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Role of Epidermal Growth Factor in Bovine Oocyte Maturation and Preimplantation Embryo Development In Vitro[1]

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ABSTRACT

Epidermal growth factor (EGF) has been shown to have a positive effect during in vitro maturation (IVM) and has been reported in follicular fluid at levels capable of stimulating meiosis in a variety of species. The aim of the present work was to study the effect on subsequent development of EGF present in defined medium during bovine 1) oocyte maturation or 2) embryo culture. The presence of EGF during IVM, irrespective of concentration (1, 10, 100 ng/ml), stimulated cumulus expansion and significantly increased the proportion of oocytes attaining metaphase II, the rate of cleavage, and the proportion of embryos reaching the 5- to 8-cell stage at 72 h postinsemination. Blastocyst rates on Days 7 and 9 were also significantly improved for oocytes matured in the presence of EGF (10% vs. 18–24% on Day 7 and 21% vs. 31–32% on Day 9, for Tissue Culture Medium 199 [M199] and M199 + EGF, respectively). The presence of fetal calf serum (FCS) during IVM resulted in similarly elevated rates of development. There was no cumulative effect when EGF and FCS were present together during IVM. The presence of EGF also altered the pattern of proteins neosynthesized during maturation. The maturation-promoting effect of EGF was evident for denuded oocytes also, suggesting that EGF may act, at least in part, directly on the oocyte. Immunofluorescence studies revealed the EGF receptor on immature cumulus-oocyte complexes. When present during postfertilization culture in defined medium (synthetic oviduct fluid), EGF stimulated development in comparison to that of the control but could not replace serum. The results suggest a physiological role for EGF in the regulation of bovine oocyte maturation and development.

INTRODUCTION

Sera included in media for in vitro maturation (IVM) frequently allow improvement in oocyte quality as assessed by developmental rates. Indeed, previous reports from our laboratory have clearly demonstrated that immature bovine oocytes matured in Tissue Culture Medium 199 (M199) supplemented with 10% fetal calf serum (FCS) are superior in terms of developmental competence to those matured in M199 alone [1]. The focus of our work is now to determine what factor(s) contained in sera are responsible for this effect.

Sera contain many components including hormones, trace elements, and growth factors. Among the latter, epidermal growth factor (EGF) has been implicated in development. In pigs, the reported serum EGF level is 8 ng/ml [2], and in humans it is 1.2–3.75 ng/ml [3]. EGF concentrations in follicular fluid (FF) vary from 2 to 15 ng/ml (human [4–6]; porcine [2]). There is a lack of data in the literature on FF EGF levels in ruminants. Indirect evidence for its presence comes from the study of Rose et al. [7], in which specific binding of EGF to bovine cumulus and small antral granulosa cells was strongly inhibited by FF from 2- to 5-mm follicles, suggesting that such FF contains EGF/EGF-like substances.

Binding sites for EGF have, in fact, been demonstrated in the ovaries of several species (bovine [8, 9]; porcine [10]; human [11, 12]; rat [13]). The number of EGF binding sites has been shown to be influenced by both gonadotropin and steroids [14–16], with the expression of EGF receptor (EGF-R) on granulosa cells being highest in the preovulatory follicle [16]. A role of gonadotropin in EGF-R regulation is further suggested by observations that EGF-R levels are higher during late proestrus and estrus than during diestrus [13].

EGF-Rs, visualized by immunofluorescence, are apparently present on bovine cumulus-oocyte complexes (COCs)/embryos at all stages of development (immature to blastocyst stage; F. Gandolfi, personal communication). Preimplantation embryos of the pig, cow, sheep, and pony bind EGF specifically [17–19]. Other authors have shown EGF binding, EGF-R, and/or EGF-R mRNA in mouse [20–24], pig [25], and rabbit [26, 27] embryos, suggesting a general biological role for the EGF-R in mammalian pre- and periimplantation development.

As to its role in the oocyte, EGF has been shown to have a positive effect during IVM in a variety of species (cattle [28–34]; pigs [35–39]; rodents [6, 16, 40–43]; humans [43, 44]).

That EGF may contribute to postfertilization embryonic development has been shown by Wood and Kaye [45], who reported that EGF stimulated total uptake of [^{3}H]-leucine into protein by mouse embryos cultured for 24 h from morulae, although no mitogenic effect was observed. Buyalos and Cai [46] noted that the percentage of fully expanded and hatched mouse blastocysts at 72 h was significantly higher

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after incubation with EGF (2–100 ng/ml). This effect was neutralized by coincubation with anti-EGF antibody. Kurachi et al. [47] described the expression of EGF/transforming growth factor α (TGFα) in human oviduct epithelium and the positive effect of coculture of mouse 2-cell embryos with human oviduct epithelial cells. Coculture significantly increased blastocyst formation, but the positive effect was completely abolished by addition of anti-EGF and/or anti-TGFα monoclonal neutralizing antibodies. These facts, coupled with the fact that EGF binding has been demonstrated on the surface of the oocyte/embryo, suggest that EGF may play a role in development.

