The effects of epidermal growth factor and insulin-like growth factor I on the metabolic activity, nuclear maturation and subsequent development of cattle oocytes *in vitro*

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The effects of epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) on the maturation and subsequent development of cattle oocytes *in vitro* were evaluated in three experiments. Cumulus–oocyte complexes (COC) were collected from cattle ovaries and matured for 20–24 h in control medium or in medium containing 50 ng EGF ml⁻¹, 100 ng IGF-I ml⁻¹, EGF + IGF-I, or 10% (v/v) fetal calf serum plus 0.1 i.u. human menopausal gonadotrophin ml⁻¹ (hMG). In Expt 1, treatment with EGF + IGF-I stimulated cumulus expansion, the metabolism of pyruvate and glutamine, and nuclear maturation. In Expt 2, only the metabolic measurements from oocytes that reached metaphase II were considered, and EGF + IGF-I stimulated pyruvate metabolism to the same extent as serum + hMG. In Expt 3, the oocytes were fertilized after maturation culture, and the resultant embryos cultured for up to 8 days. The cleavage was greater in the EGF and EGF + IGF-I groups than in the controls but less than in the serum + hMG group. Moreover, the number of blastocyst cells at 7 days after insemination and the proportion of cleaved embryos that developed to the blastocyst stage by day 8 was greater in the serum + hMG group than in the control group indicating that maturation treatment can affect early embryonic development. In conclusion, EGF + IGF-I can stimulate cumulus expansion, oxidative metabolism, nuclear maturation and cleavage after fertilization of bovine oocytes *in vitro*. The relative effects of the treatments on oocyte pyruvate metabolism in Expts 1 and 2 generally paralleled their effects on cleavage and subsequent development in Expt 3, suggesting that mitochondrial function is related to developmental potential. Further investigation is required to determine which component(s) of serum or gonadotrophin treatment is responsible for the effects on subsequent embryonic development.

Introduction

In mammals, oocytes are arrested at the diplotene stage of prophase of the first meiotic division (Tsafiriri, 1985). *In vivo*, nuclear maturation to metaphase II is stimulated by the preovulatory LH surge, but the intrafollicular mechanism is not well understood (Mattioli, 1992). *In vitro*, bovine oocytes undergo spontaneous nuclear maturation when they are removed from the follicle, but formation of the male pronucleus after fertilization and subsequent embryonic development are limited (Crozet, 1991). The addition of high concentrations of gonadotrophins, or serum containing circulating concentrations of gonadotrophins, to the maturation medium results in improved rates of embryo development (Crozet, 1991). The positive effects of gonadotrophins on oocyte maturation are mediated through the cumulus cells, but it is not clear whether

these effects result from interaction with gonadotrophin receptors on the cumulus cells, or from crossreaction with the receptors for cellular growth factors (Brackett and Zuelke, 1993; Harper and Brackett, 1993).

A variety of cellular growth factors including members of the transforming growth factor α family, transforming growth factor β , fibroblast growth factors, tumour necrosis factor, interleukin 1, insulin-like growth factor 1 (IGF-I), insulin-like growth factor 2, and epidermal growth factor (EGF) are found in the ovary and have effects on ovarian cell function (Dorrington *et al.*, 1987; Hammond *et al.*, 1991; Adashi, 1992; Stern and Coulam, 1992; Hammond *et al.*, 1993). In particular, IGF-I has been shown to stimulate or enhance the *in vitro* maturation of pig (Reed *et al.*, 1993; Xia *et al.*, 1994) and cattle (Harper and Brackett, 1993) oocytes. Epidermal growth factor has been shown to stimulate the *in vitro* maturation of mouse (Downs, 1989), rat (Benyosef *et al.*, 1992), human (Das *et al.*, 1991), pig (Reed *et al.*, 1993; Ding and Foxcroft, 1994) and

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cattle (Park and Lin, 1993; Kobayashi *et al.*, 1994; Lonergan *et al.*, 1996) oocytes. When added to maturation medium containing a relatively low concentration of FSH, EGF increased the proportion of cattle oocytes that developed to the blastocyst stage after fertilization (Harper and Brackett, 1993). Lorenzo *et al.* (1994) have shown that IGF-I and EGF together stimulate nuclear maturation in a greater proportion of cattle oocytes than does either growth factor alone.

In addition to changes in protein synthesis (Kastrop *et al.*, 1991), and in the distribution of cortical granules and mitochondria (Hyttel *et al.*, 1986; Shamsuddin *et al.*, 1993), the nuclear maturation of cattle oocytes in the presence of gonadotrophins is accompanied by increases in the metabolism of glutamine, pyruvate and glycine (Zuelke and Brackett, 1993; Rieger and Loskutoff, 1994), but not glucose (Zuelke and Brackett, 1992; Rieger and Loskutoff, 1994). The results of these studies suggest that oxidative metabolism increases, and is the major form of cellular energy production, during maturation of the cattle oocyte *in vitro*.

