# Effects of Epidermal Growth Factor, Insulin Like Growth Factor-I and Insulin on Meiotic Maturation of Bovine Denuded Oocytes

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**Abstract:** *This study was carried out to examine whether EGF, IGF-I and insulin could directly modify the meiotic maturation of bovine denuded oocytes in vitro, and to see whether the stimulatory effect of EGF on bovine oocyte meiotic maturation is mediated through the tyrosine kinase pathway. In the first three experiments, cumulus-oocyte complexes (COCs) were removed from their cumulus cells and the denuded oocytes were cultured in vitro with various concentrations of EGF, IGF-I and insulin. Nuclear maturation was examined after 24 h of culture. EGF, IGF-I as well as insulin effectively increased the proportion of oocytes developed to the metaphase II stage. The higher rates of MII oocytes were achieved with 50 ng/ml EGF (69.4%), 100 ng/ml IGF-I (66.1%) or 5 μg/ml insulin (63.1%) compared to about 48 to 54% in the controls. In experiment 4, whereas EGF significantly ($p<0.05$) increased the proportion of MII oocytes for denuded oocytes derived from the collected COCs, it had no stimulatory effect on MII formation of oocytes found cumulus-free or denuded at the time of aspiration. In experiment 5, with erbstatin, a specific tyrosine kinase inhibitor, the stimulatory effect of EGF on meiotic maturation of denuded oocytes was abolished. These results indicate that EGF, IGF-I and insulin could directly stimulate meiotic maturation of bovine denuded oocytes removed from the surrounding cumulus cells after oocyte collection. Moreover, the results also suggest that the stimulatory effect of EGF on bovine denuded oocyte meiotic maturation is mediated by the tyrosine kinase signal transduction pathway.*

**Key words:** *EGF, IGF-I, Insulin, Meiotic maturation, Bovine oocytes.*

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Several growth factors have been demonstrated to be synthesized in the ovary and to have some effects on ovarian cell function such as follicular development and proliferation of the ovarian follicle cells [1–3]. Among these growth factors, epidermal growth factor (EGF) has been shown to stimulate the in vitro maturation of cattle [4–6], pig [7, 8], mouse [9, 10], rat [11] and rabbit [12] oocytes. The stimulatory effect of IGF-I on the *in vitro* maturation of cattle [5], pig [8] and rabbit [12] oocytes has also been reported. Several reports have suggested that growth factors act through the surrounding cumulus cells to enhance oocyte maturation [5, 11–14]. We have recently found that wortmannin, a specific phosphatidylinositol (PI) 3-kinase inhibitor, blocks meiotic maturation of cumulus-oocyte coplexes as well as denuded bovine oocytes in vitro, suggesting that PI 3-kinase in the oocyte itself is involved in the process of meiotic maturation [15]. Since PI 3-kinase is an enzyme that is essentially involved with growth factors signal transduction, the present study was conducted to evaluate the effects of EGF, IGF-I and insulin, which are known to activate PI 3-kinase in somatic cells [16, 17] on meiotic maturation of bovine denuded oocytes, and to investigate whether the effect of EGF on meiotic maturation of bovine denuded oocytes is mediated by the tyrosine kinase pathway.

## Materials and Methods

### *Oocyte recovery and culture*

Bovine oocytes were obtained by aspiration of 2–5 mm follicles of ovaries collected from a slaughterhouse. Only oocytes surrounded with compact unexpanded cumulus cells and with homogeneous ooplasm were recovered under a stereomicroscope and placed in a watch glass containing Dulbecco's phosphate buffered

