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Influence of Growth Factors on the Time-Dependent Meiotic Progression of the Bovine Oocytes during their *in vitro* Maturation

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The present study examines the effects of epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), and the presence of cumulus cells on the meiotic progression of the bovine oocyte maturation. Oocytes were aspirated from 2-8 mm ovarian follicles, and the cumulus-oocyte complexes (COC) obtained (n = 2,729) were divided into COC or denuded oocytes. They were then cultured in four defined treatments comprising TCM-199 and growth-factors: control (no growth factor), IGF-I, EGF, and EGF+IGF-I. Each treatment group of oocytes was cultured during 6, 12, 18, 24, and 30 h; the oocytes were fixed and stained at the end of each 6 hour interval from 0 to 30-h, and were assessed for their nuclear maturation stage. Germinal vesicle (GV) and metaphase II (MII) stages present were used as the final parameters for data analysis. The results indicate that treatments with growth factors enhanced the incidence of GV activation and MII stage in all the culture periods. Maximal stimulation for nuclear maturation occurred with EGF+IGF-I. As regards the denuded oocytes, no positive effects on nuclear maturation rates were observed for any treatment. These results suggest that EGF and IGF-I, singly and combined, stimulate the meiotic progression significantly, and this effect can be induced by a positive signal which is transferred from cumulus cells to oocyte.

Key words: Bovine oocyte, Cumulus cells, Meiosis, EGF, IGF-I

Immature mammalian oocytes show a defined and characteristic nuclear morphology which undergoes many changes in the maturation process (17, 26). These oocytes are arrested at the dyctiate stage of the meiotic prophase, until shortly before ovulation, when the preovulatory

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gonadotrophin surge triggers the resumption of the meiotic process. The preovulatory luteinizing hormone (LH) surge is generally accepted as the endocrine process regulating induction of *in vivo* oocyte maturation, since exposure of the follicle to LH or human chorionic gonadotropin (hCG) induces maturation (13). However, more recent observations imply that LH is only one of a complex sequence of factors that appear to regulate oocyte maturation, and it is unclear whether other paracrine and autocrine factors are involved in the mechanisms of controlling the events during follicular maturation (27).

Studies with rodents have implicated some growth factors in meiotic maturation. In vitro studies with EGF showed induction of germinal vesicle breakdown (GVBD) in cumulus cell-enclosed mouse oocytes maintained in meiotic arrest with purines, dbc-AMP, or the phosphodiesterase inhibitor IBMX (5, 6). It has also been demonstrated that IGF-I participates in the regulation of many ovarian functions; for example, it is a potent mitogen for granulosa cells (12) and acts as a biological amplifier of FSH action in the ovary (16). However, there is very little information about growth-factor induced regulation of oocyte maturation in domestic species.

This study set out to examine the effects of EGF, IGF-I and cumulus cells on the progression of meiotic *in vitro* maturation in bovine oocytes, and tested the dependence of these growth factors on cumulus-generated factors to induce oocyte maturation. The results obtained may serve to shed light on some physiological functions of these growth factors with respect to bovine oocyte maturation.

Materials and Methods

Oocyte collection and in vitro culture.– Bovine ovaries were obtained from the slaughterhouse (GYPISA, Pozuelo de Alarcón, Madrid) and transported in PBS (Dulbecco's Phosphate Buffer Saline) to the laboratory within 1 hour. Follicular contents from small antral follicles (2 to 8 mm in diameter) were aspirated with an 18-gauge needle attached to a 10 ml disposable syringe. The oocytes were transferred onto a 35 mm plastic Petri dish (Bibby, England) containing 2 ml of Hepes-buffered washing medium (TCM 199 Hepes-buffered, Sigma) supplemented with 2 % FCS (Gibco Ltd), followed by five washes. According to pre-defined criteria (20), selected cumulus-oocytes were divided for culturing either as cumulus-oocyte complexes (with intact and unexpanded cumulus, COC) or denuded oocytes (after denudation with 200 IU/ml hyaluronidase, and without layers of cumulus cells, DO), placed into drops (50 µl) of maturation medium under oil and cultured on 35 mm Petri dishes at 39 °C in 5 % CO_2, 95 % air and 100 % relative humidity. The starting time of the oocytes collection from the follicular fluid was determined as time 0 for oocyte incubation. The oocytes were matured in TCM-199 (Earle's salt with sodium bicarbonate and L-glutamine, Sigma). Growth factors (Boehringer Mannheim, Germany) were added to the maturation media to form four different treatments: control (no growth factor), IGF-I (100 ng/ml), EGF (50 ng/ml), and EGF+IGF-I (50 + 100 ng/ml respectively). EGF and IGF-I, were diluted in TCM-199 medium and frozen at –70 °C for a maximum of three months. Concentrations of growth factors were chosen based on the levels found in follicular fluid (7, 11, 14), and according to our previous results (19, 23). Except in the case of washing medium, neither hormone nor serum sources were used in the maturation media.

