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Improvement of oocyte competence and *in vitro* oocyte maturation with EGF and IGF-I in Guinea pig model

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ABSTRACT

In vitro maturation (IVM) system is an alternative method to superovulation protocols to obtain mature oocytes. Epidermal Growth Factor (EGF) and Insulin-like Growth Factor I (IGF-I) have been widely used in IVM medium in different species. Although the guinea pig is a valuable animal model for reproductive studies, IVM is rarely used. We aimed to establish a suitable *in vitro* production system using EGF and/or IGF-I during IVM to improve oocyte competence. Firstly, immunolocalization of EGF and IGF-I receptors in the ovary was assessed. An IVM dose-response experiment was performed with cumulus-oocyte complexes (COCs) supplemented with: 1) EGF [0, 10, 50, 100 ng/mL or 10% fetal calf serum (FCS)]; 2) IGF-I [0, 50, 100, 200 ng/mL or 10% FCS]; or 3) the concentrations of EGF and IGF-I which showed the best IVM index in the previous experiments, with or without Fetal Calf Serum (FCS). Cortical granule and mitochondria distribution patterns were determined in *in vivo* and *in vitro*-matured oocytes for the first time in this species. Apoptotic rate after IVM and oocyte competence by *in vitro* embryo development were evaluated. Immunohistochemistry results showed positive immunostaining of EGF and IGF receptors in corpus luteum, oocytes, granulosa and theca cells in follicles in all stages of development. Supplementation of IVM medium with 50 ng/mL EGF or 100 ng/mL IGF-I or their combination with FCS successfully led to oocyte nuclear and cytoplasmic maturation and reduced the apoptotic rate. Both growth factors improved oocyte competence during IVM in this species since early embryos were *in vitro* developed, showing better results when FCS was used in the IVM medium.

1. Introduction

Guinea pig (*Cavia porcellus*) has been used as a laboratory animal since the late 18th century, and is still essential in many research areas, such as Assisted Reproductive Technologies (ARTs) [1]. However, female guinea pigs have a low ovulation rate, and superovulation procedures have not been successfully established [2,3]. Therefore, the use of *in vitro* maturation (IVM) protocols can be a useful method with this species to obtain a source of large numbers of metaphase-II oocytes *in vitro* instead of matured oocytes *in vivo* for biomedical research applications in ARTs [4,5]. In this way, *in vitro* alternatives can considerably reduce the number of animals required in the lab (3R principle) to receive hormonal treatments when obtaining MII-oocytes *in vivo*. Besides, together with the *in vitro* follicular growth procedures [6], IVM could be used to produce embryos *in vitro* for the preservation of the genetic pool of valuable guinea pig strains. Although some follicular development research has been established [3], IVM protocols are rarely developed [7], and cellular mechanisms occurring during *in vitro* oocyte maturation are unknown in this species [7]. Nevertheless, understanding guinea pig oocyte essential events during their maturation is fundamental to improve IVM protocols, and, subsequently, IVF procedures for this species.

During maturation, mammalian oocyte resumes meiosis and progresses to the metaphase stage of the second meiotic division, while

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cytoplasmic organelles are reorganized and macromolecules (such as mRNA, cAMP, and ATP-dependent phosphorylation activity of proteins) are synthetized [8]. Cortical granule (CG) migration and mitochondria redistribution are necessary events for the oocyte to acquire the capability to support fertilization and early embryonic development. Whereas CGs position themselves under the plasma membrane of the mature oocyte to prevent polyspermy [9,10], mitochondria play an important role in oocyte energy metabolism by producing most of the ATP through oxidative phosphorylation, which is necessary for maintaining oocyte maturation and the first divisions of the subsequent embryos. For this reason, the mitochondrial cytoplasmic distribution pattern has been associated with the quality and developmental capacity of mammalian oocytes and embryos [8,11]. In addition, mitochondria are also involved in the production of reactive oxygen species [12] and in cumulus cell (CCs) apoptosis [13]. Both viability and steroidogenic capacity of CCs are crucial in reaching oocyte competence during maturation [14]. In contrast, abnormal distribution of CGs and/or mitochondria clearly impairs subsequent embryo development in several species [9,15].

Establishing the IVM protocol results in a complex process, since each laboratory uses different methods and additives. Gonadotropins are the primary regulators of oocyte maturation, but, in absence of hormones, growth factors such as Epidermal Growth Factor (EGF) and Insulin-like Growth Factor I (IGF-I) can be used in serum-free IVM systems [16,17]. They bind to their specific receptors to induce a cellular response. In ovaries, they play important endocrine, paracrine, and autocrine roles in cell proliferation, differentiation, folliculogenesis, and steroidogenesis [18].

The development of IVM systems in animal models to obtain a large number of MII oocytes will be a useful tool to reduce animal number and management in the lab. In the present work, in first place, we immunolocalized EGF and IGF-I receptors in guinea pig ovaries to elucidate if these growth factors can have a direct effect in the ovarian structures. After that, we characterized, for the first time, the compatible CG and mitochondrial migration patterns in *in vitro* and *in vivo*-matured guinea pig oocytes. Finally, we established a defined-IVM medium using EGF and/or IGF-I as an alternative method to gonadotropin therapies. We assessed the effect of tested media on oocyte competence by studying the cellular events during oocyte maturation, such as nuclear maturation (MII rate); cytoplasmic maturation (CG migration and mitochondrial relocation); cumulus cells (CCs) apoptotic rate, and oocyte developmental competence to early embryo stage *in vitro*.

2. Materials and methods

Unless otherwise stated, all chemicals were purchased from Merck Sigma-Aldrich.

2.1. Animals

Healthy adult female and male guinea pigs (*Cavia porcellus*) weighing between 600 and 700 g and aged three months were maintained under standard commercial conditions in the agricultural farm of the UTPL. Male guinea pigs (n = 3) were used for sperm collection and *in vitro* fertilization. Female guinea pigs (n = 6) were used to monitor estrous cycle and for collection of *in vivo* matured oocytes for establishing cortical granule and mitochondria patterns.

Animals were housed in cages under controlled conditions, including a constant photoperiod (12 h light/dark), 20–24 °C, and 60–70% of relative humidity maintained by a forced ventilation system. Each animal had free access to food and water. They were fed an *ad libitum* vitamin-C enriched commercial diet (Pronaca CA, Ecuador), containing 18.0% crude protein, 6.0% crude fiber, and 5.0% fat. All experiments were accomplished according to ethical guidelines on Animal care [19] and accepted by the Ethics Committee for Animal Experimentation of the Universidad Técnica Particular de Loja (Ref. CBEA-001).