Against this background, the objective of the present study was to examine the effect on subsequent development of EGF present in defined medium during bovine 1) oocyte maturation or 2) embryo culture. Also, differences in protein neosynthesis patterns in oocytes matured in the presence or absence of EGF were investigated by one-dimensional PAGE and compared to those observed when oocytes were matured in the presence of serum.

MATERIALS AND METHODS

EGF from mouse submaxillary glands was purchased from Sigma Chemical Company (St. Louis, MO; cat. #E-4127).

General Procedures

Oocyte recovery and IVM. COCs were obtained by aspiration of 2- to 6-mm follicles of ovaries from slaughtered cows. All oocytes completely surrounded by unexpanded cumulus cells were used; they were washed four times in modified PBS (supplemented with 36 mg/L pyruvate, gentamycin, and 0.5 mg/ml BSA; fraction V, cat. #A-9647; Sigma). Groups of up to 50 oocytes were transferred to 4-well plates (Nunc, Roskilde, Denmark) containing 500 μl of medium for 24-h maturation at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity. After maturation, cumulus expansion was visually assessed under a stereomicroscope. To assess nuclear maturational status, oocytes were denuded by repeated pipetting, fixed in ethanol:acetic acid (3:1), and stained with orcein.

For all maturation experiments, two controls were used: M199 (cat. #041–01150H; Life Sciences, Paisley, Scotland) alone as a lower defined control medium (with no additional additives), to which the test substance was added, and M199 supplemented with 10% (v:v) FCS (cat. #011–06180H; Life Sciences) as an upper control (see [1]).

For all culture experiments, maturation of oocytes took place in M199 + 10% FCS.

In vitro fertilization (IVF). After 24-h culture, COCs were washed four times in PBS and once in fertilization medium before being transferred in groups of up to 100 into 4-well plates. Each well contained 250 μl of fertilization medium (TALP [48], containing 10 μg heparin-sodium salt [167 U/mg; Calbiochem, San Diego, CA]/ml). Motile spermatozoa were obtained by centrifugation of frozen-thawed spermatozoa on a Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient (2 ml at 45% over 2 ml at 90%) for 20–30 min at 700 × *g* at room temperature. Viable spermatozoa, collected at the bottom of the 90% fraction, were washed in TALP and pelleted by centrifugation at 100 × *g* for 10 min at room temperature. Spermatozoa were counted in a hemocytometer and diluted in the appropriate volume of TALP to give a concentration of 4×10^6 spermatozoa/ml; 250 μl of this suspension was added to each fertilization well to obtain a final concentration of 2×10^6 spermatozoa/ml. Plates were then incubated for 20–24 h in 5% CO_2 in humidified air at 39°C.

In vitro culture (IVC). For all experiments, embryo culture took place in modified synthetic oviduct fluid medium (SOF) [49], in the presence or absence of BSA and FCS depending on experimental conditions (see below), under paraffin oil in a humidified atmosphere of 5% CO_2:5% O_2:90% N_2 at 39°C. Cumulus cells were removed from presumptive zygotes by vortexing for 2 min in 2 ml PBS. The zygotes were subsequently washed twice in PBS and twice in SOF before being transferred in groups of 20–30 to the culture droplets (1 zygote/μl medium). According to experimental conditions (see below), FCS was added to the droplets (10% [v:v]) 24 h after placement in culture (i.e., 48 h postinsemination [hpi]).

Cleavage was assessed at 48 h after placement in culture, i.e., at 72 hpi (% noncleaved, 2–4 cells, 5–8 cells). The number of embryos developing to at least the expanded blastocyst stage was assessed on Days 6–8 of culture. Hatching was recorded on Day 8 of culture and expressed as a percentage of Day 8 blastocysts. In the culture experiment (experiment 4), for the estimation of total cell numbers, Day 7 blastocysts were placed on a slide, air-dried, and fixed in ethanol (100%) for 24 h. They were subsequently colored using Hoechst 33342 (10 μg/ml in 2.3% sodium citrate) and visualized with an epifluorescence microscope (Zeiss, Oberkochen, Germany).

Experiment 1. To study the effect of addition of EGF during maturation on subsequent development, COCs were pooled and randomly allocated to one of five treatments: M199 alone, M199 + EGF at one of three concentrations (1, 10, 100 ng/ml), or M199 + 10% FCS.

Experiment 2. To see whether or not there was a cumulative effect of serum and EGF together during maturation, oocytes were matured in either M199 alone, M199 + 10 ng/ml EGF, M199 + 10% FCS, or M199 + 10 ng/ml EGF + 10% FCS.

For experiments 1 and 2, postfertilization culture took place in SOF + 3 mg/ml BSA (cat. #A-9647; Sigma) + 10% FCS added at Day 2 of culture.

Experiment 3. This experiment was carried out to determine whether the effect of EGF on meiotic maturation was due to a direct action on the oocyte or to an action mediated via the cumulus cells. Thus, after recovery of COCs from the ovaries, half were denuded by gentle vortexing, and both denuded and intact COCs were then cultured for 24 h in either M199 alone, M199 + 10 ng/ml EGF, or M199 + 10% FCS.