The first objective of the present study was to determine whether the stimulation of maturation of cattle oocytes by IGF-I and EGF is similarly accompanied by changes in metabolic activity. The second objective was to determine the effects of treatment of bovine oocytes with EGF and IGF-I during maturation on the subsequent cleavage and development of embryos formed from those oocytes. Some of the data presented here have been published as abstracts (Rieger *et al.*, 1995; Gandolfi *et al.*, 1996).

Materials and Methods

The study comprised three experiments. In Expt 1, the cumulus expansion, metabolic activity, and nuclear maturation of cattle oocytes were determined immediately after recovery from the ovary or after *in vitro* maturation for 20 h in the absence or presence of EGF, IGF-I, human menopausal gonadotrophin (hMG) and serum. In Expt 2, oocytes were subjected to the same maturation treatments and the effect of treatment on metabolic activity of those oocytes that reached metaphase II was evaluated. In Expt 3, the oocytes were subjected to the same maturation treatments and then fertilized *in vitro*. The effects of the treatment on cleavage, development to the blastocyst stage and number of cells at the blastocyst stage were determined. Unless otherwise indicated, all reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Oocyte collection

Ovaries were collected from local abattoirs in sterile phosphate-buffered saline (PBS) and transported to the laboratory in a warm box at 28°C within 2 h after death of the animals. Most of the animals were of the Holstein-Friesen breed, and approximately three to ten years old.

Cumulus-oocyte complexes (COC) were isolated by slicing the surface of the ovaries with a sterile scalpel blade (Xu *et al.*, 1992) and rinsing them in 50 ml TCM 199 supplemented with 25 mmol Hepes l⁻¹, 4 g BSA l⁻¹ and 10 mg heparin l⁻¹. Selected COC with several complete layers of granulosa cells surrounding an oocyte with a homogeneous cytoplasm were

rinsed three times in 2 ml of the same medium and then randomly distributed among the treatment groups.

Maturation treatments and evaluation of cumulus expansion

The basic medium for oocyte maturation was TCM 199 containing 0.68 mmol L-glutamine l⁻¹ and supplemented with 25 mmol NaHCO₃ l⁻¹ and 0.1% (w/v) polyvinylalcohol. To this was added either (i) 50 ng EGF ml⁻¹ (Sigma), (ii) 100 ng IGF-I ml⁻¹ (Boehringer), (iii) 50 ng EGF ml⁻¹ plus 100 ng IGF-I ml⁻¹, or (iv) 10% (v/v) fetal calf serum plus 0.1 i.u. human menopausal gonadotrophin ml⁻¹ (hMG, Pergovet, Serono, Rome). The control treatment was the basic medium only.

For each treatment, droplets were prepared containing 5 to 11 COC at a concentration of 1 COC per 10 µl and cultured for 20 h at 38.5°C under paraffin oil (Sigma) in a humidified atmosphere of 5% CO₂ in air. In Expt 1, cumulus expansion was then evaluated under a stereomicroscope at × 16.

Metabolic measurements

The metabolism of individual oocytes (Expts 1 and 2) was measured using TCM 199 containing 0.68 mmol L-glutamine l⁻¹ and supplemented with 25 mmol NaHCO₃ l⁻¹, 20 mmol Hepes l⁻¹, and 0.1% (w/v) polyvinylalcohol.

After culture in the maturation treatments for 20 h, approximately 10 COC were taken at random from each maturation treatment group, placed in 10 mmol sodium citrate l⁻¹ for 5 min and pipetted up and down repeatedly to remove the cumulus cells. The denuded oocytes were then washed three times in PBS before being transferred to metabolic measurement medium. In Expt 1, COC were also recovered from fresh ovaries and denuded in the same way as the oocytes in the cultured groups. For each replicate, usually eight denuded oocytes of each group were then subjected to the metabolic measurement procedure. The remaining denuded oocytes were contemporaneously cultured in 0.5 ml of metabolic measurement medium (without radiolabelled substrates) at 38.5°C under 5% CO₂ in humidified air.