Nuclear maturation evaluation.– At the end of each 6-h interval from 0 to 30-h of culture, the oocytes were fixed and stained to ascertain the influence of growth factors on nuclear maturation *in vitro.* Cumulus cells of COC were removed with hyaluronidase (200 IU/ml) and mechanically stripped using a fine-bore pipette. The oocytes were then pipetted onto a slide. A coverslip spotted with a paraffin-vaseline (10:1) mixture at each corner was placed directly over the center of the drop containing oocytes. Fixation of oocytes was carried out by placing the slides in acetic acid-ethanol (1:3) for 24 h and staining with aceto-orcein (2 % orcein in 60 % acetic acid) for two minutes. Nuclear maturation was evaluated under phase-contrast microscope at 200 and 500x magnifications. Oocytes were classified as germinal vesicle (GV), germinal breakdown (GVBD), metaphase I (MI), metaphase II (MII) and degeneration.

Statistical analysis.– Nuclear maturation experiment was replicated at least three times with different batches of ovaries. Statistical analysis using the Chi-square test was carried out in pooled data using the Biomedical Data Program (4); when more than two groups were compared, analysis of variance followed by Duncan's range multiple test was used to determine statistical differences; P values less than 0.05 were considered significant. The presence of GV and MII stages were used as end-point parameters for assessing growth-factor effect on bovine oocyte maturation. Data are present in figures as percent mean ± SEM of oocytes having GV or MII stages.

Results

Oocyte totals, fixed and stained for maturity (n = 2,729) at each time point, are depicted in figures and tables. Degenerated oocytes (resulting in a different reaction to stain) were omitted from the results, because they represented a small percentage (less than 3 %) at each time point.

At collection time (0 h, 30-80 minutes after donor death at slaughterhouse) the majority of oocytes were at GV stage, and

Table I. *Nuclear configuration of bovine oocytes at onset (0 h) of* in vitro *culture.*
Data pooled from three independent experiments. The obtained oocytes were fixed and stained with aceto-orcein immediately after recovery. Differences were statistically observed between oocytes with or without cumulus cells.

Type of oocyte	Nuclear stage*	
	GV (%)	GVBD (%)
COC	33/35 (94.2)	2/35 (5.7)
Denuded	27/30 (90)	3/30 (10)

* Germinal Vesicle (GV) and Germinal Vesicle Breakdown (GVBD) stages are represented as number of oocytes observed/total examined. COC = Cumulus-oocyte complexes.

Table II. *Effect of the presence/absence of cumulus cells on bovine oocyte* in vitro *maturation with EGF + IGF-I at 24-h culture.*
Data pooled from three independent experiments. The oocytes were fixed and stained with aceto-orcein at 24-h culture.

Type of oocyte	Nuclear stage*	
	GV (%)	MII (%)
COC		
Control	8/30 (26.6)	13/32 (40.6)
EGF+IGF-I	0/48 (0)*	31/43 (72.1)**
Denuded		
Control	5/21 (23.8)	7/20 (35.0)
EGF+IGF-I	5/26 (19.2)	10/23 (43.4)

* Nuclear stages are represented as number of oocytes observed/total examined. Values within column in the same type of oocyte and nuclear stage with distinct superscripts are significantly different.* $p < .05$; ** $p < .01$, respect to the corresponding control.