Experiment 4. To study the effect of EGF on postfertilization development alone, presumptive zygotes, after IVM in M199 + 10% FCS and IVF, were pooled and randomly allocated to one of five treatment groups: SOF alone, SOF + EGF at one of three concentrations (1, 10, 100 ng/ml), or SOF + BSA + FCS. This experiment was conducted in two different laboratories under identical conditions (4 replicates in one, 5 in the other). As the same trends were observed for both sets of data, results were pooled and are given as the mean of nine replicates.

Experiment 5: Immunofluorescence. Rabbit polyclonal antibody (Ab-4) against human EGF-R (residues 1005–1016) was obtained from Oncogene Science Inc. (Uniondale, NY; cat. #PC19). For visualization of the EGF-R, COCs were fixed for 30 min in 3% paraformaldehyde. They were subsequently washed several times in PBS + 0.1% polyvinylpyrrolidone and then preincubated with goat serum (1:10) for 1 h to block nonspecific binding of IgG. They were next incubated with the primary antibody for 1 h (1:10). After several washes as described above, oocytes were incubated with the second antibody (goat-anti rabbit IgG-fluorescein isothiocyanate; cat. #B12107; Bio Sys, Compiegne, France) for 1 h (1:400). All incubations took place at ambient temperature. As a negative control, oocytes were incubated in nonimmune rabbit serum. After mounting, slides were stored in the dark at 4°C until observation.

Experiment 6: Radiolabeling and electrophoresis. Approximately 21 h after the initiation of maturation, COCs were removed from the maturation medium, washed twice in PBS, and incubated for 3 h in PBS supplemented with 1 mCi/ml [^{35}S]-methionine (Express Protein Labeling Mix; NEN, Boston, MA) at a ratio of 25–50 oocytes per 100 μl in a closed Eppendorf at 39°C (Kastrop et al. [60]). For the groups matured in the presence of 10% FCS, 10 ng/ml EGF, or both, the labeling medium was supplemented with 1% FCS, 1 ng/ml EGF, or both, respectively. Following the labeling period, cumulus cells were removed by repeated pipetting, and oocytes were washed twice in PBS (without BSA) and lysed individually or in groups of 10 in 15 μl of sample buffer [50]. Samples were boiled for 3 min and stored at −20°C until electrophoresis.

Thawed samples were centrifuged (13 000 × *g* for 5 min) and analyzed on SDS-PAGE homogenous slab mini gels (T = 10%; C = 2.6%) individually or in groups of 10. Gels were treated with Amplify (Amersham, Arlington Heights, IL) for 30 min, dried, and exposed for 7 days (Hyperfilm-MP; Amersham) at room temperature. Cumulus cells were pelleted (500 × *g* for 10 min), washed twice in PBS (without BSA), and lysed in sample buffer (15 μl per 10 cumulus complexes). Samples were treated in the same way as oocytes. The equivalent of 10 cumulus complexes were loaded on each lane.

Statistical Analysis

Raw data were examined with use of chi-square analysis or Fisher's exact test where appropriate and Student's *t*-test. The overall chi-square was calculated and found to be significant each time before the Fisher's exact test was performed to detect differences between treatment groups. A *p* value greater than 0.05 was considered significant.

RESULTS

Experiment 1

Results for the first experiment are presented in Table 1. Addition of EGF, irrespective of concentration, or 10% FCS to M199 stimulated cumulus expansion (Fig. 1) as well as significantly increasing the proportion of oocytes attaining metaphase II. EGF, at all concentrations studied, significantly increased the cleavage rate above that obtained with either M199 or M199 + FCS. There were significantly more embryos at the 5- to 8-cell stage at 72 hpi when maturation took place in the presence of EGF (all concentrations) or 10% FCS as compared to maturation in M199 alone. EGF at all concentrations significantly increased Day 6 blastocyst yield over that obtained with M199 alone, and EGF at 10 or 100 ng/ml significantly increased Day 6 yield over that seen with M199 + 10% FCS. Oocytes matured in M199 showed a significantly lower Day 8 blastocyst yield than those matured in all other treatments. When calculated over oocytes cleaved, only M199 and M199 + FCS were significantly different in terms of Day 8 blastocyst yield (34% vs. 45%), with M199 + EGF being intermediate irrespective of concentration. EGF at 10 ng/ml significantly increased the hatching rate over that obtained with M199 alone.

Experiment 2

Results are presented in Table 2. The trend for cleavage rate in experiment 2 was similar to that described for experiment 1; values obtained with M199 were lower than with all other treatments. Differences were not, however, significant, perhaps because the numbers were smaller. Maturation in the presence of FCS, EGF, or both increased the proportion of embryos at the 5- to 8-cell stage at 72 hpi,

FIG. 1. Bovine COCs (**A**) before maturation; after maturation for 24 h in vitro (**B**) in M199 alone, (**C**) in M199 + 10% FCS, (**D**) in M199 + 1 ng/ml EGF, (**E**) in M199 + 10% FCS + 10 ng/ml EGF; (**F**) after maturation in vivo, for comparison. × 100.

