% (EGF), 49.5% (IGF-I) and 61.4% (EGF + IGF-I) (Table 2), while the rates for denuded oocytes were 35.3%, 46.6%, 44.4% and 42.5%, respectively (Table 3). In cumulus cell-enclosed oocytes, statistically significant differences in the metaphase II stage were observed between: control and EGF treatment ($P < 0.05$), control and IGF-I treatment ($P < 0.05$), control and EGF + IGF-I treatment ($P < 0.01$). The highest metaphase II values were recorded for treatment with EGF + IGF-I. Moreover, compared with IGF-I or control treatments, this treatment resulted in significant differences in the metaphase II stage ($P < 0.05$ and $P < 0.01$, respectively).

There were no significant differences in the maturation rate for denuded oocytes ($n = 444$) among the four treatments used (Table 3).

Discussion

Cumulus oophorus expansion in bovine oocytes occurs in response to an ever-changing milieu of gonadotrophins, growth factors, steroids, factors secreted by the oocyte, and other unknown molecules (Buccione *et al.*, 1990). These compounds could be contributing to maturational changes that occur in the oocyte (Hyttel *et al.*, 1989), mediated by intracellular messengers such as cAMP, calmodulin or diacylglycerol (Gonçalves and Graves, 1992). A similar expansion criterion

was adopted in the study reported here to those used by Behnke, (1987) and Downs (1989), but the results recorded for cumulus expansion in control medium (in the absence of growth factors) were also similar to those of other authors who used similar conditions (Hensleigh and Hunter, 1985; Younis *et al.*, 1989), probably because of the absence in the maturation media of serum, hormones and other factors.

Serum-free media was used for maturation *in vitro* in an attempt to determine the relationship between growth factors and the regulation of nuclear maturation and cumulus expansion, while effectively ruling out the influence of unknown serum factor(s). It has been demonstrated that these growth factors can be synthesized by the ovary (May *et al.*, 1987). The presence of EGF or EGF-like substances has been demonstrated for porcine and human follicles at concentrations similar to those used in the study reported here (Hsu *et al.*, 1987; Hoffman *et al.*, 1990). Moreover, concentrations of IGF-I, similar to those used in the present study, have been detected in bovine and porcine follicular fluid (Hammond *et al.*, 1988; Echternkamp *et al.*, 1990).

The results indicate that EGF enhances cumulus expansion in bovine cumulus-oocyte complexes, in the same way as demonstrated by Downs (1989) for rodent oocytes. IGF-I cannot promote effective cumulus expansion, probably because it does not act via the cumulus cells, or because it interferes with the production of an 'expansion factor' produced by the oocyte (Buccione *et al.*, 1990). Zhang *et al.* (1991) used IGF-I analogues, such as insulin and they too reported poor results for bovine cumulus expansion. We demonstrated that EGF stimulates

resumption of meiosis in bovine oocytes in the absence of serum or hormones. Our data are in agreement with the results obtained for germinal vesicle breakdown in rodent oocytes (Dekel and Sherizly, 1985; Downs, 1989), as well as those obtained in bovine oocytes (Sanbuissho *et al.*, 1990) and porcine oocytes (Reed *et al.*, 1993).

The effects of the growth factors on nuclear maturation are different where each alone stimulates maturation, although the highest results were achieved with the addition of EGF plus IGF-I. This finding could indicate an additive action between the growth factors used and indicates that their actions are combined under conditions *in vivo*. When added to the maturation media, EGF plus IGF-I significantly enhanced nuclear maturation *in vitro* in bovine oocytes and they brought about specific actions on oocyte maturation, including cumulus and cytoplasmic effects. Hainaut *et al.* (1991) postulated that maturation with IGF-I is initiated upon activation of the membrane receptor for this growth factor and requires tyrosine dephosphorylation of p34, the kinase component of maturation promoting factor (MPF).

The growth factors used in the study reported here enhanced maturation in cumulus-oocyte complexes but not in denuded oocytes, and this was in accordance not only with the results observed in cattle (Sanbuissho *et al.*, 1990; Lorenzo *et al.*, 1992) and rodents (Deckel and Sherizly, 1985; Downs, 1989), but also with those obtained using TGF- α , which has similar effects to EGF *in vitro* (Brucker *et al.*, 1991), and binds to the same receptor species (Massagué, 1983; Derynck, 1986). Our hypothesis is that the growth factors act in the presence of the cumulus cells, via which a positive stimulus for maturation is transferred to the oocyte. This stimulatory signal promotes oocyte maturation and can subsequently also disrupt oocyte communication with the cumulus cells, according to a study conducted by Deckel and Sherizly (1985). The growth factors in follicular fluid may be a key factor in the regulation of intrafollicular bovine oocyte maturation. The role of IGF-I may be subtle in cytoplasmic maturation, evincing its positive effect only in fertilization or early events related to embryogenesis (Herlerr *et al.*, 1992).

It is feasible too that EGF plus IGF-I interact with gonadotrophins, steroids or other molecules to regulate oocyte follicular development *in vivo*. In conclusion, we provided evidence that EGF, either alone or in conjunction with IGF-I, stimulates cumulus expansion. The addition of EGF, IGF-I or both factors enhanced nuclear maturation in immature bovine oocytes *in vitro* and this effect is mediated by the presence of cumulus cells. These findings may explain the mechanism of agents involved in the generation of signals regulating the oocyte maturation process. Research into the effect of growth factors on bovine IVF is currently under way.

