Int. J. Dev. Biol. 42: 1169-1172 (1998)

Short Contribution

Developmental competence of immature pig oocytes under the influence of EGF, IGF-I, follicular fluid and gonadotropins during IVM-IVF processes

MARIA J. ILLERA[1], PEDRO L. LORENZO[1], JUAN C. ILLERA[1*] and ROBERT M. PETTERS[2]

[1]*Departamento de Fisiología Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain and* [2]*Reproductive Physiology Research Laboratory, Animal Science Department, North Carolina State University, Raleigh, NC, USA*

ABSTRACT Epidermal growth factor (EGF) and insulin like growth factor-I (IGF-I) were evaluated for their effects on *in vitro* maturation and fertilization in presence or absence of gonadotropin and porcine follicular fluid. Four groups were made with the addition of growth factors: none (control), EGF, IGF-I or EGF+IGF-I. Each group underwent four predefined treatments with gonadotropin (FSH and LH), follicular fluid, a combination of both, or none (as control). Porcine cumulus-oocyte complexes (COCs) were matured in media containing the above-mentioned treatments for 42-44 h prior to fertilization with fresh sperm capacitated for 2.5 h. At the end of the fertilization period, the presumable embryos were fixed, stained and examined as whole-mounts to ascertain their nuclear status. The addition of EGF alone or in combination with IGF-I, significantly increased the proportion of monospermic oocytes forming 2 normal pronuclei. Also, supplementation with both growth factors together enhanced the percentages of pronucleus formation and total penetration. In addition, treatments with EGF+IGF-I significantly decreased ($P<0.01$) the incidence of degeneration in fertilized oocytes. However, no significant differences in the proportions of COCs undergoing polyspermy were observed among all treatments. These results suggest a stimulatory effect of tested growth factors in maturation and fertilization of pig oocytes. Furthermore, gonadotropins and follicular fluid can be replaced by the addition of EGF and IGF-I to the maturation media with positive effects on fertilization rate.

KEY WORDS: *growth factor, oocyte development, maturation, fertilization, gonadotropins, follicular fluid*

Pig oocytes initiate meiosis during fetal life which is arrested at the diplotene stage (germinal vesicle) of the prophase immediately after birth. These oocytes undergo resumption of meiosis at the time of the prepuberal gonadotropin surge prior to ovulation. This process is commonly known as oocyte maturation (Edwards, 1965). Gonadotropins are the primary initiators of follicular development and reactivation of meiosis in growing oocytes (Motlik *et al.*, 1986) sustaining ooplasmic maturation by stimulating steroidogenesis in granulosa cells. Thus, traditionally they have been added to *in vitro* oocyte maturation media (Funahashi and Day, 1997; Singh *et al.*, 1997). However, more recent observations imply that gonadotropins is only one of a complex sequence of factors, such as growth factors, which appear to regulate ovarian function (Tonetta and DiZerega, 1989). Among them, epidermal growth factor (EGF) induces protein synthesis in the porcine oocyte (Singh *et al.*, 1997). Besides, insulin-like growth factor-I (IGF-I) is a potent mitogen for granulosa cells (Hernández *et al.*, 1988), and acts as a biological amplifier of the action of FSH in the ovary (Hsu and Hammond, 1987). Some information about growth-factor induced regulation of oocyte maturation in animal species such as mice (Brucker *et al.*, 1991), pigs (Reed *et al.*, 1993; Ding and Foxcroft, 1994), and cattle (Harper and Brackett, 1993; Lorenzo *et al.*, 1993) has been described. In addition, IGF-I contributes to enhance bovine oocyte fertilization *in vitro* (Herlerr *et al.*, 1992) and stimulates maturation in rabbit oocytes (Lorenzo *et al.*, 1996). On the other hand, as-yet-undefined follicular factors, in the form of follicular supplementation or granulosa/tecal cell co-culture systems, influence oocyte maturation, sperm penetration, and sperm nuclear decondensation (reviewed by Nagai, 1994; Funahashi and Day, 1997). Yoshida *et al.* (1992b) observed that porcine follicular fluid from medium size follicles seemed to stimulate the ability of male pronucleus formation. Therefore, a harmonious oocyte maturation is essential to produce viable male pronucleus and porcine embryos efficiently (Funahashi and Day, 1997).

Abbreviations used in this paper: IVM, *in vitro* maturation; IVF, *in vitro* fertilization; COC, cumulus-oocyte complex; pFF, porcine follicular fluid; PBS, phosphate buffer saline.