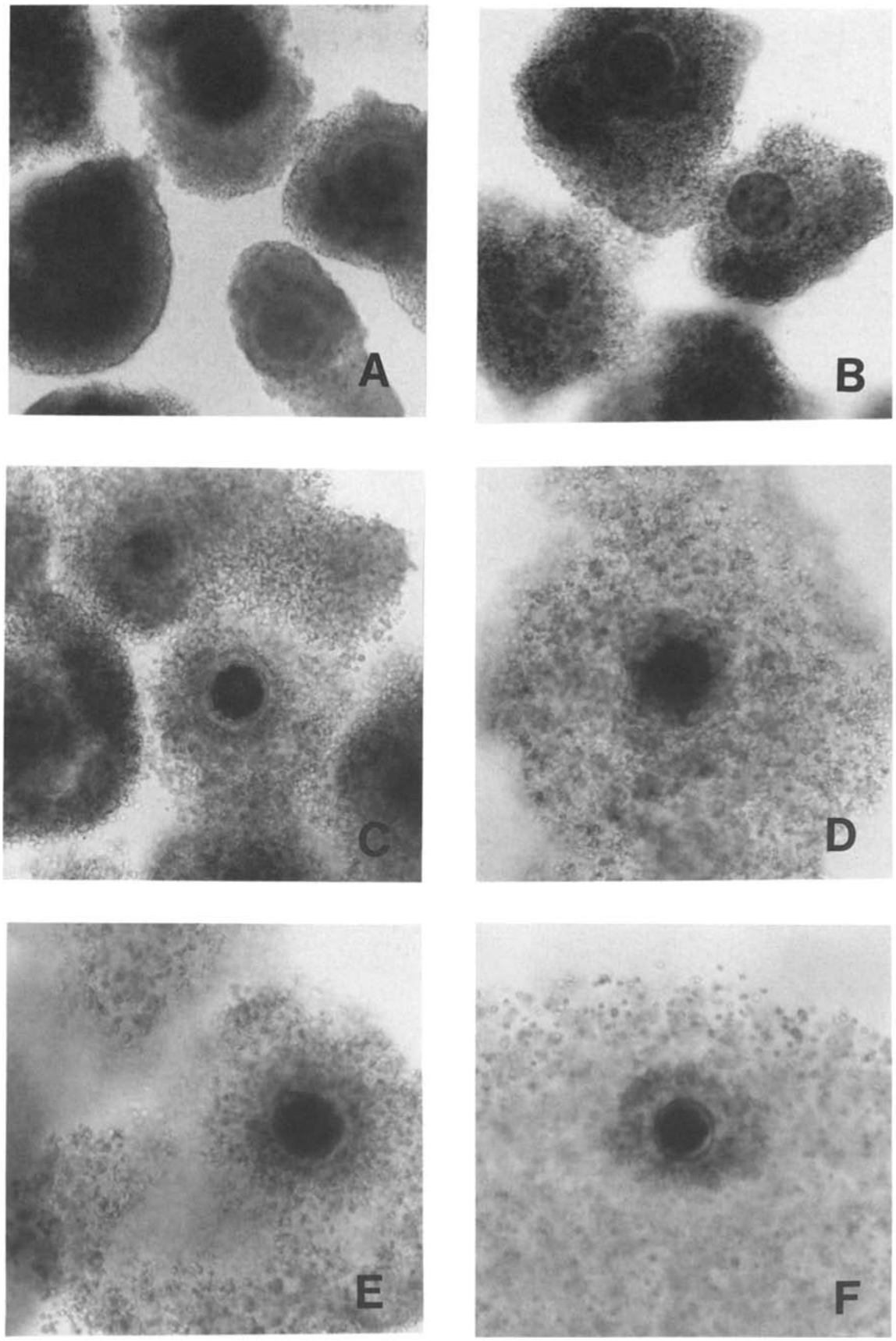

TABLE 1. Effect of EGF during IVM of bovine embryos.*

Treatment	Oocytes reaching Metaphase II		IVM/IVF Oocytes	Cleaved		5–8 Cell		Blastocyst yield					Hatching rate†	
								Day 6		Day 8		Day 8‡		
	No.	(%)	(n)	No.	(%)	No.	(%)	No	(%)	No.	(%)	(%)	No.	(%)
M199	46/72[a]	(64)	247	157[ac]	(64)	99[a]	(40)	24[a]	(10)	53[a]	(21)	(34)[a]	20[a]	(38)
+EGF														
1 ng/ml	49/55[b]	(89)	342	277[b]	(81)	197[b]	(58)	60[bc]	(18)	107[b]	(31)	(39)[ab]	56[ab]	(52)
10 ng/ml	32/33[b]	(97)	349	280[b]	(80)	203[b]	(58)	79[b]	(23)	109[b]	(31)	(39)[ab]	61[b]	(56)
100 ng/ml	50/55[b]	(91)	317	253[b]	(80)	177[b]	(56)	76[b]	(24)	102[b]	(32)	(40)[ab]	44[ab]	(43)
M199+FCS (Ctrl)	48/55[b]	(87)	290	208[c]	(72)	142[b]	(49)	44[ac]	(15)	93[b]	(32)	(45)[b]	44[ab]	(47)

*Mean of 4 replicates.
†As a percentage of Day 8 blastocysts.
‡As a percentage of oocytes cleaved.
[a,b,c]Values in the same column with different superscripts differ significantly ($p < 0.05$).

this effect being significant in the case of FCS (44% vs. 56%). Oocytes matured in M199 alone yielded a significantly lower Day 6 blastocyst rate compared to oocytes matured in all other treatments, in agreement with the results of experiment 1. There was no evidence of an additive effect of EGF and FCS. Similarly, addition of FCS, EGF, or both to M199 increased the blastocyst yield on Day 8. There was, however, no additive effect of both EGF and FCS. No differences in hatching rate were observed.