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References

- Beckne JE (1987) *Bovine Sperm Capacitation and Oocyte in vitro Maturation*. PhD Thesis, Minnesota University
- Brucker C, Alexander NJ, Hodgen GD and Sandow BA (1991) Transforming growth factor- α augments meiotic maturation of cumulus cell-enclosed mouse oocytes *Molecular Reproduction and Development* **28** 94–98
- Buccione R, Vanderhyden BC, Caron PJ and Eppig JJ (1990) FSH-induced expansion of the mouse cumulus oophorus *in vitro* is dependent upon a specific factor(s) secreted by the oocyte *Developmental Biology* **138** 16–25
- Dekel N and Sherizly I (1985) Epidermal growth factor induces maturation of rat follicle enclosed oocytes *Endocrinology* **116** 512–516
- Derynck R (1986) Transforming growth factor- α : structure and biological activities *Journal of Cell Biochemistry* **32** 293–304
- Dixon WJ, Brown MB, Engelman L, Hill MA and Jennrich RI (1990) *BMDP Statistical Software Manual*. University of California Press, Los Angeles
- Downs SM (1989) Specificity of epidermal growth factor action on maturation of the murine oocyte and the cumulus oophorus *in vitro Biology of Reproduction* **41** 371–379
- Echternkamp SE, Spicer LJ, Gregory KE, Channing 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
- Edwards RG (1965) Maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes *Nature* **208** 349–351
- Gonçalves PB and Graves CN (1992) Interaction between cAMP, diacylglycerol and Ca²⁺–calmodulin pathways in the regulation of cumulus expansion *Biology of Reproduction* **46** (Supplement 1) 69
- Gospodarowicz D and Bialecki H (1979) Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine and human origin *Endocrinology* **104** 757–764
- Hainaut P, Giorgetti S, Kowlaski A, Ballotti R and Van Obberghen E (1991) Antibodies to phosphotyrosine injected to *Xenopus laevis* oocytes modulate maturation induced by insulin/IGF-I *Experimental Cell Research* **195** 129–136
- Hammond JM, Hsu CJ, Klindt J, Tsang BK and Downey BR (1988) Gonadotropins increase concentrations of immunoreactive insulin-like growth factor-I in porcine follicular fluid *in vivo Biology of Reproduction* **38** 304–308
- Hensleigh HC and Hunter AG (1985) *In vitro* maturation of bovine cumulus enclosed primary oocytes and their subsequent *in vitro* fertilization and cleavage *Journal of Dairy Science* **68** 1456–1562
- Herlerr A, Lucas-Hann A and Niemann A (1992) Effects of insulin-like growth factor-I on *in vitro* production of bovine embryos *Theriogenology* **37** 1213–1224
- Hernández ER, Resnick CE, Svoboda ME, Van Wyk JJ, Payne DW and Adashi EY (1988) Somatomedin-C/insulin-like growth factor I as an enhancer of androgen biosynthesis by cultured rat ovarian cells *Endocrinology* **122** 1603–1612
- Hoffmann GE, Scott RT, Jr, Brzyski RG and Jones HW, Jr (1990) Immunoreactive epidermal growth factor concentration in follicular fluid obtained from *in vitro* fertilization *Fertility and Sterility* **54** 303–307
- Hsu C and Hammond JM (1987) Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine cells *in vitro Endocrinology* **120** 198–207
- Hsu CJ, Holmes SD and Hammond JM (1987) Ovarian epidermal growth factor-like activity: concentrations in porcine follicular fluid during follicular enlargement *Biochemical and Biophysical Research Communications* **147** 242–247
- Hsueh AJW, Welsh TH and Jones PBC (1981) Inhibition of ovarian and testicular steroidogenesis by epidermal growth factor *Endocrinology* **108** 2002–2004
- Hyttel P, Greve T and Callesen H (1989) Ultrastructural aspects of oocyte maturation and fertilization in cattle *Journal of Reproduction and Fertility Supplement* **38** 35–47
- Lorenzo P, Illera MJ, Sánchez J, Silván G and Illera JC (1992) The effect of EGF on cumulus expansion and bovine oocyte maturation *in vitro Theriogenology* **37** 250
- Massagué J (1983) Epidermal growth factor-like transforming growth factor II. Interaction with epidermal growth factor receptor in human placenta membranes and A431 cells *Journal of Biological Chemistry* **258** 13614–13620
- May JV, Buck PA and Shomberg DW (1987) Epidermal growth factor enhances ¹²⁵Iodo-follicle stimulating hormone binding by cultured porcine granulosa cells *Endocrinology* **120** 2413–2420
- Reed ML, Estrada JL, Illera MJ and Petters RM (1993) Effects of epidermal growth factor, insulin-like growth factor-I, and dialyzed porcine follicular fluid on porcine oocyte nuclear maturation *in vitro Journal of Experimental Zoology* **266** 74–78
- Sanbuissho A, Coskum S and Lin YC (1990) Stimulatory action of epidermal growth factor (EGF) on *in vitro* bovine oocyte maturation *Assisted Reproduction Technology and Andrology* **1** 143–153

- Tonetta SA and DiZerega GS (1989) Intraovarian regulation of follicular maturation *Endocrine Reviews* **10** 205–229
- Veldhuis JD, Rodgers RJ and Furlanetto RW (1986) Synergistic actions of estradiol and the insulin-like growth factor somatomedin-C on swine ovarian (granulosa) cells *Endocrinology* **119** 530–538
- Westergaard LG, Andersen CY and Byskov AG (1990) Epidermal growth factor (EGF) in small antral ovarian follicles of pregnant women *Journal of Endocrinology* **127** 363–367
- Younis AI, Brackett BG and Fayrer-Hosken RA (1989) Influence of serum and hormones on bovine oocyte maturation and fertilization *in vitro Gamete Research* **23** 189–201
- Zhang L, Blakewood EG, Deninston RS and Godke RA (1991) The effect of insulin on maturation and development of *in vitro*-fertilized bovine oocytes *Theriogenology* **35** 301