Except for the medium, the technique used to measure oocyte metabolism was as described by Rieger and Loskutoff (1994). Mixtures of D-[5-³H]glucose (specific activity = 16.6 Ci mmol⁻¹, Amersham International, Little Chalfont) and L-[¹⁴C(U)]glutamine (271 mCi mmol⁻¹, Amersham), or D-[5-³H]glucose and [2-¹⁴C]pyruvate (31.1 mCi mmol⁻¹, NEN Products Division, Dupont, Nemours), or [2-¹⁴C]pyruvate alone were dried by vacuum evaporation. The radiolabels were then taken up in metabolic measurement medium to give concentrations of 0.12 µCi µl⁻¹, 0.18 µCi µl⁻¹, and 0.14 µCi µl⁻¹, for D-[5-³H]glucose, L-[¹⁴C(U)]glutamine and [2-¹⁴C]pyruvate, respectively.

Individual oocytes were taken up in 2 µl of metabolic measurement medium and placed in the cap of a sterile 2.5 ml polypropylene screw-cap microtube together with 2 µl of radiolabelled substrate mixture (total culture volume 4 µl) to yield final concentrations (labelled plus unlabelled) of 5.56 mmol glucose l⁻¹, 1.0 mmol glutamine l⁻¹, and 2.1 mmol pyruvate l⁻¹. The cap was quickly fitted onto the tube, which

had been loaded with 1.5 ml of 25 mmol $\text{NaHCO}_3 \text{ l}^{-1}$ equilibrated with a gas mixture of 5% O_2 , 5% CO_2 and 90% N_2 . Five or six sham preparations, containing all reagents but no oocyte, were included for each preparation of labelled substrates. These served to control for all non-specific counts due to machine background, chemiluminescence, bacterial contamination, and spontaneous breakdown of the labelled substrate. At the end of the 3 h measurement period, the caps were removed and the bicarbonate was mixed with 15 ml of scintillation fluid and counted for 5 min in a liquid scintillation counter. The total d.p.m. of labelled substrate was determined by mixing 2 μl of the labelled substrate solution with 1.5 ml of 25 mmol $\text{NaHCO}_3 \text{ l}^{-1}$, and counting in the same way. The amount of each substrate metabolized by each oocyte was calculated as described for cattle embryos (Rieger *et al.*, 1992).

Evaluation of nuclear maturation

Immediately after the metabolic measurements, the denuded oocytes were washed three times and then fixed by immersion for 24 h in a solution of acetic acid and ethanol (1:3). After staining with 1% (v/v) Lacmoid, the nuclear morphology was examined at $\times 250$, and classified as immature (germinal vesicle or germinal vesicle breakdown stage), intermediate (metaphase I, anaphase I or telophase I), or mature (metaphase II) as described by Sirard and Bilodeau (1990). Oocytes that could not be identified as being at any of these stages were classified as degenerate. In Expt 1, the oocytes from each replicate of each treatment were fixed, stained and evaluated as a group. In Expt 2, the oocytes were fixed, stained and evaluated individually.

In vitro fertilization and embryo culture

In Expt 3, COC were matured for 20 h using the treatments described above. A straw containing cryopreserved bull spermatozoa was thawed in water at 25°C and the cells were layered on top of a 45–90% Percoll gradient made with modified Tyrode's medium (TALP); the gradient was centrifuged for 30 min at 600 g. The sperm pellet was counted and diluted to a final concentration of 1×10^6 spermatozoa ml^{-1} medium TALP, supplemented with 0.3% BSA fatty acid free, 10 μg heparin ml^{-1} and 5 mmol caffeine l^{-1} . Microdrops of 90 μl were made with the sperm suspension and mineral oil was layered on top.

Groups of 30 oocytes were denuded by vortexing for 4 min in 500 μl of Hepes-buffered TALP medium and washed twice before being transferred into the fertilization drops. Between 8 and 12 oocytes, in a volume of 10 μl , were added to each drop and were incubated at 38.5°C in an humidified atmosphere of 5% CO_2 in air for 18–20 h.

Bovine oviducts were obtained from the abattoir at the same time as the ovaries, and the epithelial cells were isolated by scraping the lumen of the ampullary region with a Pasteur pipette and then were washed twice in 5 ml Hepes-buffered (25 mmol l^{-1}) medium 199, supplemented with 0.4% BSA. Clusters of cells were cultured for 24–48 h in medium 199 supplemented with 100 mg glutamine l^{-1} , 75 mg kanamycin l^{-1} , 2.1 g sodium bicarbonate l^{-1} and 10% (v/v) fetal calf

serum, before being divided into 20 μl aliquots containing approximately 5000 cells. At the end of the fertilization period putative zygotes were rinsed twice in medium 199 and then transferred in groups of ten in the culture drops. Incubation was performed as described above and the cleaved eggs were transferred to a second drop containing previously prepared oviduct cells, after 2 days of culture. On day 7 after insemination, the embryos judged to have reached the blastocyst stage were removed from culture. The metabolism of glutamine and glucose by individual embryos was measured over 3 h, by culture in a 4 μl hanging microdrop containing the radio-labelled substrates, over 1.5 ml of 25 mmol $\text{NaHCO}_3 \text{ l}^{-1}$, as described above for the oocytes. They were then stained as described above for an accurate determination of number of nuclei. The remaining embryos were left in culture for a further 24 h (day 8) and those that were judged to have reached the blastocyst stage were fixed and stained in the same way.