saline (PBS) supplemented with 0.05% polyvinyl pyrrolidone (PVP) (Sigma, Lot 93H03175, St. Louis, MO) and 100 μg/ml kanamycin. Unless otherwise mentioned, all denuded oocytes used in this study were prepared by gentle vortexing of the recovered cumulus-oocyte complexes (COCs) for 1 min in an Eppendorf tube containing 0.5 ml TCM-199 supplemented with 0.1% hyaluronidase, and the remaining cumulus cells were mechanically removed. Ten to 20 denuded oocytes were transferred to 100 μl droplets of culture medium covered with mineral oil (Sigma) in a 35-mm polystyrene culture dish (Falcon Plastics, no. 1008, Becton Dickinson and Company, Lincoln Park, NJ). The basic culture medium was TCM-199 with Earle's salts (Gibco BRL, Grand Island, NY) supplemented with 0.3% PVP, 0.2 mM Na-pyruvate (Sigma) and 50 μg/ml gentamicin. Culture was carried out for 24 h in a humidified atmosphere of 5% $CO_2$ in air at 39°C. To assess the stage of meiosis, the cultured denuded oocytes were fixed for 48 h in acetic acid and ethanol (1:3), stained with 1% lacmoid in 45% acetic acid, and examined under a phase-contrast microscope. Oocytes were classified as being at the germinal vesicle (GV), prometaphase I (PMI), metaphase I (MI), anaphase I (AI), telophase I (TI) or metaphase II (MII) stage. The statistical analysis showed no effect of the treatment on the proportion of degenerated oocytes (ranged from 1.7 to 3%, 2.1 to 4.8%, 1.8 to 3.9%, 1.8 to 5% and 2.1 to 3.9% in Experiments 1, 2, 3, 4 and 5, respectively), and therefore these oocytes were not included in the calculations.

*Preparation of erbstatin*

An Erbstatin analoge, Methyl-2,5-dihydroxycinnamate (ICN Biomedicals, Lot. 88760, Aurora, Ohio) was used as tyrosine kinase inhibitor. The drug was first dissolved in dimethyl sulfoxide (DMSO) (Sigma) to 100 mM and kept at −4°C. The solution was diluted again in DMSO and readjusted to the required concentration with the culture medium. The final DMSO concentration in the culture medium was 0.049%. For all experiments, a medium containing the corresponding volume of DMSO was included and served as a control.

*Experimental design*

In the first experiment, the effect of EGF on meiotic maturation of bovine denuded oocytes was examined. Denuded oocytes were cultured in culture medium supplemented with 0 (control), 1, 10, 50 and 100 ng/ml EGF (E-4127, from mouse submaxillary glands, Sigma, Lot. 065H8821, St. Louis, MO). At the end of the culture period the oocytes were examined for the stage of meiotic maturation.

In the second Experiment, the effect of the addition of IGF-I to the culture medium on meiotic maturation of bovine denuded oocytes was examined. Denuded oocytes were matured in the presence of 0 (control), 50, 100 and 200 ng/ml IGF-I (I-3769, Human, Recombinant, Sigma, Lot. 056H6696. St. Louis, MO) before being examined for meiotic progression.

Experiment 3 was carried out to examine the effect of insulin on meiotic maturation of bovine denuded oocytes. Denuded oocytes were cultured in a medium supplemented with 0 (control), 1, 5 and 25 μg/ml insulin (I-5500, from bovine pancreas, Sigma, Lot. 125H0097. St. Louis, MO).

Experiment 4 was conducted to compare the effect of EGF on meiotic maturation of bovine denuded oocytes which have been derived from the collected COCs (COC-DO) to that on those found denuded at the time of oocyte aspiration (DO). Both types of cumulus-free oocytes were cultured with or without 50 ng/ml EGF.

In Experiment 5, to examine whether the stimulatory effect of EGF on oocyte meiotic maturation is mediated by the tyrosine kinase pathway, denuded oocytes were cultured in the following treatments: 50 ng/ml EGF, 50 ng/ml EGF+ 20 μM erbstatin, 50 ng/ml EGF+ 30 μM erbstatin or without any treatment (control).

*Statistical analysis*

Results are presented as the mean percentages ± SEM. Data were statistically compared by chi-square test, and a probability level of $p<0.05$ was considered significant.

## Results

Data from Experiment 1 (Table 1) showed that EGF increased the proportion of denuded oocytes which developed to MII. At 1 ng/ml, EGF had no positive effect. The percentage of MII oocytes was significantly ($P<0.05$) higher in the presence of 50 ng/ml EGF than the control. With 100 ng/ml EGF, the rate of MII formation was relatively lower than that with 50 ng/ml but remained higher than the control. With 50 ng/ml EGF, a significantly smaller number of oocytes remained at the PMI and MI stages compared to the control.