In addition, the ovaries for the immunohistochemistry analysis of EGF and IGF-I receptors in guinea pigs (n = 18) and those for subsequent *in vitro* experiments (n = 110) were collected from animals slaughtered for commercial purposes at a local abattoir, since guinea pig meat is highly appreciated in Ecuador for human consumption.

2.2. Experimental design

First, an immunohistochemical study to evidence EGF and IGF-I receptors (EGF-R and IGF-IR, respectively) was performed. Second, to establish CG and mitochondria migration patterns, *in vivo* ovulated oocytes were used. For that purpose, the estrous cycles of females were monitored by vaginal cytology, and ovulated cumulus–oocyte complexes (COCs) were recovered by flushing the reproductive tract. Later, an IVM dose-response experiment with different concentrations of EGF, IGF and EGF-IGF was performed. Nuclear oocyte maturation, CG and mitochondrial distribution patterns and CC apoptosis were evaluated to assess oocyte maturation (Fig. 1). Oocyte competence was assessed as the capacity of the oocyte to achieve the early stages of embryo development by *in vitro* fertilization and *in vitro* culture system.

2.3. Immunohistochemistry of EGF and IGF-I receptors in Guinea pig ovaries

Immunohistochemical staining was performed using the Avidin-Biotin-Complex (ABC) system as previously described by Arias-Álvarez et al. [20] with certain modifications. Ovary samples were fixed in 4% buffered neutral paraformaldehyde (PF) solution (pH 7.4) and embedded in paraffin blocks, which were cut in 5 μm sections and mounted on glass slides. Briefly, endogenous peroxidase activity was blocked in deparaffinized sections with normal goat serum (1:10, Santa Cruz Biotechnology). Rabbit polyclonal IgG EGF-R and IGF-IR (both 1:100, Santa Cruz Biotechnology) antibodies were incubated overnight at 4 °C, whereas for negative control sections PBS was used instead of primary antibody. Subsequently, sections were incubated with a secondary biotinylated anti-rabbit IgG antibody (1:200, Vector Laboratories) and then ABC (Vector Elite kit, Vector Laboratories) was used. After chromogen incubation (Vector Nova RED substrate Kit for Peroxidase, Vector Laboratories), sections were counter-stained with hematoxylin, dehydrated and mounted with Depex medium. Finally, they were observed under a light microscope equipped with a digital camera (F550; Leica). The ovarian follicles were classified according to the morphology of the follicular cells as primordial, primary, secondary and antral, as described by Sadeu et al. [4]. The intensity of immunohistochemical staining was graded as follows: (−) no immunostaining; (+) weak staining; (++) moderate staining, and (+++) strong staining.

2.4. Monitoring of estrous cycle and collection of in vivo matured oocytes of Guinea pigs

Daily vaginal smears were performed which made it possible to determine the day of ovulation (Day 0) [21]. Subsequently, the vaginal smears continued once every 12 h for every female. Vaginal smears to evaluate the different phases of the estrous cycle were performed with a sterile swab, which was previously moistened with saline solution and gently inserted into the vagina at an angle of approximately 45°. When the swab was fully inserted, the end was rotated 2 to 3 turns, allowing the cotton tip to pick up an adequate load of cells. The smears were prepared immediately after withdrawal of the swab by rolling the cotton tip along the length of a glass slide. For fixation, slides were dipped 10 min in a container of methanol (Panreac) at room temperature. The slides were then allowed to dry completely and were stained with Giemsa 10% for 15 min and rinsed in distilled water. The examination was done under an optical microscope (Olympus, CX31R). Estrus was confirmed by the presence of the maximum cornification of the superficial cells and before the appearance of leukocytes; the metaestrus by