Statistical analysis

In Expt 1, the effect of treatment on cumulus expansion was evaluated by first calculating the arcsines of the proportion of expanded COC in each replicate ($n = 25$ or 26 per treatment). These values were subjected to one-way analysis of variance, followed by Duncan's multiple-range tests to compare individual treatment means. The effect of treatment on the metabolism of each substrate was analysed by one-way analysis of variance, followed by Duncan's multiple-range tests to compare individual treatment means. The effect of the metabolic measurement procedure on nuclear maturation was evaluated by Chi-square analyses of the proportion of mature oocytes following metabolic measurement or contemporaneous culture. The arcsines of the proportion of oocytes in each class of nuclear maturation in each replicate ($n = 7$ per treatment) were calculated and these values were subjected to one-way analysis of variance, followed by Duncan's multiple-range tests to compare individual treatment means.

In Expt 2, only the metabolic measurements from oocytes that had reached metaphase II were included in the statistical analysis. These data were subjected to one-way analysis of variance, followed by Duncan's multiple-range tests to compare individual treatment means.

In Expt 3, the arcsines of the proportions of oocytes that cleaved or reached the blastocyst stage by day 7 or 8 in each replicate ($n = 12$ per treatment) were subjected to one-way analysis of variance, followed by Duncan's multiple-range tests to compare individual treatment means. The effects of treatment on the metabolism of glutamine and glucose by individual blastocysts, and on the number of cells in the blastocysts, were analysed by one-way analysis of variance, followed by Duncan's multiple-range tests to compare individual treatment means.

Results

Experiment 1

A total of 1148 COC was used to evaluate the effects of maturation treatment on cumulus expansion. Overall, there was

Table 1. The effect of maturation treatment on the metabolism of glucose, glutamine and pyruvate by individual denuded cattle oocytes (Expt 1)

Treatment	Glucose		Glutamine		Pyruvate	
	Number of oocytes	(pmol per oocyte per 3 h)	Number of oocytes	(pmol per oocyte per 3 h)	Number of oocytes	(pmol per oocyte per 3 h)
Non-cultured	24	4.8 ± 1.2	16	0.9 ± 0.3 ^a	12	4.6 ± 2.1 ^a
Control	48	5.6 ± 1.0	32	2.4 ± 0.3 ^b	24	7.4 ± 1.4 ^a
EGF	44	3.7 ± 1.1	28	2.9 ± 0.2 ^{bc}	24	13.2 ± 1.5 ^b
IGF-I	44	5.3 ± 0.9	29	2.9 ± 0.2 ^{bc}	19	19.7 ± 1.6 ^c
EGF + IGF-I	46	3.8 ± 0.7	30	3.2 ± 0.2 ^c	20	17.5 ± 1.3 ^{bc}
Serum + hMG	45	4.6 ± 0.9	30	3.3 ± 0.2 ^c	23	20.4 ± 1.7 ^c

The oocytes were denuded and metabolism measured for 3 h, either immediately after recovery from the ovaries (non-cultured), or after culture for 20 h (all other groups). EGF: epidermal growth factor; IGF-I: insulin-like growth factor I; hMG: human menopausal gonadotrophin.

Values are means ± SEM. ^{abc}Within columns, values with different superscripts are significantly different ($P < 0.05$).

Table 2. The effect of maturation treatment on the nuclear maturation of cattle oocytes (Expt 1)

Treatment	Total number of oocytes	Immature (%)	Intermediate (%)	Mature (%)	Degenerate (%)
Control	66	4.4 ± 2.1	24.6 ± 5.7	27.7 ± 4.8 ^a	43.2 ± 6.1 ^a
EGF	63	1.4 ± 1.4	20.1 ± 8.1	54.0 ± 9.5 ^{ab}	24.5 ± 4.9 ^b
IGF-I	57	3.2 ± 2.1	17.0 ± 7.2	51.1 ± 7.8 ^{ab}	28.6 ± 8.7 ^{ab}
EGF + IGF-I	66	2.9 ± 1.8	12.0 ± 6.7	61.3 ± 5.8 ^b	23.8 ± 5.0 ^b
Serum + hMG	63	0.0 ± 0.0	34.3 ± 10.4	51.8 ± 12.5 ^{ab}	13.8 ± 2.9 ^b

The oocytes were cultured for 20 h, and then denuded of cumulus cells and subjected to metabolic measurement or culture for 3 h before being fixed and stained. EGF: epidermal growth factor; IGF-I: insulin-like growth factor I; hMG: human menopausal gonadotrophin. Proportions are means ± SEM for seven replicates.