Experiment 3

Figure 2 presents the results for the third experiment. With respect to the effect of EGF on the proportion of oocytes reaching metaphase II, the findings were similar to those for experiment 1. Addition of EGF to M199 significantly increased the proportion of oocytes extruding the first polar body after 24 h of culture. This effect was the same for both denuded and cumulus-intact oocytes. All COCs exhibiting a polar body, irrespective of culture conditions, also exhibited a normal metaphase plate, as did denuded oocytes matured in the presence of FCS. Interestingly, however, of the denuded oocytes matured either in M199 alone or in M199+EGF that extruded a polar body, approximately 50% were still at telophase.

Experiment 4

Results for experiment 4 are presented in Table 3. There was no significant difference in cleavage rate or in the percentage of embryos at the 5- to 8-cell stage at 72 hpi. While culture in the presence of EGF increased blastocyst yields on Days 6 and 7, the effect was not as marked as that seen in the presence of BSA and FCS. EGF had no effect on blastocyst cell number. Culture in SOF + BSA + FCS yielded blastocysts with significantly higher total cell numbers than all other treatments.

Experiment 5

A representative example of results are shown in Figure 3. More than 20 COCs were analyzed, and all were positive for EGF-R by indirect immunofluorescence.

Experiment 6

Figure 4A shows the effect that the presence of FCS, EGF, or both in the maturation medium (M199) had on the oocyte protein neosynthesis pattern. The intensity of the 45-kDa band was reduced in the three treatment groups compared to the control. A band of 48 kDa was reduced in the EGF and EGF+FCS groups whereas bands of 46 and 47 kDa appeared in the presence of EGF. None of these bands were present in FCS, and only the 47-kDa band appeared weakly in the EGF+FCS group. These observations were repeated in 4 different groups of 10 oocytes coming from 3 different

TABLE 2. Effect of EGF and/or FCS during IVM of bovine embryos.*

Treatment	(n)	Cleaved		5–8 Cell		Blastocyst yield					Hatching rate†	
						Day 6		Day 8		Day 8‡		
		No.	(%)	No.	(%)	No.	(%)	No.	(%)	(%)	No.	(%)
M199	171	117	(68)	75[a]	(44)	26[a]	(15)	37[a]	(22)	(32)[a]	18	(49)
+FCS (10%)	160	121	(76)	90[b]	(56)	46[b]	(29)	58[b]	(36)	(48)[b]	34	(59)
+EGF (10 ng/ml)	174	127	(73)	90[ab]	(52)	41[b]	(24)	52[ab]	(30)	(41)[ab]	31	(60)
+FCS+EGF	176	132	(75)	93[ab]	(53)	45[b]	(26)	56[b]	(32)	(42)[ab]	33	(59)

*Mean of 3 replicates.
†As a percentage of Day 8 blastocysts.
‡As a percentage of oocytes cleaved.
[a,b]Values in the same column with different superscripts differ significantly ($p < 0.05$).

TABLE 3. Effect of EGF during culture of bovine embryos in vitro.*

		Cleaved		5–8 Cell		Blastocyst yield					Hatching Rate†		Cell number	
						Day 6		Day 7		Day 7‡				
Treatment	(n)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	(%)	No.	(%)	mean ± SD	(n)
SOF	254	199	(78)	138	(54)	29[a]	(11)	60[a]	(24)	(30)[a]	5	(8)	97 ± 28[a]	(58)
+EGF														
1 ng/ml	278	219	(79)	155	(56)	53[b]	(19)	91[b]	(33)	(42)[b]	13	(14)	96 ± 26[a]	(79)
10 ng/ml	259	210	(82)	147	(57)	39[ab]	(15)	78[ab]	(30)	(37)[ab]	11	(14)	98 ± 29[a]	(75)
100 ng/ml	274	213	(78)	161	(59)	36[ab]	(13)	87[b]	(32)	(41)[b]	10	(11)	100 ± 33[a]	(66)
SOF+BSA+FCS	296	235	(79)	172	(58)	103[c]	(35)	111[b]	(37)	(47)[b]	27	(24)	117 ± 44[b]	(81)

*Mean of 9 replicates.
†As a percentage of Day 8 blastocysts.
‡As a percentage of oocytes cleaved.
[a,b]Values in the same column with different superscripts differ significantly ($p < 0.05$).

ovary batches, as well as in 5 oocytes analyzed individually (not shown). None of these changes were noted in cumulus cell proteins (Fig. 4B).