***Address for reprints:** Departamento de Fisiología Animal, Facultad de Veterinaria, Universidad Complutense, Madrid 28040, Spain. FAX: 34 91 394 3864. e-mail: vefis07@sis.ucm.es

0214-6282/98/$10.00

Printed in Spain

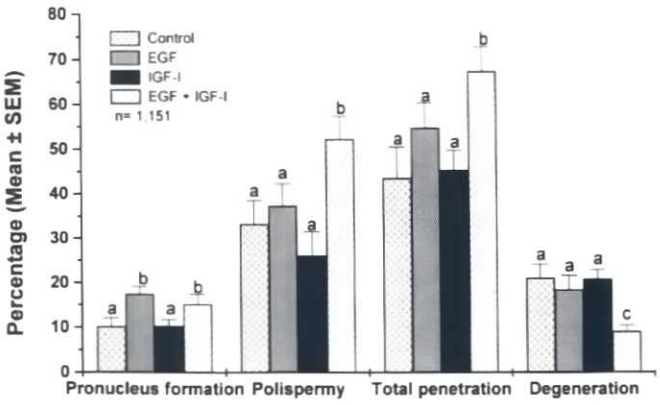

Fig. 1. Effect of growth factors (EGF and IGF-I) on IVM-IVF of pig oocytes. *Percentages in pronuclear formation, polyspermy, total penetration and degeneration stages in porcine embryos achieved after maturation and fertilization procedures under the influence of the tested growth factors: none (as control), EGF (10 ng/ml), IGF-I (100 ng/ml) and EGF+IGF-I. Each histogram indicates the mean ± S.E.M. of five experiments. Bars with different letters within the same stage group mean significant difference (a vs. b,c, P<0.05).*

A total of 3,698 porcine cumulus-oocyte complexes were used to evaluate the effects of epidermal growth factor and/or insulin like growth factor I in *in vitro* fertilization of pig oocytes in the presence or absence of gonadotropin and porcine follicular fluid. According to the results obtained, treatments with both growth factors (without supplement of gonadotropin and pFF) showed an increase in the percentages of the parameters measured compared to treatments without them (Fig. 1). In addition, supplementation with EGF stimulated significantly (P<0.05) the incidence of pronucleus formation while addition of both growth factors together showed an increase in the percentages of total penetrations and polyspermy.

As shown in Figure 2A, in the group I (control-group without supplementation of growth factors but with the addition of gonadotropins plus pFF) the values of polyspermy (P<0.05) and total of penetrations (P<0.05) were stimulated compared to control. A significantly higher proportion of oocytes showed higher percentages of metaphase II (the remained unfertilized oocytes) in control compared to oocytes supplemented with gonadotropins or pFF (P<0.05). Results obtained in the group II (with EGF and with/without gonadotropins and/or pFF), revealed no significant differences in the proportions of oocytes undergoing male pronucleus formation between media with gonadotropins plus pFF (15±2.1%) and media with EGF alone (Fig. 2B), where the maximal percentage of pronucleus formation was reached (17.4±1.7%). No significant differences were observed in the proportions of polyspermy, total of penetrated oocytes and degeneration compared to control-group I.

With respect to the group III (with IGF-I and with/without gonadotropins and/or pFF), as shown in Figure 2C, no significant effects were observed among treatments in the IGF-I-group in the endpoints analyzed. However, addition of IGF-I to control maturation medium showed significantly lower percentages in male pronucleus formation than EGF-treatment (P<0.01; Fig. 1). In addition, no significant differences in the proportions of COCs undergoing total penetrations, polyspermy or degeneration were observed after treatments with IGF-I compared to EGF- and control-groups. Lastly, in the group IV, the effect of EGF plus IGF-I with or without gonadotropins and/or pFF during oocyte IVM-IVF was investigated. Use of EGF in conjunction with IGF-I, without any supplementation of gonadotropins or pFF, provides significantly greater percentages of male pronucleus formation (P<0.05), than treatments with gonadotropins or pFF. Moreover, the highest oocyte penetration rates throughout the study (67.3±5.4%) were obtained when EGF was used together with IGF-I, but they were not significantly different compared to treatments with gonadotropin or pFF supplementation (Fig. 2D). In addition, the incidence of degeneration – in this group IV – was significantly decreased by addition of both growth factors together (P<0.05), contrary to results found in the rest of groups including EGF, gonadotropins and/or pFF (Table 1). Finally, the highest percentages of cumulus expansion (more than 90% of full expansion) were reached in treatments with both growth factors and gonadotropins, alone or in combination. However, addition of IGF-I alone (group III) provided a very low stimulation in full expansion (lower than 12%, P<0.05) compared to treatments including EGF, gonadotropin or pFF.