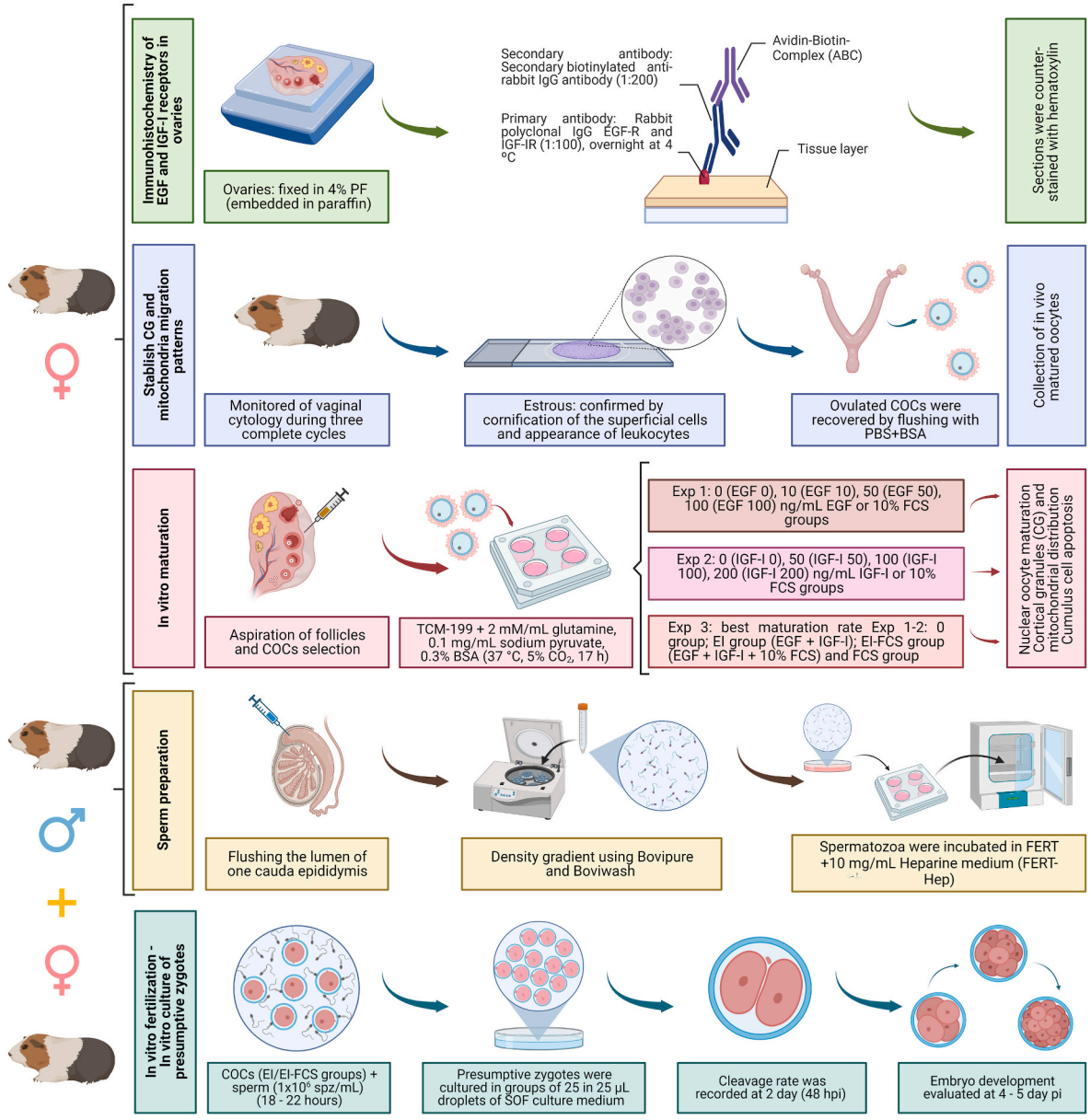

Fig. 1. Experimental design: A suitable *in vitro* production (IVP) system using EGF and/or IGF-I during *in vitro* maturation (IVM). First, immunolocalization of EGF and IGF-I receptors in the ovary was carried out. Then an IVM dose-response experiment was performed with COCs supplemented with the following: In Experiment 1 (Exp 1), with EGF (0, 10, 50, 100 ng/mL or 10% FCS); in Experiment 2 (Exp 2), with IGF-I (0, 50, 100, 200 ng/mL or 10% FCS); and in Experiment 3 (Exp 3), with the concentrations of EGF and IGF-I which showed the best index of oocyte maturation in the experiments 1 and 2, with or without serum. To assess cytoplasmic oocyte maturation, cortical granule and mitochondria distribution patterns in *in vivo* and *in vitro*-matured oocytes were determined. In addition, we assessed the oocyte competence to develop as an embryo. Sperm preparation and IVF procedure are outlined. EGF: Epidermal Growth Factor, IGF-I: Insulin-like Growth Factor I, COCs: Cumulus–oocyte complexes, PF: Paraformaldehyde, CG: Cortical granule, PBS: phosphate buffered saline, BSA: bovine serum albumin, FCS: fetal calf serum, SOF: synthetic oviductal fluid, hpi: hours post-insemination.

the presence of cornified squamous epithelial cells and small stained leukocytes; the diestrus by the presence of the intermediate cells with a large number of leukocytes; and the proestrus by the presence of round, nucleated epithelial cells (Supplementary Fig. S1). The estrous cycle was monitored during three complete cycles for each female, and finally animals were sacrificed after ovulation. Ovulation occurs 1–1.5 days after vaginal opening, which is the moment when the epithelium covering the vaginal orifice breaks down [3]. Ovulated COCs were recovered by flushing the reproductive tract with phosphate buffered saline (PBS) plus 0.3% (w/v) bovine serum albumin (BSA).

2.5. *In vitro maturation*

In vitro maturation protocol was performed as described by Arias-Álvarez et al., [22,23] with some modifications. Ovaries were transported from the abattoir to the laboratory at 37 °C in PBS supplemented with gentamicin. COCs were collected by aspirating antral follicles (>700 µm diameter) [3] on the ovary surface under a stereoscopic microscope. The maturation medium consisted of Tissue Culture Medium (TCM-199) enriched with 2 mM/mL glutamine, 0.1 mg/mL sodium pyruvate and 0.3% (w/v) BSA. Groups of 30–32 COCs with intact and unexpanded CCs were cultured at 37 °C in 5% CO_2 air and at maximum humidity for 17 h in each replicate.

A total of 1746 COCs were used in three experiments, and each

experiment was repeated at least four times. In *Experiment 1*, 622 COCs underwent IVM with 0, 10, 50, 100 ng/mL EGF or 10% (v/v) FCS. In *Experiment 2*, 624 COCs underwent IVM with 0, 50, 100, 200 ng/mL IGF-I or 10% (v/v) FCS. In *Experiment 3*, 500 COCs were supplemented with the concentrations of EGF and IGF-I which showed the best index of oocyte maturation in experiments 1 and 2 (50 ng/mL EGF and 100 ng/mL IGF-I), with or without FCS, as follows: 0 group (non-supplemented); EI group (EGF + IGF-I); EI-FCS group (EGF + IGF-I + 10% (v/v) FCS), and FCS group (10% (v/v) FCS) (Fig. 1).

After IVM, oocytes (n = 1588) were mechanically denuded by repeated aspiration with a fine-bore pipette and fixed in 4% PF solution. Chromosomes were stained with Hoechst 33342 (10 μg/mL), and nuclear maturation was measured as MII rate.

2.6. Cortical granule (CG) and mitochondrial distribution

A total of 594 oocytes were also stained to visualize CGs (n = 382) and mitochondria (n = 212). Visualization of CGs and mitochondria was performed according to Arias-Álvarez et al. [24] with minor modifications; oocytes were treated with 0.5% pronase after CC removal, fixed in 4% PF, and stored in PBS at 4 °C. Then, oocytes were treated with 0.02% v/v Triton X-100 and 0.75% BSA in PBS. CGs were stained with FITC of *Lens culinaris* (100 μg/mL) and with 180 nM MitoTrackerRed CMX Ros (Molecular Probes Inc) for mitochondrial visualization. Oocytes were examined under a confocal laser-scanning microscope (Leica, TCS SP2) at 488 and 546 nm to visualize CGs and mitochondria, respectively. Format, laser, gain and offset were kept constant for every sample. Sections of 5 μm each were made for each oocyte and a maximum projection was accomplished for each one.

Furthermore, a total of 40 immature (before maturation) and 33 *in vivo*-matured guinea pig oocytes were also used to verify CG (n = 20 and 16, respectively) and mitochondrial migration patterns (n = 20 and 17, respectively). These migration patterns were described based on previous studies in other species for CGs [24] and mitochondria [15].