Results of Experiment 2 are shown in Table 2. There was no significant difference between any IGF-I treatment and the control regarding the number of oocytes which remained at the PMI and MI stages. At both 100 and 200 ng/ml, IGF-I significantly ($P<0.05$) increased the rate of MII oocytes than the control. No significant

**Table 1.** Meiotic maturation of bovine denuded oocytes cultured *in vitro* with EGF for 24 h

| Conc. of EGF (ng/ml) | No. of oocytes | GV[1)] | PMI, MI[1)] | AI, TI[1)] | MII[1)] |
|---|---|---|---|---|---|
| 0 | 139 | 2.9 ± 0.4 | 34.5 ± 1.9[a] | 8.6 ± 0.2[a] | 53.9 ± 1.9[a] |
| 1 | 107 | 2.8 ± 1.9 | 28.9 ± 0.5[ab] | 12.2 ± 1.3[a] | 56.1 ± 1.3[a] |
| 10 | 119 | 3.3 ± 0.6 | 26.1 ± 1.1[ab] | 5.9 ± 0.5[a] | 64.7 ± 0.9[ab] |
| 50 | 124 | 1.6 ± 0.8 | 20.9 ± 0.7[b] | 8.1 ± 0.4[a] | 69.4 ± 0.5[b] |
| 100 | 100 | 4.0 ± 1.6 | 23.0 ± 0.9[ab] | 11.0 ± 1.1[a] | 62.0 ± 1.4[ab] |

[1)] Values are mean percentages ± SEM. [a,b] Different superscripts within the same column are significantly different (P<0.05). Four replicates of each concentration were done.

**Table 2.** Meiotic maturation of bovine denuded oocytes cultured *in vitro* with IGF-I for 24 h

| Conc. of IGF-I (ng/ml) | No. of oocytes | GV[1)] | PMI, MI[1)] | AI, TI[1)] | MII[1)] |
|---|---|---|---|---|---|
| 0 | 98 | 4.1 ± 0.5 | 32.7 ± 1.4[a] | 12.2 ± 1.3[a] | 51.0 ± 0.6[a] |
| 50 | 80 | 1.2 ± 1.3 | 23.8 ± 0.8[a] | 12.5 ± 0.5[a] | 62.5 ± 1.2[ab] |
| 100 | 109 | 3.6 ± 2.0 | 22.0 ± 2.6[a] | 8.3 ± 1.9[a] | 66.1 ± 1.3[b] |
| 200 | 96 | 2.1 ± 1.1 | 22.9 ± 1.3[a] | 9.4 ± 0.5[a] | 65.6 ± 1.8[b] |

[1)] Values are mean percentages ± SEM. [a,b] Different superscripts within the same column are significantly different (P<0.05). Three replicates of each concentration were done.

**Table 3.** Meiotic maturation of bovine denuded oocytes cultured *in vitro* with insulin for 24 h

| Conc. of insulin (μg/ml) | No. of oocytes | GV[1)] | PMI, MI[1)] | AI, TI[1)] | MII[1)] |
|---|---|---|---|---|---|
| 0 | 92 | 2.2 ± 1.2 | 40.2 ± 1.3[a] | 8.7 ± 0.4[a] | 48.9 ± 0.9[a] |
| 1 | 127 | 3.9 ± 0.6 | 27.6 ± 1.6[b] | 11.8 ± 1.9[a] | 56.7 ± 1.0[ab] |
| 5 | 103 | 3.9 ± 1.7 | 23.3 ± 1.1[b] | 9.7 ± 0.8[a] | 63.1 ± 0.6[b] |
| 25 | 114 | 3.5 ± 0.7 | 26.3 ± 0.6[b] | 9.7 ± 1.5[a] | 60.5 ± 0.8[ab] |

[1)] Values are mean percentages ± SEM. [a,b] Different superscripts within the same column are significantly different (P<0.05). Three replicates of each concentration were done.

differences between all IGF-I treatments were recorded in the percentages of MII oocytes.