DISCUSSION

In terms of blastocysts produced, the most successful bovine IVM systems have employed bovine serum to optimize oocyte developmental capacity. As pointed out by Harper and Brackett [30], EGF in serum is possibly one of the undetermined components contributing to enhanced oocyte maturation.

The experiments described here were performed in the absence of any protein other than EGF. This was a deliberate choice made in order to exclude possible confounding factors introduced by BSA and/or serum. It could be argued that the EGF was used by the oocytes/embryos as a source of protein, with resulting alterations in metabolism and development. However, in the study of Wood and Kaye [45], the addition of equimolar BSA to the culture medium failed

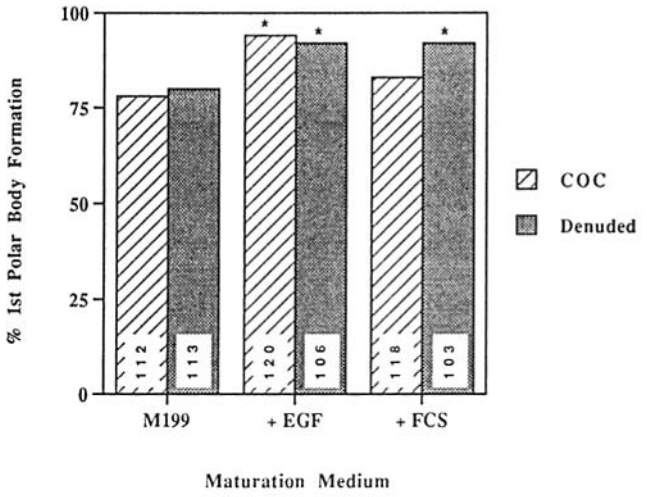

FIG. 2. Effect of maturation medium on the proportion of oocytes extruding the first polar body following IVM for 24 h. Asterisk indicates a significant difference ($p < 0.05$) from the control treatment (M199) for denuded and cumulus-enclosed oocytes. Four replicates were performed. The total numbers of oocytes in each group are given in the graph.

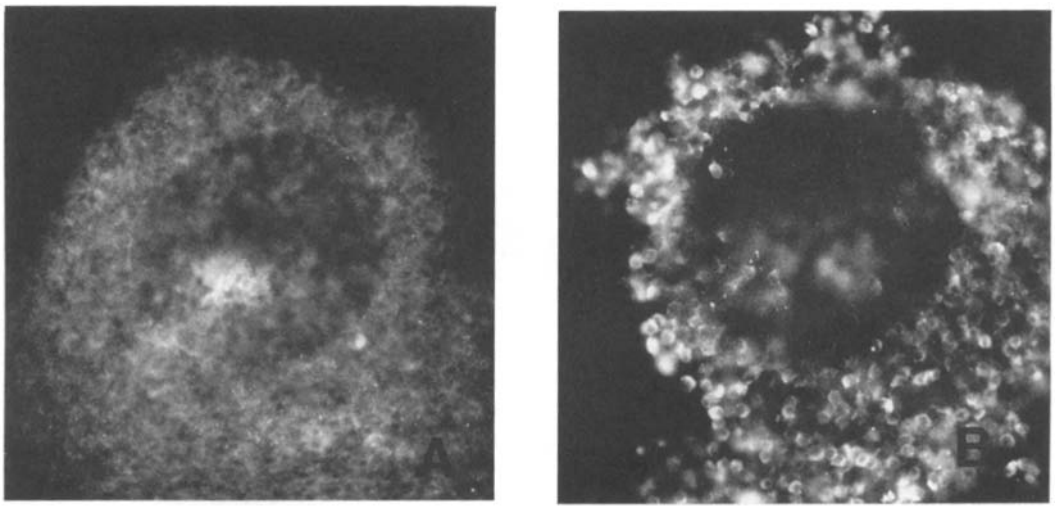

FIG. 3. Visualization of EGF-R by indirect immunofluorescence. **A**) Control; **B**) COC showing positive labeling. ×186