2.7. Cumulus cell apoptosis

The rest of the COCs after IVM (n = 158) were fixed in 4% PF solution for 1 h at room temperature. Apoptosis was analyzed as previously described by Arias-Álvarez et al. [23] using the In Situ Cell Death Detection Kit, POD (Roche Diagnostics), according to the manufacturer's instructions. COCs were treated with RNAses before staining. As a negative control, samples were incubated with the label solution of the TUNEL reaction mixture without the enzymatic solution. COCs were counterstained with Hoechst 33342 (10 μg/mL), mounted (ProLong Gold antifade reagent, Invitrogen) and observed under a fluorescent microscope (F550; Leica). Samples were analyzed using IMAGE J/FIJI 1.46 software. The apoptosis index was calculated from the relation between green area and blue area × 100, and was $\log_{10}$-transformed before statistical analysis to achieve normal distribution (Supplementary Fig. S2).

2.8. Sperm preparation and in vitro fertilization (IVF)

Guinea pig spermatozoa were obtained as previously described by Mújica and Ruiz [25] by flushing the lumen of each cauda epididymis with 0.154 M NaCl (2 mL per vas) at 37 °C. Then, sperm was selected and capacitated following a protocol from Cañón-Beltrán et al. [26]. Sperm was centrifuged for 10 min at 250×*g* through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure (Nidacon Laboratories AB), according to the manufacturer's instructions. The sperm pellet was isolated and washed in 3 mL of Boviwash (Nidacon Laboratories AB) by centrifugation at 250×*g* for 5 min. The pellet was re-suspended in the remaining 300 μL of Boviwash. Later, spermatozoa were incubated in 500 μL of fertilization FERT-TALP medium (Merck) supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL fatty acid-free BSA and 10 μg/mL heparin sodium salt (Calbiochem). The final concentration of spermatozoa was adjusted to 1×10^6 spermatozoa/mL.

A total of 300 COCs from the IVM groups with the combination of the best concentrations of EGF and IGF, with or without the FCS, were used to assess oocyte competence for fertilization and early embryo development *in vitro* (EI = 50 ng/mL EGF + 100 ng/mL IGF-I and EI-FCS = 50 ng/mL EGF + 100 ng/mL IGF-I + 10% (v/v) FCS). COCs were co-incubated for 18–22 h in 500 μL fertilization media (Tyrode's medium) in four-well cell culture plates in groups of ~30 COCs per well under an atmosphere of 5% CO_2 in the air, with maximum humidity and at 38.5 °C. Three replicates were performed.

2.9. In vitro culture of presumptive zygotes

At 18–22 h post-insemination (hpi), presumptive zygotes from each experimental group (EI n = 131 and EI-FCS n = 129), were mechanically denuded as described before, and then cultured in groups of 25 in 25 μL droplets of culture medium (synthetic oviductal fluid (SOF) with 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 μL/mL basal medium eagle (BME) amino acids, 10 μL/mL minimum essential medium (MEM) amino acids and 1 g/mL phenol red, and 5% FCS under mineral oil. Plates were incubated at 38.5 °C under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 with maximum humidity (Thermo Fisher Scientific, USA). Cleavage rate was recorded at Day 2 (48 hpi) and its subsequent development was observed on Days 4 and 5 post-insemination. The cleavage and developmental rate were calculated based on the total number of presumptive zygotes in the culture.

2.10. Statistical analysis

Data were analyzed using the Statistical Analysis System software (SAS, v.9.4). Mean values were subjected to Chi-square test to compare nuclear maturation, CG and mitochondria migration rates of IVM oocytes among experimental groups and experiments. The apoptotic index was analyzed by a one-way ANOVA test using Duncan post-hoc test. Data on cleavage rates and embryo yield were analyzed using Chi-square test and were obtained with the SigmaStat software package. Differences were considered significant when $P < 0.05$, and trend to significance when $P < 0.1$.

3. Results

3.1. Immunolocalization of EGF and IGF-I receptors in the ovary

As depicted in Fig. 2, positive immunostaining for EGF-R (panel a) and IGF-IR (panel b) was immunolocalized in guinea pig ovaries. Corpus luteum showed moderate immunoreactivity (++) for EGF-R (2a1), and it was weak (+) in IGF-IR (2b1). EGF-R showed weak staining (+) in the oocytes and granulosa cells of the primordial and primary follicles (2a2). In secondary follicles, EGF-R showed moderate staining (++) in oocytes and granulosa cells, and weak staining (+) in theca cells (2a3), whereas immunoreaction increased in antral follicles (+++) (2a4). For IGF-IR, primordial and primary follicles showed moderate staining (++) in oocytes and granulosa cells (2b2), as well as in the secondary follicles (++), but most of the theca cells showed weak staining (+) in such follicles (2b3). In antral follicles, oocytes showed moderate staining (++), whereas granulose and theca cells showed strong (+++) immunostaining (2b4). For both receptors, non-specific immunoreactions were observed when staining was performed in the negative control.

3.2. Nuclear maturation, CG and mitochondrial distribution patterns in oocytes before and after ovulation or IVM

Before IVM, all the immature oocytes presented GV nuclear morphology, and 100% of *in vivo*-matured oocytes reached the MII configuration.