Although matured porcine oocytes can be penetrated *in vitro* by spermatozoa under appropriate conditions, low rates of pronuclear formation and a high incidence of polyspermy have been reported as the main problem by many authors (reviewed by Funahashi and Day, 1997). The results obtained show that medium composition for *in vitro* maturation of pig oocytes affects the ability of oocytes to form the male pronucleus after sperm penetration. In a current study made by us, the addition of pFF to maturation media was effective for enhancing the rate of nuclear maturation (Reed *et al.*, 1993). While these results are consistent and similar to other previous observations (Yoshida *et al.*, 1992a), even using media supplemented with cysteine or glutathione (Yoshida, 1993), they are in conflict with the report of Naito *et al.* (1988), which indicates that pFF did not enhance male pronucleus formation. These contradictory results might be due to differences in culture conditions or to interactions of pFF with specific components that make up the media used in the experiments. According to Yoshida (1993), it has been suggested that an acidic substance(s) with molecular mass between 10 and 200 kDa improves not only nuclear maturation, but also male nuclear formation and normal development of pig oocytes. This can be the explanation of the high rates of pronuclear formation observed in medium supplemented with pFF but without gonadotropins or growth factors. On the other hand, beneficial effects of gonadotropins on nuclear maturation of pig oocytes and cumulus expansion have been

TABLE 1

INFLUENCE OF GROWTH FACTORS (EGF AND IGF-I), GONADOTROPINS AND PFF IN DEGENERATION DURING IVM-IVF OF PIG OOCYTES

		Growth Factor treatments			
Gonadotropins	pFF	Control	EGF	IGF-I	EGF+ IGF-I
+	+	14.1 2.0a	13.2 ± 2.0a	14.1 ± 1.6	12.0 ± 1.8a
+	-	25.7 ± 4.1b	22.9 ± 2.2b	16.5 ± 2.0	11.1 ± 2.5a
-	+	20.2 ± 2.1b	22.4 ± 4.1b	18.1 ± 2.5	11.9 ± 4.1a
-	-	20.7 ± 3.1b	18.3 ± 3.2b	20.7 ± 2.1	9.1 ± 3.1b

Percentages of degeneration in porcine embryos (n= 645) after maturation and fertilization procedures across the study. Each value indicates the mean± S.E.M. of almost five experiments. Different letter within the same column means significant difference (a vs. b, P<0.05; a vs c, P<0.01).

reported by many authors (Nagai, 1994). Mattioli *et al.* (1991) showed that FSH and LH, separately or in combination, induced cumulus expansion and accelerated nuclear maturation of pig oocytes matured *in vitro*. The role of gonadotropins on *in vitro* matured oocytes and their capacity to undergo fertilization has not been yet completely clarified.

As the present report shows, gonadotropin and follicular fluid can be replaced by the addition of EGF and IGF-I to the maturation media with no adverse effect on fertilization rate. However, the use of these growth factors did not prevent polyspermy, whose incidence through the present study was as high as other authors reported (Yoshida *et al.*, 1992a; Yoshida, 1993). In standard procedures with no oviduct cell involvement, high rates of penetration were accompanied by equally high incidences of polyspermy. However, studies carried out by Abeydeera *et al.* (1997) indicate that, when pig oocytes are matured in the presence of pFF, the incidence of polyspermy can be reduced. These reports are in agreement with our results, showing a reduction of polyspermy in treatment with pFF and without other supplementation. Rath (1992), reported that there is a high correlation between the incidence of polyspermy and the absolute number of spermatozoa and oocyte present at fertilization *in vitro*; if the number of spermatozoa per oocyte is diminished, polyspermy could be minimized. These data are also coincident with those described by Abeydeera and Day (1997). However, these results should be carefully considered, because when minimizing the sperm oocyte ratio may be accompanied by a reduction in penetration rate. The growth factors used in the study reported here enhanced maturation and fertilization in cumulus-oocyte complexes but not in the denuded oocytes (data not shown). This was in accordance not only with our own results observed in cattle (Lorenzo *et al.*, 1993) and rabbits (Lorenzo *et al.*, 1996), but also with those obtained using TGFα, which has similar effects to EGF *in vitro* (Brucker *et al.*, 1991) and binds to the same receptor species (Massagué, 1983). A study in porcine oocytes concluded that the action of EGF is mediated via the cumulus cells and gap junctions with the oocyte (Coskum and Lin, 1993). Since IGF-I or EGF were unable to enhance maturation and fertilization of denuded oocytes, either alone or together, our data support the hypothesis that the growth factors act in the presence of the cumulus cells, through which a positive stimulus for maturation is transferred to the oocyte.