Data of Experiment 3 (Table 3) revealed that insulin, at all levels tested, significantly (P<0.05) decreased the proportion of oocytes which remained at PMI and MI stages compared to the control. With 5 μg/ml insulin treatment, the rate of MII was significantly increased compared to the control (P<0.05). No significant differences between all insulin treatments were observed in the proportion of oocytes developing to MII.

The results of Experiment 4 are shown in Table 4. EGF significantly (P<0.05) decreased the number of denuded oocytes derived from the collected COCs (COC-DO) which remained at PMI and MI stages in comparison to those cultured without EGF supplementation. The proportion of COC-DO which progressed to MII stage was significantly increased with EGF (P<0.05). EGF had no positive effect on the proportion of denuded oocytes found at the time of aspiration (DO) which develped to MII.

In Experiment 5, the possibility that the effect of EGF on denuded oocyte meiotic maturation is mediated by the receptor-associated tyrosine kinase was examined. Whereas EGF significantly increased the proportion of denuded oocytes which developed to the MII stage in comparison to the control, erbstatin, at 20 μM, partially blocked this stimulatory effect of EGF. When the concentration of erbstatin was increased to 30 μM, it

**Table 4.** Meiotic maturation of bovine denuded oocytes obtained from COCs (COC-DO) or found denuded at the time of aspiration (DO) cultured *in vitro* with 50 ng/ml EGF

| EGF 50 ng/ml | NO. of oocytes | GV[1)] | PMI, MI[1)] | AI, TI[1)] | MII[1)] |
|---|---|---|---|---|---|
| − | 118 (COC-DO) | 2.6 ± 1.1 | 36.4 ± 0.4[a] | 10.2 ± 1.7[a] | 50.8 ± 0.4[a] |
| − | 92 (DO) | 7.6 ± 0.1 | 43.5 ± 0.6[a] | 8.7 ± 0.9[a] | 40.2 ± 1.1[a] |
| + | 102 (COC-DO) | 1.9 ± 1.1 | 21.6 ± 1.4[b] | 8.8 ± 0.9[a] | 67.6 ± 1.2[b] |
| + | 112 (DO) | 7.1 ± 1.2 | 40.2 ± 0.9[a] | 6.3 ± 0.6[a] | 46.4 ± 1.1[a] |

[1)] Values are mean percentages ± SEM. [a,b] Different superscripts within the same column are significantly different ($P<0.05$). Four replicates of each concentration were done.

**Table 5.** Effect of erbstatin on EGF-induced meiotic maturation of bovine denuded oocytes *in vitro*

| Treatment | No. of oocytes | GV[1)] | PMI, MI[1)] | AI, TI[1)] | MII[1)] |
|---|---|---|---|---|---|
| TCM | 85 | 5.9 ± 1.5 | 34.1 ± 1.7[a] | 10.6 ± 1.4 | 49.4 ± 0.4[a] |
| TCM + EGF | 97 | 2.1 ± 1.1 | 20.6 ± 1.7[b] | 10.3 ± 1.0 | 67.0 ± 0.9[b] |
| TCM + EGF + 20 μM erbstatin | 104 | 4.8 ± 1.4 | 26.9 ± 1.4[ab] | 9.6 ± 1.6 | 58.7 ± 1.0[ab] |
| TCM + EGF + 30 μM erbstatin | 93 | 5.4 ± 0.7 | 33.3 ± 1.5[a] | 9.7 ± 1.1 | 51.6 ± 0.9[a] |

[1)] Values are mean percentages ± SEM. [a,b] Different superscripts within the same column are significantly different ($P<0.05$). Three replicates of each concentration were done.

significantly suppressed the stimulatory effect of EGF on meiotic maturation of the denuded oocytes ($p<0.05$).

## Discussion

Many investigators have failed to find any positive effect of growth factors on meiotic maturation of denuded oocytes [5, 11–14] and therefore suggested that cumulus cells are required for growth factors to exert their stimulatory effects. The results of the present study showed that EGF, IGF-I and insulin, when added to a serum-free medium, could stimulate meiotic maturation of bovine denuded oocytes, indicating that these growth factors may act, at least in part, directly on the oocyte.