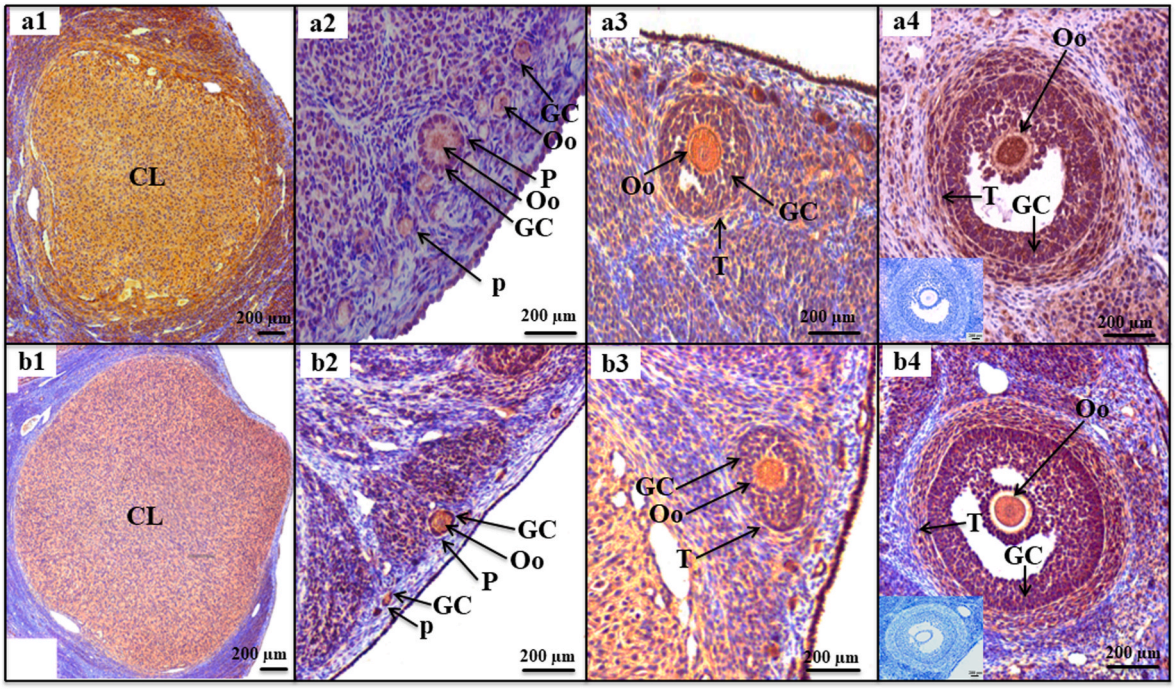

Fig. 2. Immunolocalization of EGF-R (a) and IGFI-R (b) in guinea pig ovary. Red staining denotes receptor localization. a1) Corpus luteum (CL: ++), a2) Primordial and primary follicles (GC, Oo: +), a3) Secondary follicles (GC, Oo: ++ and T: +), a4) Antral follicles (GC, Oo and T: +++), b1) Corpus luteum (CL: +), b2) Primordial and primary follicles (GC, Oo: ++), b3) Secondary follicles (GC, Oo: ++; T:+), b4) Antral follicles (Oo: ++ and GC, T: +++). GC: granulosa Cells; Oo: oocyte; T: theca cells; CL: corpus luteum; p: primordial follicle; P: primary follicle. Representative photomicrographs are shown. Inserts in a4 and b4 images show non-specific immunoreaction in the negative control. Scale bar is 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The CG patterns observed in guinea pig oocytes before and after ovulation or IVM (Fig. 3a) were defined as: non-migrated (3a1) (CGs distributed throughout the cytoplasm and considered non-cytoplasmically matured); partially migrated (3a2) (most of the CGs spread throughout the cortical area); and migrated (3a3) (CGs adjacent to the plasma membrane and peripheral distribution that is considered cytoplasmically matured).

CGs migrated to the periphery were the main pattern observed in the

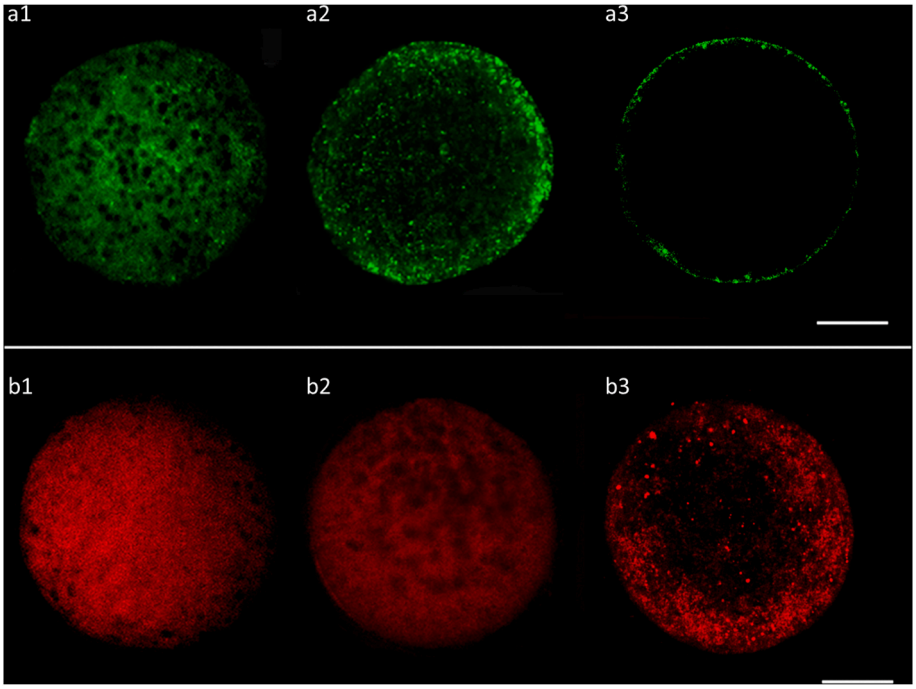

Fig. 3. Representative images of (a) cortical granule (CG) and (b) mitochondrial distribution patterns in guinea pig oocytes after *in vitro* maturation visualized under confocal laser microscopy. (a1, a2, a3) CG migration patterns, (a1) homogeneous distribution, (a2) cortical distribution, (a3) peripheral distribution, (b1, b2, b3) mitochondrial migration patterns, (b1) non-migrated (homogeneous), (b2) clustered, (b3) migrated (polarized). Scale bar 30 μm.

oocytes after IVM with EGF and IGF-I supplementation in the media (71.9–76.9%, respectively), as well as in *in vivo* matured oocytes (87.5%). The remaining 12.5% of *in vivo* matured oocytes were partially migrated. In immature oocytes, homogeneous distribution of GCs was prevalent (70%), being the rest partially migrated (20%) and migrated (10%).

The mitochondrial patterns (Fig. 3b) were classified as non-migrated (homogeneous) (3b1) when the mitochondria were homogeneously distributed throughout the cytoplasm (considered non-cytoplasmically matured); clustered (3b2) when the mitochondria were heterogeneously distributed with granular aggregations; and migrated (polarized) (3b3) when the mitochondria were relocated to the cortical area (considered cytoplasmically matured).

Migrated distribution of mitochondria is the most abundant pattern observed in the oocytes after EGF and IGF-I supplementation in the maturation media (80.0–73.3%, respectively) and in *in vivo* matured oocytes (82.4%). For the rest of *in vivo* matured oocytes, 11.8% were clustered and 5.9% non-migrated patterns. In immature oocytes, the main pattern found was mitochondria distributed throughout the cytoplasm (60%). The remaining 40% of immature oocytes showed clustered (20%) and migrated patterns (20%).