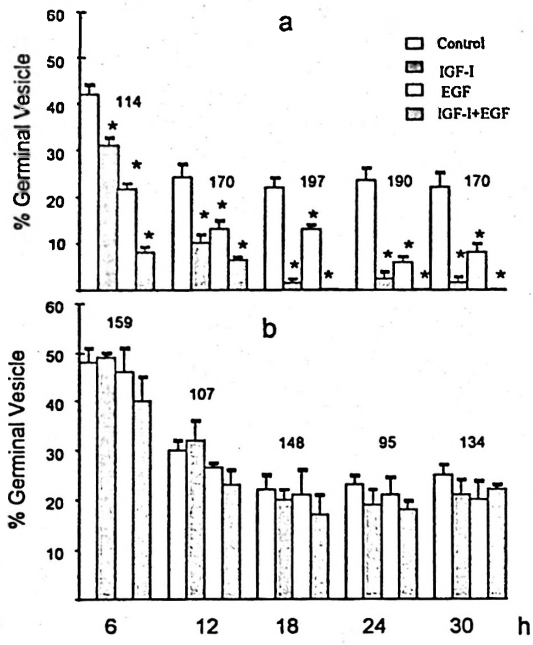

Fig. 1. *Effect of EGF and/or IGF-I on % germinal vesicles of bovine oocytes matured* in vitro.
Oocytes were cultured in TCM-199 medium (control) and containing IGF-I (100 ng/ml), EGF (50 ng/ml), or EGF+IGF-I (50 and 100 ng/ml). (a) Cultured cumulus-oocyte complexes, and (b) cultured denuded oocytes. Values above bars show the total number of oocytes examined in each time-point from three experiments. Germinal Vesicle stage was assessed after 6, 12, 18, 24, and 30 h of oocyte culture. Bars indicate the percentage of oocytes that remain at GV stage after each time-culture. In each time point, bars with asterisks indicate significant difference compared to the control group (* $p<0.05$). There were no difference between control and growth factor-treated groups in denuded oocytes.

a small number of the oocytes at germinal vesicle breakdown (GVBD) stage, as shown in table I. For COC, in the control medium at 6 h of culture, the 42.0% were mainly at GV stage (fig. 1a), but no oocytes in metaphase I stage were observed. With regard to COC, in 12 and 18 h cultured oocytes, the percentage of M I stage was higher than that at 6 h, with significant differences ($p < 0.05$) between the growth factor-treated groups and control (data not shown). In 24 h cultured oocytes, metaphase II rates of all treatments with growth factors were greater than for the treatment without growth factors. The highest metaphase II values were recorded in response to EGF+IGF-I treatment and proved significantly different ($p < 0.01$) from those recorded for control treatment. Also, COC cultured in EGF+IGF-I showed a complete maturation activation since no oocytes at GV stages appeared at the end of 24 h of culture (fig. 1a). Following further culture (30 h), a declining, but not a significant, percentage of MII stage occurred after the 24 h peak in all treatments (fig. 2a).

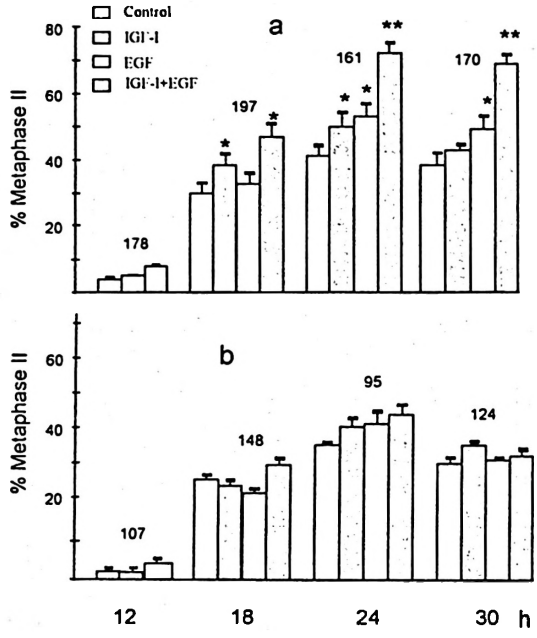

Fig. 2. *Effect of EGF and/or IGF-I on % metaphase II of bovine oocytes matured* in vitro.
Oocytes culture were as indicated in figure 1. Values above bars show the total number of oocytes examined in each time-point from three experiments. Metaphase II stage was assessed after 12, 18, 24, and 30 h of oocyte culture. Bars indicate the percentage of oocytes that reach at metaphase II stage after each time-culture. In each time point, bars with asterisks indicate significant difference compared to the control group (* $p < 0.05$; ** $p < 0.01$). There were no differences between control and growth factor-treated groups in denuded oocytes.

No significant differences among treatments at the recorded GV and MII stages, were observed regarding the maturation rate for denuded oocytes ($p > 0.05$). At 24 h culture, COC exhibited higher maturation values than denuded oocytes in all treatments, especially in the EGF + IGF-I treatment (72.1 % versus 43.0 %, $p << 0.01$), as it is shown in table II.

Discussion

The time sequence of *in vitro* bovine oocyte nuclear maturation has been previously studied by some authors (18, 22, 24, 30), but none of them used growth factors in their maturation media. Immature oocyte development in the ovarian follicle to mature oocyte (metaphase II stage) depends on many regulatory factors (25, 29). It has been previously demonstrated that growth factors can be synthesized by the ovary (10, 21). 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 (14, 15). Moreover, concentrations of IGF-I, similar to those used in the present study, have been detected in bovine and porcine follicular fluid (7, 11).