In support of our findings, Das *et al.* [10] demonstrated that EGF stimulates nuclear maturation in both COCs and denuded murine and human oocytes, Vorobéva and Nikitin [18] reported that EGF is able to stimulate meiotic reinitiation of cumulus-free mouse oocytes blocked with cAMP at the dictyotene stage, and more recently Lonergan *et al.* [4] reported a direct evidence of a maturation-promoting effect for EGF on bovine denuded oocytes. The only use of denuded oocytes that were derived from the healthy collected COCs rather than those found denuded at the time of aspiration might be the reason why the results of this study contradict those of investigators who have been able to demonstrate the stimulatory effect of EGF only in the presence of cumulus cells [5, 11–13].

Lonergan *et al.* [4] have also excluded oocytes that were found denuded at the time of aspiration from their maturation treatments, and they suggested that such types of oocytes are inherently less competent. To test this hypothesis, we compared the effect of EGF on meiotic maturation of both types of denuded oocytes. The results revealed that whereas EGF significantly improved meiotic maturation of denuded oocytes derived from the aspirated COCs, it had no effect on those found denuded at the time of aspiration, so that either the latter type of denuded oocytes are inherently less competent, as suggested by Lonergan *et al.* [4], or they have lost their receptors for EGF. Another possibility is that the necessary signaling transduction pathway for EGF might have been broken in such oocytes.

On the basis of these results, the failure to find positive effects of growth factors on meiotic maturation of denuded oocytes reported by other investigators [5, 11–14] could be attributed to the type of oocytes used in their studies rather than to the absence of cumulus cells. Consistent with this suggestion, Singh *et al.* [19] provided evidence that both EGF and its receptor are synthesized by the oocyte, and the existence of IGF-I receptor mRNA in porcine and rat oocytes was reported [20, 21]. Moreover, insulin and IGF-I stimulate meiosis in xenopus oocytes which are normally not surrounded by cumulus cells [22, 23], and insulin induces germinal

vesicle breakdown in xenopus oocytes freed of their vitelline envelope [24]. This is further supported by the fact that the stimulatory effect of EGF on meiotic maturation of mouse oocytes is independent of cumulus expansion [25], and that the inhibitory effect of Müllerian inhibitory factor, through an action on tyrosine kinase, on meiosis of denuded rat oocytes was reversed by EGF [26].

EGF is a polypeptide growth factor that exerts its biological effects by binding to a membrane receptor, and the binding of EGF to its receptor will lead to the activation of the receptor-associated tyrosine kinase which is a first step in the EGF signal transduction pathway [27]. The stimulatory effect of EGF on meiotic maturation of bovine COCs was blocked by erbstatin, a specific tyrosine inhibitor, indicating that this growth factor acts through a tyrosine-kinase pathway to stimulate oocyte maturation [28].

In the present study, we examined whether the stimulatory effect of EGF on meiotic maturation of bovine denuded oocytes could also be abolished with erbstatin. The results showed that erbstatin blocked the positive effects induced by EGF on denuded oocyte maturation, indicating that tyrosine kinase is required for EGF to stimulate denuded oocyte maturation. In another experiment conducted in our laboratory (unpublished data), it has been found that EGF-induced meiotic maturation of both COCs and denuded bovine oocytes could be inhibited with wortmannin, a specific PI 3-kinase inhibitor. Taken together, these findings indicate that cumulus cells do not mediate the inhibitory effects of either wortmannin or erbstatin on EGF-mediated meiotic maturation, so that PI 3-kinase, and tyrosine kinase in the oocyte itself, are likely to be involved in mediating the stimulatory effects of EGF on bovine oocyte maturation. This could be cited as indirect evidence that EGF as well as other growth factors with receptor-associated tyrosine kinase, including insulin and IGF-I, act directly on the oocyte to stimulate its meiotic maturation.

In conclusion, the results of this study indicate that EGF, IGF-I and insulin would modify meiotic maturation of bovine denuded oocytes *in vitro*, and that oocytes found denuded at the time of aspiration are meiotically less competent than those derived from the collected COCs. The results also showed that erbstatin could suppress the stimulatory effect of EGF on denuded oocyte meiotic maturation, suggesting a possible role of the receptor-associated tyrosine kinase in the oocyte itself in mediating the stimulatory effect of EGF on oocyte meiotic maturation.

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