3.3. Experiment 1: effect of EGF on in vitro oocyte maturation and apoptosis index in cumulus cells

A higher MII rate was found when the oocytes underwent IVM with 50 ng/mL EGF, compared to the rest of experimental groups ($P < 0.05$) (Table 1). Also, this group showed a higher percentage of oocytes with a peripheral migration pattern of CGs and a migrated mitochondrial distribution, compared to the group without EGF ($P < 0.05$). Apoptosis rate was significantly lower for groups with 50 ng/mL EGF, compared with those cultured without EGF ($P < 0.05$). There were no significant differences among groups in the other CG and mitochondrial migration patterns studied, although FCS supplemented group tended to show higher values in the percentage of oocytes showing migrated CGs ($P < 0.1$) and also a lower apoptosis rate ($P < 0.05$), compared to the control group (Group 0).

Table 1
Nuclear and cytoplasmic maturation rates in guinea pig oocytes and apoptotic index of COC IVM with different concentrations of Epidermal Growth Factor (EGF).

	0 ng/mL EGF	10 ng/mL EGF	50 ng/mL EGF	100 ng/mL EGF	10% FCS
MII rate n (%)	115 (43.5)^{a}	114 (51.8)^{a}	116 (75.9)^{b}	108 (53.7)^{a}	111 (50.5)^{a}
Cytoplasmic maturation					
Cortical granules					
Migrated n (%)	10 (32.3)^{a}*	14 (45.2)^{ab}	23 (71.9)^{b}	12 (50.0)^{ab}	16 (64.0)^{ab}*
Partially migrated n (%)	15 (48.4)	11 (35.5)	7 (21.9)	10 (41.7)	7 (28.0)
Non-migrated n (%)	6 (19.4)	6 (19.4)	2 (6.3)	2 (8.3)	2 (8.0)
Mitochondrial distribution					
Migrated n (%)	5 (27.8)^{a}	8 (57.1)^{ab}	12 (80.0)^{b}	5 (31.3)^{a}	10 (66.7)^{ab}
Clustered n (%)	4 (22.2)	3 (21.4)	1 (6.7)	6 (37.5)	1 (6.7)
Non-migrated n (%)	9 (50.0)	3 (21.4)	2 (13.3)	5 (31.3)	4 (26.7)
Apoptotic index n (%)	13 (28.7 ± 1.4)^{a}	11 (21.1 ± 1.2)^{ab}	11 (17.2 ± 0.9)^{b}	13 (19.7 ± 1.2)^{ab}	10 (16.0 ± 1.2)^{b}

Data are mean ± s.e.m. within rows; different superscript letters indicate significant differences ($P \leq 0.05$) between treatments. Data with * show a trend ($P < 0.1$).

3.4. Experiment 2: effect of IGF-I on in vitro oocyte maturation and apoptosis index in cumulus cells

As shown in Table 2, supplementation with 100 ng/mL IGF-I significantly stimulated nuclear oocyte maturation in guinea pig oocytes ($P < 0.05$). This group showed a higher percentage of CG peripheral patterns, compared with the group without IGF-I or with 50 ng/mL IGF-I ($P < 0.05$). The lowest rates of non-migrated CGs were found in the groups supplemented with 100 and 200 ng/mL IGF-I, compared to the non-supplemented group ($P < 0.05$). No significant differences were found in the rest of mitochondrial patterns between experimental groups. The apoptotic index of the CCs was significantly lower in the groups with 50 or 100 ng/mL IGF-I than in the group without IGF-I ($P < 0.05$).

3.5. Experiment 3: effect of EGF plus IGF-I on in vitro oocyte maturation and apoptosis index in cumulus cells

Metaphase II rate was significantly increased in oocytes undergoing IVM with the combinations of both growth factors (50 ng/mL EGF and 100 ng/mL IGF-I) alone (EI group) or with 10% FCS (EI-FCS group), compared to the group without growth factors (0 group) and the group supplemented with only 10% FCS (FCS group) ($P < 0.05$) (Table 3). The group supplemented with FCS tends to show an increase in the MII rate as well ($P < 0.1$), compared to the control group (0 group). The highest percentage of oocytes with migrated CG distribution was shown in the EI-FCS group ($P < 0.05$), whereas the 0 group showed the highest rate of oocytes with a non-migrated pattern of CGs ($P < 0.05$). No significant differences were found in the mitochondrial distributions studied between groups. The percentage of apoptosis in the CCs of the EI-FCS group was significantly lower, compared to the rest of the experimental groups ($P < 0.05$).

3.6. Experiment 4: effect of EGF plus IGF-I with or without fetal calf serum in embryo development

No differences were observed in the cleavage rate 48 h post-incubation between the EI and the EI-FCS groups (74.1 and 79.8%, respectively). In addition, early embryo development rate at Day 4 was

Table 2
Nuclear and cytoplasmic maturation rates in guinea pig oocytes and apoptotic index of COC IVM with different concentrations of Insulin-like growth factor (IGF-I).

	0 ng/mL IGF-I	50 ng/mL IGF-I	100 ng/mL IGF-I	200 ng/mL IGF-I	10% FCS
MII rate n (%)	113 (38.9)^{a}	114 (48.2)^{a}	112 (72.3)^{b}	112 (45.5)^{a}	117 (50.4)^{a}
Cytoplasmic maturation					
Cortical granules					
Migrated n (%)	9 (31.0)^{a}	13 (43.3)^{a}	23 (76.7)^{b}	16 (55.2)^{ab}	15 (53.6)^{ab}
Partially migrated n (%)	10 (34.5)	15 (50.0)*	6 (20.0)*	12 (41.4)	10 (35.7)
Non- migrated n (%)	10 (34.5)^{a}	2 (6.7)^{ab}	1 (3.3)^{b}	1 (3.4)^{b}	3 (10.7)^{ab}
Mitochondrial distribution					
Migrated n (%)	6 (40.0)	8 (53.3)	11 (73.3)	7 (46.7)	9 (60.0)
Clustered n (%)	5 (33.3)	3 (20.0)	4 (26.7)	4 (26.7)	3 (20.0)
Non-migrated n (%)	4 (26.7)	4 (26.7)	0 (0.0)	4 (26.7)	3 (20.0)
Apoptotic index n (%)	11 (34.6 ± 1.5)^{a}	12 (14.5 ± 1.1)^{bc}	11 (12.8 ± 0.9)^{b}	11 (21.7 ± 1.2)^{ac}	11 (17.5 ± 1.2)^{abc}

Data are mean ± s.e.m. within rows; different superscript letters indicate significant differences ($P \leq 0.05$) between treatments. Data with * show a trend ($P < 0.1$).