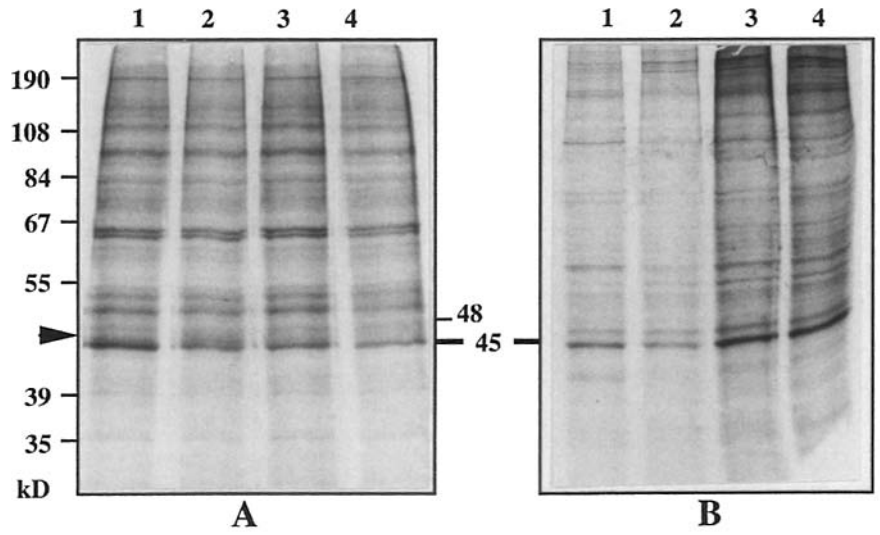

FIG. 4. SDS-PAGE analysis of protein neosynthesis in cattle oocytes labeled after IVM in M199 alone (lane 1), M199 + 10 ng/ml EGF (lane 2), M199 + 10% FCS (lane 3), and M199 + 10 ng/ml EGF + 10% FCS (lane 4). The arrowhead indicates the position of 46-, 47-kDa bands activated in the EGF group. Positions of molecular mass markers (SDS-7B; Sigma) are indicated on the left. **A**) Groups of 10 oocytes; **B**) cumulus cells from 10 oocytes.

to evoke the effect on embryonic protein synthesis observed with EGF. Also, in the study of Buyalos and Cai [46], the positive effect of EGF on embryonic development was shown to be due solely to specific mitogenic effects of EGF as evidenced through use of anti-EGF antibody. In view of these findings, we conclude that the observed effects of EGF are from its binding to specific receptors, known to be present (current study; F. Gandolfi, personal communication), and not from nonspecific nutritive effects on the oocytes/embryos.

The present data demonstrate that supplementation of M199 with EGF alone during IVM at physiological concentrations stimulates cumulus cell expansion and improves the percentage of oocytes undergoing nuclear maturation as well as the proportion of embryos attaining the blastocyst stage. All levels of EGF tested provided similar results. EGF also altered the pattern of proteins neosynthesized during IVM. These findings would suggest a physiological role for EGF/EGF-like molecules in the intrafollicular regulation of oocyte maturation. In support of this, Das et al. [6], testing the specific contribution of the EGF component of human FF on oocyte maturation using an in vitro mouse model, found that the stimulatory effect of FF was lost after extraction of FF EGF by immunoprecipitation. This was reversible by addition of 5 ng/ml EGF back to the FF.

It has been reported that the major site of action of growth factors that regulate oocyte maturation is the cumulus cells [37, 51, 52]. The present study provides the first direct evidence of a maturation-promoting effect of EGF on denuded bovine oocytes. Similar evidence has been obtained in the mouse [43]. EGF was found to stimulate polar body formation to a similar extent in denuded and cumulus-enclosed bovine oocytes. This would suggest that the effect of EGF may be, in part, independent of the cumulus cells. As pointed out by Das et al. [43], several lines of evidence support this possibility: EGF-R-associated tyrosine kinase activation triggers meiosis in *Xenopus* oocytes [53]; Müllerian inhibitory factor inhibits meiosis in denuded rat oocytes by an action on tyrosine kinase, an effect reversed by EGF [41]; the meiosis-stimulating effect of EGF on the mouse COC is associated with minimal changes in cAMP production and is independent of cumulus expansion [42].

Relatively few reports have been published on the maturation of denuded bovine oocytes [31, 54–57]. Many of these reports refer to oocytes found to be nude on aspiration and not to normal COCs that were subsequently denuded as in the present study. Such oocytes are probably inherently less competent. Lorenzo et al. [30] reported a significant improvement over control values when bovine COCs were matured in the presence of EGF, insulin-like growth factor-I, or both. This effect was not apparent for denuded oocytes. However, their denuded group consisted of oocytes nude on aspiration, as mentioned above. Also, the maturation rate in their control cumulus-enclosed oocytes was extremely low (35%). Dominiko and First [57] reported that a cumulus monolayer can support polar body formation in denuded oocytes and that presence of a polar body can be indicative of developmental potential. Zhang et al. [58] reported a metaphase II rate of 4–26% (without and with coculture) for bovine oocytes denuded before culture. Why such a low rate was observed is unclear, but the reason may be related to the method of cumulus cell removal. The authors reported that most denuded oocytes were degenerate after culture. In our experience, when oo-

cytes were denuded by repeated vigorous pipetting, many were degenerate after culture. However, after cumulus removal by gentle vortexing, the vast majority remained viable. As mentioned above, a certain proportion of denuded oocytes matured in the absence of serum were at telophase at 24 h with the polar body extruded. This is in agreement with the study of Homa [59]. Whether this finding was due to a modification of the kinetics of metaphase II attainment or modifications in the oocyte is now under study.

EGF has been reported to induce cumulus expansion independent of its effect on oocyte meiosis [42]. This is in agreement with the present study. Indeed, a previous report from our laboratory [1] has shown that FSH, which stimulates cumulus expansion to a degree similar to that observed with EGF, does not improve subsequent development. It is clear that while perhaps not physiological, cumulus expansion is not necessary for normal development. This would suggest that EGF imparts competence in some other fashion and would be consistent with the idea that EGF acts directly on the oocyte in addition to its action on the cumulus.