Oocyte maturation has been judged by many different criteria according to the goals of specific studies. In studies testing the ability of growth factors to stimulate the meiotic maturation, the end-points are usually the occurrence of the presence of GV and MII stages during the maturation period (6, 23). Our results about the oocyte's nuclear configuration at 0 h, agree with those shown by other authors. At this moment (from 30 to 80 minutes after the animal's death) the majority of analyzed oocytes had an intact nucleus (GV) and a small percentage at GVBD stage, which agree with XU *et al.* (30). First polar body abstriction (MII) was observed at 12 h, and the peak level was reached at 24 h according to SIRARD *et al.* (24). A decline in the rates of MII stage at 30 h culture (after the peak at 24-h) may be explained by the degeneration of the polar body itself or, as XU *et al.* (30) postulated, by the fact that with the whole mounting technique used to stain the oocytes (28), the first polar body degenerates, disappears, and the MII stage can be classified as MI.

Serum and hormone-free media was used for *in vitro* maturation in an attempt to explain the relationship between growth factors and the regulation of nuclear maturation, while effectively ruling out the influence of unknown serum and hormonal factor/s. The highest, EGF-induced, nuclear maturation percentages correspond to those obtained in rodent oocytes (3, 5). Such high rates can be attributed to the fact that the main agent for meiotic detention/activation in these species' oocytes is the stimulation of cAMP-dependent protein kinase. However, in their response to protein kinase stimulation via cAMP accumulation, bovine oocytes seem to differ from those of the above species (25), possessing other meiotic activation inhibiting substances (e.g., purines) upon which EGF, in all likelihood, fails to act in the same way. In our maturation experiment, the highest results were achieved with the addition of EGF + IGF-I, such data being in line with the recent results obtained in porcine (23) and human oocytes (8). This could indicate that, under *in vivo* conditions, there may be an additive element at work in the way in which these two growth factors combine their respective actions. They bring about specific actions on oocyte maturation, including cumulus and cytoplasmic effects (19). HAINAUT *et al.* (9)

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). Otherwise, the IGF-I in the above cited reports, did not stimulate GVBD of mouse oocytes but did stimulate the resumption of meiosis for rat oocytes.

The ovarian and intrafollicular mechanisms for the regulation of oocyte growth and maturation are complex. The mechanisms whereby growth factors regulate or modulate resumption of meiosis in oocytes may be mediated via the granulosa and/or cumulus cells (1, 3). The growth factors used in this study enhanced maturation in cumulus-oocyte complexes, but not in denuded oocytes. This was in accordance not only with the results observed in rodents (3, 6), but also with those obtained using other growth factors such TGF-a (1, 2). This indicates that the growth factors act in presence of cumulus cells, by which a positive stimulus for nuclear maturation is transferred to the oocyte.

In conclusion, this paper provides evidence that EGF and IGF-I, singly and combined, are potent inducers of meiotic progression in bovine oocytes sourrounded by cumulus cells.

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P. L. LORENZO, M. J. ILLERA, J. C. ILLERA y M. ILLERA. *Influencia de factores de crecimiento sobre la progresión meiótica de la maduración* in vitro *de oocitos bovinos.* Rev. esp. Fisiol. (J. Physiol. Biochem.), 51 (2), 77-84, 1995.

Se examinan los efectos del factor de crecimiento epidérmico (EGF), del factor de crecimiento similar a la insulina de tipo I (IGF-I) y de las células del cúmulo sobre la progresión meiótica de la maduración de los oocitos bovinos. Estos se obtuvieron mediante aspiración de folículos ováricos de 2-8 mm, y parte de los complejos oocito-cúmulo (COC) recogidos se denudaron (DO). Seguidamente se cultivaron durante 6, 12, 18, 24 y 30 horas en cuatro tratamientos compuestos de TCM-199 y factores de crecimiento, control (sin factores de crecimiento), IGF-I, EGF y EGF + IGF-I. Los oocitos se fijaron y tiñeron para averiguar el estadio de maduración alcanzado al final de cada intervalo de 6 horas, desde 0 hasta 30 h. Los estadios de vesícula germinal (GV) y de metafase II (MII) se utilizaron como parámetros para los análisis estadísticos. Los resultados indican que la utilización de factores de crecimiento aumenta los valores obtenidos de GVBD y MII en todos los períodos de cultivo, alcanzando el máximo valor al usar EGF + IGF-I. Los oocitos denudados no muestan diferencias de la maduración nuclear en ninguno de los tratamientos utilizados. Los resultados sugieren que el EGF y el IGF, solos o combinados, estimulan la progresión meiótica. Además, este efecto es inducido por una señal que se transfiere desde las células del cúmulo hacia el oocito.

Palabras clave: Oocito bovino, Células del cúmulo, Meiosis, EGF, IGF-I.

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