Growth factors may be a "key" factor in the regulation of intrafollicular oocyte maturation. It is feasible too that EGF and IGF-I interact with gonadotropin, steroids and/or other molecules to regulate oocyte follicular development *in vivo*. The successful development of a defined or semidefined *in vitro* maturation protocol for pig follicular oocytes would facilitate both basic and applied research programs by reducing the number of animals needed for a given protocol and providing larger number of developmentally staged oocytes and embryos for procedures such as nuclear and gene transfer.

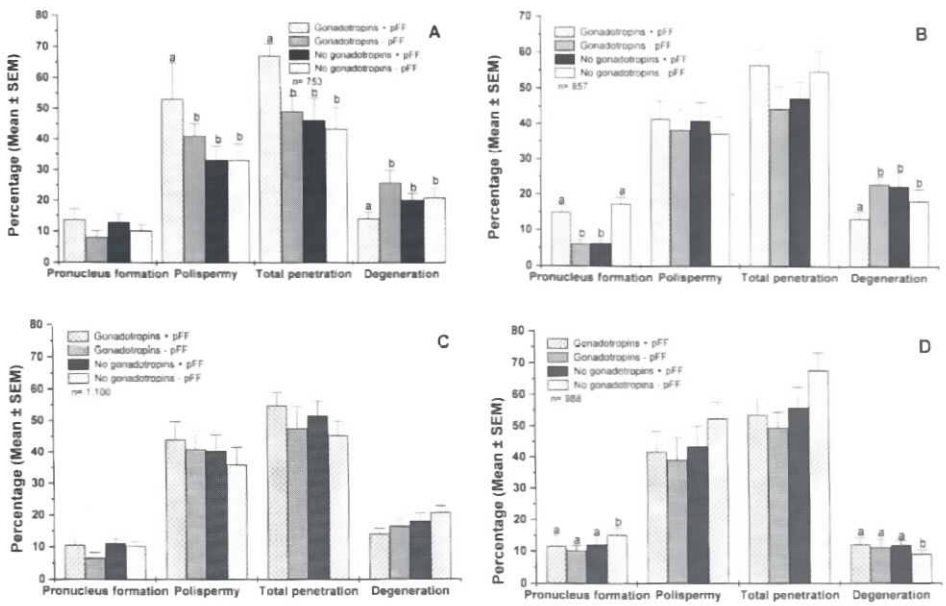

Fig. 2.- Effect of growth factors and/or gonadotropins and pFF on IVM-IVF of pig oocytes. *Percentages in pronuclear formation, polyspermy, total penetration and degeneration stages in porcine embryos achieved after maturation and fertilization procedures under the influence of:* **(A)** *gonadotropins and pFF, alone or in combination,* **(B)** *EGF (10 ng/ml) and/or gonadotropins and pFF,* **(C)** *IGF-I (100 ng/ml) and/or gonadotropins and pFF,* **(D)** *EGF plus IGF-I and/or gonadotropins and pFF. Each histogram indicates the mean ± S.E.M. of almost five experiments. Bars with different letters within the same stage group mean significant difference (a vs. b, P<0.05; a vs. c, P<0.01).*

Experimental procedures

Reagents and culture media

All reagents and media were purchased by Sigma Chemical Co. (St. Louis, MO USA) except the IGF-I (Human Recombinant, Boehringer Mannheim, Germany). Medium for oocyte washing consisted of low bicarbonate-TALP medium (Parrish *et al.* 1988). The basal oocyte maturation medium was TCM-199, supplemented with sodium pyruvate (0.100 mg/ml) and calcium lactate (0.900 mg/ml). IGF-I and EGF stock solutions were diluted and added to TCM-199 for maturation culture as appropriate. They were reconstituted in a PBS solution containing 3 mg/ml BSA, and stored at -20°C until use. The final concentration of BSA in the maturation medium was 0.003% w/v. Gonadotropins used were pFSH (5 ng/ml, USDA-B-1), pLH (5 ng/ml, USDA-B-1), prepared and stored according to USDA specifications and previous reports (Reed *et al.*, 1993). Media for pre-incubation of sperm and fertilization were as described by Yoshida *et al.* (1992a), supplemented with 10% FCS.