^{ab}Within columns, values with different superscripts are significantly different ($P < 0.05$).

a highly significant effect of treatment on the proportion of COC that expanded ($P < 0.0001$). There was no cumulus expansion in the control group ($0.0 \pm 0.0\%$, mean ± SEM, $n = 234$). The proportion of expanded COC in the IGF-I group ($13.9 \pm 3.5\%$, $n = 224$) was not different from that in the control group. Conversely, the proportion of expanded COC in the EGF ($31.1 \pm 5.1\%$, $n = 227$), EGF + IGF-I ($56.8 \pm 6.3\%$, $n = 229$) and serum + hMG ($100.0 \pm 0.0\%$, $n = 234$) groups were all significantly different ($P < 0.05$) from that in the control group, and from each other.

Metabolic measurement were made on a total of 300 oocytes. The data from 13 oocytes (4%) were judged to be erroneous and were therefore excluded from the analysis. The remaining 287 oocytes yielded 538 metabolic values (Table 1). There was a very highly significant overall effect ($P < 0.0001$) of treatment on both glutamine and pyruvate metabolism, but no significant effect ($P > 0.60$) on glucose metabolism.

Glutamine metabolism was significantly less ($P < 0.05$) in the non-cultured oocytes than in all other groups, but not different among the control, EGF and IGF-I groups or among the EGF, IGF-I, EGF + IGF-I and serum + hMG groups. However, glutamine metabolism in the EGF + IGF-I and serum + hMG groups was significantly greater than that in the control group.

Pyruvate metabolism was significantly greater in the EGF, IGF-I, EGF + IGF-I and serum + hMG groups than in the non-cultured and control groups, which were not different. The metabolism of pyruvate by the EGF + IGF-I group was not different from either the EGF or IGF-I group, but was significantly greater in the serum + hMG group than in the EGF group.

Of the 34 non-cultured oocytes, 47.1% were immature, 35.3% were intermediate, 0.0% were mature, and 17.7% were degenerate. For the cultured treatment groups, the overall proportions of mature oocytes were not different between those subjected to the metabolic measurement procedure (120/246, 48.8%) and those that were contemporaneously cultured (37/69, 53.6%).

There was no overall effect of treatment on the proportions of immature ($P > 0.40$) or intermediate ($P > 0.30$) oocytes, and no differences between individual treatment means (Table 2).

There was also no significant overall effect of treatment on the proportions of mature oocytes ($P > 0.17$). However, the proportion of mature oocytes in the EGF + IGF-I group was significantly greater than that in the control group. The proportions of mature oocytes in the EGF, IGF-I and serum + hMG groups were not different from the control group, the EGF + IGF-I group, or each other.

Table 3. The effect of maturation treatment on the metabolism of glucose, glutamine and pyruvate by individual denuded cattle oocytes that had matured to metaphase II (Expt 2)

Treatment	Glucose		Glutamine		Pyruvate	
	Number of oocytes	(pmol per oocyte per 3 h)	Number of oocytes	(pmol per oocyte per 3 h)	Number of oocytes	(pmol per oocyte per 3 h)
Control	26	4.7 ± 0.5	19	2.8 ± 0.3	18	9.6 ± 1.3 ^a
EGF	25	4.8 ± 0.5	19	2.6 ± 0.3	23	11.6 ± 0.9 ^{ab}
IGF-I	16	3.9 ± 0.6	20	2.7 ± 0.3	20	14.2 ± 1.1 ^{bc}
EGF + IGF-I	24	5.2 ± 0.6	18	3.1 ± 0.5	26	16.7 ± 0.9 ^{cd}
Serum + hMG	35	4.2 ± 0.5	23	3.4 ± 0.3	25	18.1 ± 1.3 ^d

After culture for 20 h in the indicated maturation treatments, the oocytes were denuded and metabolism measured for 3 h. The oocytes were then fixed and stained to evaluate the stage of nuclear maturation. EGF: epidermal growth factor; IGF-I: insulin-like growth factor I; hMG: human menopausal gonadotrophin. Metabolism values are means ± SEM.

^{abcd}Within columns, values with different superscripts are significantly different ($P < 0.05$).