Table 3
Nuclear and cytoplasmic maturation rates in guinea pig oocytes and apoptotic index of COCs IVM with the base medium (0 group), the combination of 50 ng/mL epidermal growth factor (EGF) and 100 ng/mL Insulin – like growth factor (IGF –I) (EI), the combination of 50 ng/mL EGF, 100 ng/mL IGF-I and 10% fetal calf serum (EI-FCS), or 10% FCS alone.

	0	EI	EI-FCS	10% FCS
MII rate n (%)	112 (38.4)^{a*}	115 (78.3)^{b}	116 (83.7)^{b}	113 (55.8)^{a*}
Cytoplasmic maturation				
Cortical granules				
Migrated n (%)	5 (23.8)^{a*}	13 (59.1)^{ab*}	20 (76.9)^{b}	12 (50.0)^{ab}
Partially migrated n (%)	6 (28.6)	7 (31.8)	5 (19.2)	11 (45.8)
Non- migrated n (%)	10 (47.6)^{a}	2 (9.1)^{b}	1 (3.8)^{b}	1 (4.2)^{b}
Mitochondrial distribution				
Migrated n (%)	5 (35.7)	8 (53.3)	10 (66.7)	8 (53.3)
Clustered n (%)	4 (28.6)	2 (13.3)	2 (13.3)	4 (26.7)
Non-migrated n (%)	5 (35.7)	5 (33.3)	3 (20.0)	3 (20.0)
Apoptotic index n (%)	11 (25.8 ± 1.4)^{a}	11 (22.7 ± 1.3)^{a}	11 (6.6 ± 0.7)^{b}	11 (22.8 ± 1.3)^{a}

Data are mean ± s.e.m. within rows; different superscript letters indicate significant difference ($P \leq 0.05$) between treatments. EI = 50 ng/mL EGF and 100 ng/mL IGF-I; EI-FCS = 50 ng/mL EGF, 100 ng/mL IGF-I and 10% (v/v) FCS. Data with * show a trend ($P < 0.1$).

significantly higher ($P < 0.05$) for EI-FCS, compared to the EI group (Table 4, Fig. 4).

4. Discussion

In the present work, we approach to IVM system with a non-hormonal IVM medium with EGF and IGF-I supplementation [22,23] applied to a guinea pig model. Furthermore, for the first time in the guinea pig oocyte, we characterized the compatible distribution patterns of CGs and mitochondria as a method to study oocyte cytoplasmic maturation. In addition, we analyzed apoptotic rate in granulosa cells as a measure of COC quality in this species [23]. Finally, successful IVF and early embryo development were described for the first time in this species.

It is known that the effect of growth factors EGF and IGF-I are mediated by their receptors. These are membrane receptors found in different locations of the body, including the reproductive tract [27]. In the present study, we observed EGF-R expression in the follicles from the primordial to the antral stage, and this was similar to that described for humans [28]. In granulosa cells, EGF promotes Ca^{2+} efflux, improves their expansion during maturation, and stimulates estrogen production through aromatase activation [29]. In theca cells, staining also increased when the follicle achieved the antral state. Thecal cells may be an important synthesis site of growth factors involved in cell-to-cell interactions, since they secrete androgens, which indeed stimulate granulosa cells growth [30].

In guinea pigs, IGF-IR expression was also found in granulosa and theca cells of follicles at different developmental stages. These findings agree with those reported in mice [31] and humans [32]. The IGF family plays an important role in proliferation and differentiation of granulosa cells [32], follicle development, steroidogenesis and follicular atresia [31]. Therefore, the strong immunolocalization of EGF-R and IGF-IR in the granulosa and theca cells, especially in the antral follicles, suggests that both EGF and IGF-I may act directly on the final follicular development and maturation process of guinea pig oocytes, as suggested in other species [28,31,32].

Table 4
Effect of EGF and IGF-I supplementation with or without 10% FCS on *in vitro* maturation of guinea pig oocytes and subsequent early embryonic development.

Groups	Total No. of presumptive zygotes in culture (n)	Cleavage rate n (%)	Morulae at Day 4 n (%)
EI	131	97 (74.1)	28 (21.3)^{a}
EI-FCS	129	103 (79.8)	38 (29.5)^{b}

n: number of presumptive zygotes per group. EI oocytes cultured in presence of 50 ng/mL EGF and 100 ng/mL IGF-I, and EI-FCS oocytes cultured in media supplemented with 50 ng/mL EGF, 100 ng/mL IGF-I and 10% FCS. Data are mean ± s.e.m. Values with different superscript in each column indicate significant differences ($P < 0.05$).

Regarding the oocyte patterns characterized, our findings showed that, in most of the oocytes after IVM, the CGs migrated to the periphery, whereas a homogeneous distribution of CGs was prevalent in immature oocytes. These findings are similar to those found in rabbit [23] and sea urchin oocytes [33]. Conversely, bovine oocytes showed an asymmetric distribution of CGs [10]. With respect to mitochondrial patterns, we determined that a great number of the oocytes after IVM showed a migrated distribution as well. However, homogeneous mitochondria distribution throughout the cytoplasm was found mainly in immature oocytes. This pattern matches those previously described in mice [34] and sheep [8], but contrasts with cattle [11], where non-matured oocytes contain small mitochondrial clumps located at the cytoplasm periphery. Upon maturation, the intensity and size of mitochondrial clumps increase, and staining is observed in the central parts of the cytoplasm. In contrast, dogs [15] display diverse mitochondrial patterns in oocytes, including diffused tubular networks spread throughout the cytoplasm and tubular networks localized in the pericortical region, with a finely granular appearance in the inner part of the cytoplasm. Differences in mitochondria distribution patterns between species may be influenced by several factors, and mainly depend on the oocyte energy requirements of each species. It is well-established that mitochondrial distribution plays a role in the concentration of ATP or Ca^{2+} within specific cellular regions in oocytes to engage in processes such as oocyte maturation, fertilization and early embryo development [11,23]. Furthermore, different studies reported that mitochondrial dysfunctions in the oocyte may be a critical determinant in embryonic developmental competence by inducing chromosomal defects, and such dysfunctions also trigger apoptosis in the embryo [11,35].