Collection, maturation of oocytes and IVF procedure

Procedures for pig oocyte *in vitro* maturation were carried out as previously described by Reed *et al.* (1993) with some modifications. Briefly, ovaries were collected from prepuberal gilts at the slaughterhouse. Cumulus-oocyte complexes (COCs) were obtained by aspiration of 2-5 mm selected follicles with a 10-ml syringe and a 18G needle. All the collected oocytes were transferred into 35-mm plastic Petri dishes containing washing medium. According to predefined criteria (Lorenzo *et al.*, 1994), healthy cumulus-oocytes complexes were selected for quality and quantity of granulosa-cumulus cells and for uniformity of granulated cytoplasm. The selected oocytes were washed 6 times in washing medium, followed by two washes in a maturation medium. Finally, oocytes were matured in 35-mm Petri dishes at 38.5°C in 5% CO_2 in air and 100% humidity for 40-42 h under treatments as indicated below. Groups of 20-25 oocytes were cultured in each ml of medium. The oocytes collected were pooled and assigned to treatment groups at random.

The basic medium (TCM-199) was supplemented with growth factors to form four treatment groups: group I (no growth factors addition, as control), group II (with 10 ng/ml of EGF), group III (with 100 ng/ml of IGF-I), and group IV (with the addition of 10 ng/ml of EGF plus 100 ng/ml of IGF-I). Each group then underwent four different, predefined treatments with gonadotropins and/or porcine follicular fluid: control (no addition), with gonadotropins, with 10% of porcine follicular fluid (pFF) and with gonadotropins plus pFF.

Porcine follicular fluid was collected from 2-5 mm follicles and centrifuged at room temperature at 1500g for 45 min to pellet debris and then dialyzed three times against the maturation medium, using 6000-8000 MW separation membrane as previously described (Reed *et al.*, 1993).

The sperm-rich fraction was collected from a mature boar (crossbred) using the gloved hand method. Semen samples were diluted with an equal volume of Kiev extender and centrifuged at 50g for 3 min. Then, the supernatant was washed twice with Sperm preincubation medium with 10% FCS, by centrifugation at 550g for 5 min. The washed spermatozoa were subsequently diluted to a final concentration of $2x10^8$ cell/ml in capacitation media with FCS and incubated for 2.5 h at 37°C (Rath, 1992). Matured oocytes were transferred to plastic dishes (Costar, Ma.# USA) containing the fertilization medium, supplemented with 10% of FCS and adjusted to pH 7.4. The spermatozoa were introduced in the fertilization dishes with a final concentration of $2x10^6$ and up to 70% progressive motility.

Assessment of cumulus expansion, maturation and fertilization and statistical analysis

Before fertilization, cumulus-oocyte complexes were evaluated at the end of the maturation period to assess the effect of growth factors on cumulus expansion. The cumulus expansion was scored based on the subjective scale of 0 to 3, in which, 0 indicates no detectable response, and +3 shows full expansion, where all layers of cumulus cells expanded, even those closest to the oocyte. At the end of the fertilization period, the oocytes were fixed and stained to ascertain the influence of growth factors, gonadotropins and pFF on fertilization *in vitro* as described by Lorenzo *et al.* (1994). The oocytes were then pipetted onto a slide. A coverslip spotted with silicone at each corner was placed directly over the center of the drop containing oocytes. Fixation of oocytes was carried out by their placing in acetic acid-ethanol, and staining with aceto-orcein. Nuclear structures were evaluated under a phase contrast microscope at 200x and 500 magnifications. Oocytes were assessed for fertilization failure (i.e., maturation stages such as intact germinal vesicle, germinal vesicle breakdown, metaphase II and degeneration), and fertilization (polyspermy, male pronucleus formation and total penetration). Statistical analysis of endpoints was carried out in pooled data by using the Biomedical Data Program (BMDP, Dixon *et al.*, 1990). Mean values were subjected to analysis of variance using the 7d procedure (one- and two-way ANOVA) and Bonferroni post-test to ascertain statistical differences between treatments. A P value less than 0.05 was accepted as denoting significant differences.

Acknowledgments

The authors thank The Lundy Packing Company, Inc., Clinton (NC, USA) for their cooperation. We also thank Bruce Collins and Zola Turner for their help in the collection and processing of ovaries and oocytes.

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Received: July 1998
Accepted for publication: September 1998