Table 4. The effect of maturation treatment of cattle oocytes on the cleavage rate, development to the blastocyst stage, and number of cells in blastocyst (Expt 3)

Treatment	Number of oocytes	Cleaved (%)	Embryos that developed to the blastocyst stage							
			By day 7				By day 8			
			<i>n</i>	Percentage of oocytes	Percentage of cleaved	Number of cells	<i>n</i> *	Percentage of oocytes*	Percentage of cleaved*	Number of cells [†]
Control	268	43.4 ± 3.5 ^a	21	8.3 ± 1.8 ^a	18.8 ± 4.2	77.9 ± 6.8 ^a	32	12.3 ± 2.5 ^a	27.3 ± 5.3 ^a	81.7 ± 11.5
EGF	271	53.9 ± 3.7 ^b	31	11.3 ± 2.6 ^{ab}	19.3 ± 4.1	70.8 ± 4.3 ^a	50	18.4 ± 2.4 ^{ab}	32.8 ± 2.9 ^{ab}	74.0 ± 7.0
IGF-I	268	51.3 ± 3.7 ^{ab}	19	7.3 ± 0.9 ^a	15.9 ± 2.6	72.8 ± 5.6 ^a	32	12.8 ± 1.9 ^{ab}	28.5 ± 6.0 ^{ab}	67.6 ± 6.9
EGF + IGF-I	276	57.9 ± 3.6 ^b	32	12.3 ± 2.2 ^{ab}	21.0 ± 3.2	80.1 ± 5.1 ^a	56	21.0 ± 2.0 ^b	37.7 ± 3.9 ^{ab}	74.6 ± 5.9
Serum + hMG	267	73.5 ± 3.5 ^c	45	16.4 ± 2.1 ^b	22.7 ± 2.6	95.1 ± 4.3 ^b	82	30.3 ± 3.4 ^c	40.9 ± 4.1 ^b	82.1 ± 5.5

The oocytes were matured for 20 h in the indicated treatments, fertilized *in vitro*, and then co-cultured with bovine oviductal epithelial cells until 7 or 8 days after insemination. EGF: epidermal growth factor; IGF-I: insulin-like growth factor I; hMG: human menopausal gonadotrophin. Proportions are mean ± SEM for 12 replicates.

*Numbers and mean proportions include the blastocysts identified and removed from culture at day 7.

[†]Mean cell numbers are for only the embryos identified as blastocysts at day 8.

^{abc}Within columns, values with different superscripts are significantly different ($P \leq 0.05$).

There was a significant overall effect of treatment on the proportions of degenerate oocytes ($P < 0.05$). The proportion of degenerate oocytes in the control group was significantly greater than that in the EGF, EGF + IGF-I, and serum + hMG groups. There were no differences in the proportion of degenerate oocytes among the EGF, IGF-I, EGF + IGF-I, and serum + hMG groups, or between the control and IGF-I groups.

Experiment 2

The effects of maturation treatment on the metabolism of glucose, glutamine and pyruvate by oocytes that matured to metaphase II are shown in Table 3. There was no significant overall effect of treatment group and no significant differences among treatments on glucose or glutamine metabolism. There was a highly significant overall effect of treatment on pyruvate metabolism ($P < 0.0001$). There was no significant difference in

pyruvate metabolism between the EGF and control groups, between EGF and IGF-I, IGF-I and EGF + IGF-I, or EGF + IGF-I and serum + hMG. However, pyruvate metabolism by the IGF-I and EGF + IGF-I groups was significantly greater ($P < 0.05$) than for the controls, and was greater in the serum + hMG group than in all other groups except EGF + IGF-I.

Experiment 3

There was a highly significant overall effect of maturation treatment on cleavage rate ($P < 0.0001$): it was lowest in the controls, intermediate in the EGF, IGF-I and EGF + IGF-I groups, and highest in the serum + hMG group (Table 4).

There was a significant overall effect of treatment on the proportion of oocytes that developed to the blastocyst stage by day 7 ($P < 0.02$), but only the serum + hMG group was significantly different from the controls. However, there was no

Table 5. The effect of maturation treatment of cattle oocytes on the metabolism of glutamine and glucose at the blastocyst stage (Expt 3)

Treatment	n	Glutamine (pmol per embryo per 3 h)	Glucose (pmol per embryo per 3 h)
Control	13	0.3 ± 0.1	46.9 ± 4.7
EGF	18	0.3 ± 0.0	56.0 ± 4.7
IGF-I	18	0.4 ± 0.0	51.8 ± 4.7
EGF + IGF-I	14	0.3 ± 0.1	49.8 ± 4.8
Serum + hMG	31	0.4 ± 0.0	61.3 ± 4.4

The oocytes were matured for 20 h in the indicated treatments, fertilized *in vitro*, and then co-cultured with bovine oviductal epithelial cells until 7 days after insemination. EGF: epidermal growth factor; IGF-I: insulin-like growth factor I; hMG: human menopausal gonadotrophin. Values are means ± SEM.

effect of treatment on the proportion of cleaved embryos that reached the blastocyst stage by day 7. The mean number of cells of day 7 blastocysts was significantly greater in the serum + hMG group than in all other treatment groups but there were no differences in glutamine or glucose metabolism among the treatment groups (Table 5).