Our results showed that supplementation with 50 ng/mL EGF in guinea pig maturation media improved MII rate and cytoplasmic maturation, both in terms of CG migration and mitochondrial redistribution; also, the quality of the CCs seemed to be improved, since a lower apoptotic rate was found in them. These results agree with those of previous authors reporting that EGF at this concentration promotes nuclear and cytoplasmic maturation [5,6,16], improves oocyte developmental competence and mitochondrial activity [6], and stimulates granulosa cell proliferation [36], acting as a mitogen and apoptotic survival factor during early embryo development [37].

Higher rates of MII and CG migration in oocytes and a lower index of apoptosis in CCs were also found when 100 ng/mL IGF-I were added to the maturation media. Previous studies have also demonstrated substantial improvements in nuclear [14,16] and cytoplasmic maturation, in terms of CG migration rates [9,38], when IGF-I was added to the maturation medium. Ergin et al. [31] showed that IGF-I regulates rat luteal steroidogenesis and acts as an anti-apoptotic factor during oocyte maturation in bovine [13] and porcine oocytes [39]. However, other authors failed to detect any stimulatory effect of IGF-I on murine [18] and sheep oocytes [4].

As we expected, the addition of both growth factors, together with FCS, improved nuclear maturation and CG migration rate, and reduced apoptotic index, compared to those COCs matured *in vitro* without growth factors. The stimulatory effects of EGF and IGF-I on the aromatase activity of CCs have also been described in other species [30,40]. The interaction between growth factors and CCs can modify steroid production, which indirectly affects oocyte maturation and CC proliferation [11]. In fact, the concentrations at which both growth factors improve nuclear oocyte maturation in guinea pig oocytes are similar to those found in rabbit [16] and bovine oocytes [17]. Surprisingly, both

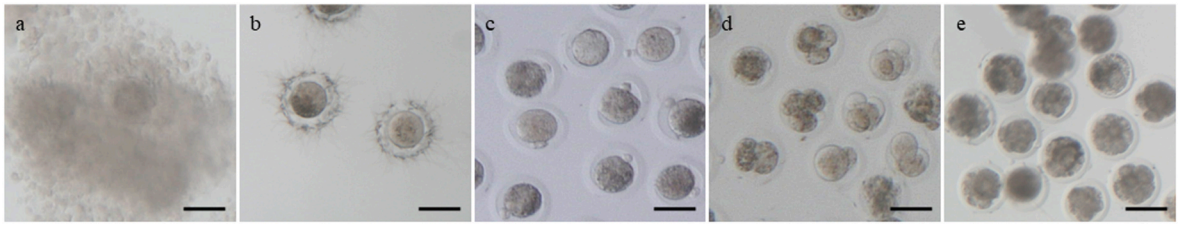

Fig. 4. Representative images of IVF in guinea pig. (a) Expanded cumulus oocytes complexes (COCs) after IVM, (b) sperm-oocyte binding during IVF, (c) presumptive zygotes (arrow shows polar body), (d) cleaved embryos at Day 2 (48 hpi), (e) morula-stage embryos at Day 4. Scale bar 100 μm.

growth factors together did not have a synergic effect on oocyte maturation, compared to when they were added individually, as shown in rabbits [16]. Moreover, no significant improvements were found in mitochondria patterns, unlike when IVM media was supplemented only with IGF-I. The cellular and molecular mechanisms by which IGF-I alone or together with EGF influence mitochondrial migration are unknown for all species.

Improvement in oocyte quality by EGF plus IGF-I supplemented with FCS (EI-FCS group) was reflected in the increased early embryo development rate at Day 4, compared to the EI group. Studies in other species have shown that the quality of the embryos is determined by the first cleavage division, embryo genome activation (EGA), and compaction of the morula. The intrinsic quality of oocytes is closely linked to the successful progression of events leading up to early EGA [41].

In guinea pigs, it is unknown when EGA occurs, and there are only a few studies assessing embryonic development *in vitro*, with no promising results. Suzuki et al. [42] showed that 2-cell embryos cultured in Whitten's medium developed to the 4-cell and morula stages, respectively, whereas comparable embryos cultured in Kane's medium arrested development at the 2-8-cell stages. Shi et al. [21], showed that morulae or blastocysts collected on Day 6 of pregnancy and IVC in a mixture of RPMI 1640 and Dulbecco's Modified Eagle's Medium (DMEM, high glucose modification, and BSA) during 8 days achieved 100% trophoblast outgrowth. In our system, we obtained *in vitro* MII oocytes (~75% IVM rate) capable of developing into early embryos from non-gonadotropin-treated laboratory guinea pigs, achieving better results than those from the study by Yao et al. [43], where they reported an IVM success rate of 69% using guinea pig oocytes obtained by *in vivo* ovarian stimulation with human chorionic gonadotropin (hCG). Our results showed that oocyte maturation with growth factors IGF and EGF helps the fertility process, since high cleavage rates were found in both experimental groups, similar to those obtained in cat [37], mouse [44] and bovine [45]. This could be related to the successful CG migration percentages achieved. Hosoe and Shioya [10], and Hoodbhoy et al. [46] demonstrated that proteins released by the CGs are necessary for preimplantation embryo development. Our study demonstrates that IVM oocytes are competent to maintain the first embryo divisions. As we expected, supplementation with FCS during IVM even improves these results, as in other species [22]. However, further studies are needed to find the optimum media and complementary markers of oocyte developmental competence to improve the early embryo development *in vitro* into the blastocyst stage in this species.

In conclusion, guinea pig oocytes respond to EGF and IGF-I, since receptors have been found in all ovarian compartments, including antral follicles. The proposed medium with 50 ng/mL EGF or 100 ng/mL IGF-I or a combination of both successfully led to oocyte maturation and reduced the apoptotic rate by improving COCs quality. The addition of both growth factors supplemented with FCS improved cleavage and early embryonic development, although further studies should be conducted in order to optimize the IVC system. Finally, the cytoplasmic patterns characterized contribute to improve understanding of oocyte maturation mechanisms in the guinea pig.

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CRediT authorship contribution statement

Karina Cañón-Beltrán: Conceptualization, Methodology, Design of the experiments, Writing – review & editing, Funding acquisition. **Rosa M. García-García:** Conceptualization, Methodology, Design of the experiments, Writing – review & editing, Funding acquisition. **Yulia N. Cajas:** Methodology, Formal analysis, Writing – original draft. **Natacha Fierro:** Methodology, Formal analysis, Writing – original draft. **Pedro L. Lorenzo:** Conceptualization, Methodology, Design of the experiments, Writing – review & editing, Funding acquisition. **María Arias-Álvarez:** Conceptualization, Methodology, Design of the experiments, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

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