More indirect evidence for a possible direct effect on the oocyte comes from our investigation of the protein synthesis patterns of oocytes matured in the presence or absence of EGF. We have investigated whether the degree of development following a particular IVM treatment could be confirmed at the level of protein synthetic capacity. The differences between immature (not shown) and in vitro-matured oocytes revealed changes in protein synthesis during oocyte maturation, in agreement with previous studies [60]. More interestingly, these modifications differed for the different IVM treatments. No changes were noted in the protein synthetic patterns of the cumulus cells originating from these oocytes. Although this pattern of protein synthesis observed at 21 h does not exclude a role of a cumulus-mediated effect earlier during maturation, it would suggest again that the oocyte may be the target of EGF. Whether these changes in protein synthesis are linked to competence remains unknown. Analysis of these proteins by two-dimensional SDS-PAGE is ongoing in our laboratory.

There are relatively fewer reports in the literature on the effect of EGF on bovine embryo culture. Keefer et al. [61] reported that EGF stimulated hatching in bovine embryos cultured singly from the 8-cell stage, but had no effect on blastocyst development. In the study of Flood et al. [62], of all the growth factors tested, only EGF had a stimulatory effect in terms of blastocyst rate, although it was not significant. Lee and Fukui [63] reported that transient exposure of morulae/early blastocysts improved later development, although this effect was not evident for earlier embryonic stages. Here we have demonstrated a stimulatory effect of EGF on postfertilization bovine embryonic development independent of that observed when the EGF is added during IVM. At a concentration as low as 1 ng/ml, the presence of EGF induced a significantly greater proportion of 1-cell presumptive zygotes to develop to the blastocyst stage by Day 7 of culture. There was no effect of EGF on blastocyst cell number, which is in agreement with the findings of Yang et al. [64] but in contrast to those of Lee and Fukui [63]. Our results are consistent with studies in the mouse reporting that EGF caused a more rapid accumulation of blastocoel fluid [65] as well as an increased rate of protein synthesis [45] and blastocyst development and hatching [22, 46].

In order to address the question of the half-life of the compound, a separate experiment was carried out in which the culture medium was renewed every 48 h (results not shown). No improvement in development was noted.

Recently, Chia et al. [66] described the concurrent expression of EGF, TGFα, and EGF-R in unfertilized human eggs and early embryos. This is in contrast to the situation in the mouse, cow, pig, and sheep, where EGF is apparently absent throughout preimplantation development [25, 67–69]. Nor was EGF found in bovine oviduct cells in vitro [68]. However, Pohland and Tiemann [9], using immunohistochemistry, detected immunoreactive EGF and EGF-R in the bovine ovary, oviduct, and uterus. In the ovary, important concentrations of EGF were found in theca externa cells of antral follicles. The intensity of staining was decreased in granulosa and theca interna cells. EGF-R was detected in granulosa cells of antral follicles. Porcine oviduct fluid contains significant amounts of EGF [70], and porcine oviduct cells secrete EGF in culture [71].

Of the other ligands that bind to the extracellular domain of the EGF-R (TGFα, amphiregulin, vaccinia virus growth factor, Schwannoma-derived growth factor) [72], TGFα has been shown to stimulate oocyte maturation in rats and mice [52, 73], cattle [32], and pigs [37]. TGFα is present in the mouse in eggs and later from the 8-cell stage [67]. In cattle, transcripts for TGFα have been detected from the 1-cell to the blastocyst stage and in oviduct cells [68].

Does EGF have a role in vivo in cattle? The action of EGF on oocyte maturation as shown here supports the notion of a role in vivo for it or for an EGF-like substance. It is possible that EGF acts in the follicle in an endocrine fashion, as it has been reported in serum and FF of other species at concentrations high enough to affect meiosis. It has been suggested that FF EGF may represent a passive diffusion into the follicle from the circulation [3]. It may also be produced locally and act in an autocrine/paracrine manner in the developing follicle. Evidence for the latter stems from the facts that 1) growth factors have been produced by cultured ovarian cells; 2) FF contains significant amounts of EGF; 3) there are binding sites for EGF in the ovary [9] that flux in relation to the maturational state of the follicle; and 4) EGF has a well-established mitogenic effect on cultured granulosa cells and dramatically modifies their response to other hormones [74]. Thus, while EGF may act alone, it could interact with other gonadotropins, steroids, and growth factors to affect maturation.

In conclusion, the present study demonstrates that EGF alone present during IVM is capable of increasing the proportion of oocytes reaching the blastocyst stage following IVF and IVC. Although some of this effect is undoubtedly due to an increased proportion of oocytes reaching metaphase II in its presence, a role in the improvement of cytoplasmic maturation cannot be excluded, since even when corrected for cleavage rate, the data indicate an improved blastocyst yield in the presence of EGF. While EGF was also shown to have a stimulatory effect postfertilization, it could not replace serum. The results suggest that EGF might be one of the major follicular factors responsible for stimulating oocyte cytoplasmic as well as nuclear maturation.

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