There was a significant overall effect ($P < 0.05$) of treatment on the proportion of oocytes that developed to the blastocyst stage by day 8 (Table 4). This proportion was significantly greater in the EGF + IGF-I group than that in the control group and significantly less than that in the serum + hMG group. There was also a significant overall effect on the proportion of cleaved embryos that developed to the blastocyst stage by day 8: the proportion in the serum + hMG group was significantly greater than that in the control group. There were no differences in the number of cells in the day 8 blastocysts among the treatment groups.

Discussion

None of the COC in the control group, and all of the COC in the serum + hMG group underwent cumulus expansion, indicating that expansion does not occur spontaneously under the culture conditions used, but that the COC used were fully able to expand. There was no significant effect of IGF-I alone, while 31.1% of COC in the EGF group expanded. Moreover, 56.8% of the COC in the EGF + IGF-I group expanded, suggesting that the effects of the two growth factors are additive. In the mouse, IGF-I similarly had no effect on cumulus expansion while EGF stimulated expansion as did FSH (Downs, 1989). Kobayashi *et al.* (1994) report that EGF, like LH + FSH, stimulates expansion of 99–100% of cattle COC, compared with 22.9% by insulin and 10.2% of control COC. Lonergan *et al.* (1996) found that as little as 1 ng EGF ml⁻¹ significantly stimulated cumulus expansion of cattle COC. Lorenzo *et al.* (1994) also observed that EGF stimulated expansion of cattle COC, but IGF-I had no effect, while there was an additive effect of the two growth factors together.

Glutamine and pyruvate metabolism were significantly greater in oocytes that had been cultured for 20 h in the

presence of serum + hMG than in non-cultured oocytes, and in the control oocytes. Conversely, glucose metabolism was unaffected by time and culture treatment. These results are in close agreement with our previous observations (Rieger and Loskutoff, 1994) and with those of Zuelke and Brackett (1992, 1993).

There were no differences in glutamine or pyruvate metabolism among the EGF, IGF-I and EGF + IGF-I groups. Moreover, neither EGF nor IGF-I alone had any effect on glutamine metabolism compared with the control group. However, EGF + IGF-I significantly stimulated both glutamine and pyruvate metabolism compared with the control group, to values that were not different from that measured in the serum + hMG group. Mean glutamine metabolism was 38% greater in the EGF + IGF-I group than in the control group, which compares favourably with the observation that mean glutamine metabolism by oocytes cultured with 10 µg LH ml⁻¹ was approximately 26% greater than by oocytes cultured in medium alone (Zuelke and Brackett, 1993). These observations suggest that EGF and IGF-I together can mimic the stimulatory effects of gonadotrophins on oxidative metabolism in cattle oocytes.

The combined EGF + IGF-I treatment stimulated oocyte maturation over that seen in the control group. This finding suggests that these growth factors are capable of stimulating the maturation of cattle oocytes as well as do gonadotrophins, and is consistent with the reports of Park and Lin (1993), Kobayashi *et al.* (1994), Lorenzo *et al.* (1994) and Lonergan *et al.* (1996). We used exactly the same concentrations of EGF and IGF-I as Lorenzo *et al.* (1994) and observed treatment means for maturation to metaphase II (MII) that were very similar to their results. Notably, the proportion of MII oocytes in the EGF + IGF-I group were 8–10% greater than in the EGF and IGF-I groups in both studies.

In Expt 2 there was no effect of maturation treatment on glucose metabolism of oocytes that had reached metaphase II. Neither was there any significant effect of treatment on glutamine metabolism, although the trend resembled that seen in Expt 1; the mean values were greater in the EGF + IGF-I and serum + hMG groups than in the control, EGF and IGF-I groups. Pyruvate metabolism was very similar to that seen in Expt 1 and was significantly greater in the IGF-I, EGF + IGF-I and serum + hMG groups than in the control group. Most significantly, pyruvate metabolism was greatest in the EGF + IGF-I and serum + hMG groups and they were not different from each other. The results of Expt 2 demonstrate that, in addition to increasing the proportion of oocytes that reach metaphase II, the combination treatment of EGF + IGF-I also increases the oxidative metabolism of those oocytes that reach this stage.

Gonadotrophins are often used to stimulate the maturation of cattle oocytes *in vitro* (Crozet, 1991; Brackett and Zuelke, 1993), but at very high concentrations: for example 5 µg LH ml⁻¹ or 500 ng FSH ml⁻¹ (Kobayashi *et al.*, 1994), 500 ng LH or FSH ml⁻¹ (Harper and Brackett, 1993), 5 µg LH ml⁻¹ and 500 ng FSH ml⁻¹ (Pinyopummintr and Bavister, 1991; Rosenkrans *et al.*, 1993), 45 µg FSH ml⁻¹ (Barnes *et al.*, 1993). Similarly, Zuelke and Brackett (1993) used 10 µg LH ml⁻¹ to demonstrate its effect on glutamine metabolism in cattle oocytes. However, Dieleman *et al.* (1983) found mean peak

concentrations of 7 ng LH ml⁻¹ and 1.6 ng FSH ml⁻¹ in bovine follicular fluid *in vivo* at 4 h after the plasma peak of the preovulatory LH surge. These peak follicular concentrations are 71 and 313 times lower than even the 'low' concentrations used by Harper and Brackett (1993) for the *in vitro* maturation of cattle oocytes.

In contrast, the stimulation of cumulus expansion, oxidative metabolism and nuclear maturation of cattle oocytes was achieved using concentrations of 50 ng EGF ml⁻¹ and 100 ng IGF-I ml⁻¹ in the present study and by Lorenzo *et al.* (1994) and 100 ng ml⁻¹ EGF in the study by Kobayashi *et al.* (1994). Concentrations of 23–168 ng IGF-I ml⁻¹ have been measured in bovine follicular fluid (Spicer *et al.*, 1988; Spicer *et al.*, 1992; Simpson *et al.*, 1994). We are not aware of any published values for EGF concentrations in bovine follicular fluid, but concentrations of 5–13 ng ml⁻¹ have been measured in porcine follicular fluid (Hsu *et al.*, 1987).

In Expt 3, EGF alone or in combination with IGF-I significantly stimulated cleavage, consistent with the effects on maturation observed in Expt 1 and with the observations of Lonergan *et al.* (1996). However, the cleavage rate was significantly greater in the serum + hMG group than in all other treatment groups, whereas the maturation rate in the serum + hMG group was greater only than the controls in Expt 1. Similarly, the proportion of oocytes that reached the blastocyst stage by day 7 was greater in the serum + hMG group than in the control group, and there were more blastocyst cells in the serum + hMG group than in all other maturation treatment groups. The proportion of oocytes and cleaved embryos that reached the blastocyst stage by day 8 was significantly greater in the serum + hMG group than in the control group. Lonergan *et al.* (1996) observed that development to the blastocyst stage was significantly greater for oocytes matured in the presence of fetal calf serum than for those matured in control medium or in the presence of up to 100 ng EGF ml⁻¹. These observations suggest that hMG, or some component of fetal calf serum, stimulates or favours cytoplasmic maturation of the oocyte, independent of its effect on nuclear maturation and oxidative metabolism of the oocyte, and cleavage of the early embryo. There were no differences in blastocyst metabolism of glucose or glutamine among the treatment groups, indicating that the maturation treatment had no long term effects on metabolic activity.

In conclusion, a physiological concentration of IGF-I combined with a near-physiological concentration of EGF stimulates cumulus expansion, oxidative metabolism and nuclear maturation of cattle oocytes *in vitro*. Maturation in the presence of EGF alone, or in combination with IGF-I, significantly increased the cleavage rate compared with the control treatment, but even the combined growth factor treatment was less effective than serum + hMG. Similarly, the number of blastocyst cells at day 7 and the blastocyst rate at day 8 were significantly greater in the serum + hMG group than for all other treatments. Although the experimental design does not permit a direct comparison, it is noteworthy that the effects of the maturation treatments on oocyte pyruvate metabolism generally paralleled their effects on cleavage and subsequent development. Maturation treatment with serum + hMG produced the greatest stimulation of pyruvate metabolism, and the highest rates of nuclear maturation, cleavage and development

to the blastocyst stage, and the greatest number of blastocyst cells. The effects of the growth factor treatments on these parameters were intermediate between the control treatment and treatment with serum + hMG. This finding suggests that the stimulation of mitochondrial function favours, or is predictive of, developmental potential. Further investigation is required to determine which component(s) of serum or gonadotrophin treatment is responsible for its effects on cleavage rate and number of blastocyst cells, and the exact relationship between oocyte oxidative metabolism and subsequent development.

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