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Cumulus cells steroidogenesis is influenced by the degree of oocyte maturation

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Abstract

Background: The possibility to predict the ability of a germ cell to properly sustain embryo development in vitro or in vivo as early as possible is undoubtedly the main problem of reproductive technologies. To date, only the achievement of nuclear maturation and cumulus expansion is feasible, as all the studies on cytoplasmic maturation are too invasive and have been complicated by the death of the cells analyzed. The authors studied the possibility to test the cytoplasmic quality of pig oocytes by evaluating their ability to produce steroidogenesis enabling factor(s). To this aim, oocytes matured under different culture conditions that allowed to obtain gradable level of cytoplasmic maturation, were used to produce conditioned media (OCM). The secretion of the factor(s) in conditioned media was then recorded by evaluating the ability of the spent media to direct granulosa cells (GC) steroidogenesis.

Methods: In order to obtain germ cells characterized by a different degree of developmental competence, selected pig oocytes from prepubertal gilts ovaries were cultured under different IVM protocols; part of the matured oocytes were used to produce OCM, while those remaining were submitted to in vitro fertilization trials to confirm their ability to sustain male pronuclear decondensation. The OCM collected were finally used on cumulus cells grown as monolayers for 5 days. The demonstration that oocytes secreted factor(s) can influence GC steroidogenesis in the pig was confirmed in our lab by studying E_2 and P_4 production by cumulus cells monolayers using a radioimmunoassay technique.

Results: Monolayers obtained by growing GC surrounding the oocytes for five days represent a tool, which is practical, stable and available in most laboratories; by using this bioassay, we detected the antiluteal effect of immature oocytes, and for the first time, demonstrated that properly matured germ cells are able to direct cumulus cells steroidogenesis by inhibiting E_2 production ($P < 0.01$). Nevertheless, only fully competent oocytes were able to suppress estrogens production, while those cultured under unfavourable conditions were unable to exert any inhibitory effect on the functions of cumulus cells ($P < 0.01$).

Conclusion: These results demonstrated that good quality oocytes can be easily selected on the basis of their ability to affect granulosa cell steroidogenesis thus reducing failures in reproductive technologies due to the transfer of fertilized oocytes with a scarce ability to sustain embryo development.

Background

Oocyte developmental competence, which involves the ability of a germ cell to produce a normal and viable embryo after fertilization, is a condition that results from both nuclear and cytoplasmic maturation. Under natural conditions, the occurrence of maturation is characterized by a high developmental competence of the cell but when this process is carried out in experimental conditions (in vitro or by in vivo superovulation techniques), oocyte quality drastically falls despite all the advances made in this field [1–4]. There is experimental evidence that oocyte developmental competence strictly depends on the process of maturation and that the quality of the oocytes produced is necessarily predicated upon the cultural methods used [5–7]. Hence, different systems in vitro may yield oocytes of different quality and the same variability is to be expected during maturation in vivo under different superovulation programs [3]. In reproductive biotechnology it is thus important that the developmental competence of a female germ cell be predicted not only as soon as possible during maturation in vivo or in vitro, but also without interfering with the function or viability of the gamete itself. So far, parameters used as markers of oocyte quality, such as the GSH (glutathione) content of the oocyte [8–11], the redistribution of cortical granules [12–15], the reprogramming of protein synthesis [16,17], the translocation of mitochondria [18], and the ability to store adequate intracellular Ca concentrations [19,20] have all proved to have limited practical applications because of the difficulties encountered in monitoring a single cell without compromising its integrity.

About thirty years ago El-Fouly and colleagues demonstrated that the removal of the oocyte from Graafian follicles of a rabbit promotes spontaneous luteinization and P_4 production [21]. It took more than 20 years before another study showed that oocytes secrete a factor that modulates E_2 and P_4 production in vitro [22]. Since then, a growing body of evidence has suggested that many functions of somatic follicular cells can be affected by the oocyte; and because the response to oocyte signals does not require a physical contact between the cells, the active substance must be a soluble factor secreted in the medium by the germ cell. For example, mouse, cow and pig oocytes – although at a different stage of development – secrete a cumulus expansion enabling factor that has been found to stimulate mucification and the production of hyaluronic acid, a component of cumulus mass extracellular matrix [23–28]. Moreover, soluble factors produced by germ cells can affect a variety of functions, namely granulosa cell proliferation [29,30], dimeric inhibin A and B secretion [31], inhibition of plasminogen activator production [32], and expression of LH receptors [33]. Finally, there is evidence that the oocyte plays an active role in the steroidogenic function of GC in many species [22,28,34–37]. Although the effects of these oocyte-secreted factors have been extensively studied and many of them can be mimicked by the growth differentiation factor-9 (GDF-9) [38,39], less is known about their nature [27,35] and their physiological role.

Thus, because indirect information may be obtained on the status of the germ cell by observing the function of granulosa cells, our hypothesis was to exploit the presence of signals from the oocytes to the somatic compartment of the follicle to predict the developmental competence of the germ cell with a non-invasive marker. A cell line was identified which was characterized by a detectable, stable and repeatable production of steroid hormones and would permit observation of the effect of porcine oocyte-secreted factors on follicular progesterone (P_4) and estradiol 17β (E_2) production during maturation, from GV until MII phase, and finally by mature cells differing on the basis of their degrees of cytoplasmic maturation.

Methods

Cumulus cell isolation and culture

Ovaries of prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory within 2 h while kept at a temperature of 25°C. After washing in normal saline, the ovaries were mechanically dissected under sterile conditions in Dulbecco's phosphate buffered saline (D-PBS, Sigma) containing 0.4% bovine serum albumin (BSA fraction V, Sigma) and 70 mg/L of kanamicin (Sigma). Selected healthy follicles of 4 to 5 mm in diameter were opened and everted to recover the cumulus-oocyte complexes (COCs). After oocyte removal, cumulus cells were mechanically disaggregated, pooled, and washed three times through a series of centrifugations at 800 g for 5 min in D-PBS/BSA. The final pellets were resuspended in TCM 199 (Sigma) supplemented with 10% heat inactivated foetal calf serum (FCS, Sigma), 5 mg/L insulin, 5 mg/L transferrin and 5 μg/L sodium selenite (ITS, Sigma) and 1.25 μM Androstenedione (4-androstene-3,17-dione, Sigma). After evaluating the viability of the cells with Trypan blue staining (0.2% w/v, Sigma), cumulus cells were plated in a 96-well plate (Greiner) at a concentration of 1×10^4 viable cells/well (5×10^4 viable cells/mL) to be cultured at 38.5°C in a humidified incubator at 5% CO_2 for up to 7 days. In order to determine the most adequate culture conditions to sustain a stable and detectable steroid production, the saturating level of FSH required to stimulate steroidogenesis was evaluated at the beginning of our experiments by exposing cumulus cells to different concentrations of porcine FSH, from 10 ng/mL up to 1 μg/mL (USDA pFSH B-1). Estradiol 17β (E_2) and progesterone (P_4) levels were assayed every day throughout the culture period, which did not exceed 7 days of incubation so that GC maintained in vitro would not lose their ability to secrete estro-

gens due to the decline of aromatase activity during functional luteinization [40].

The cell cultures were always observed by a single operator and the medium replaced daily until the radioimmunoassay (RIA) was performed to detect steroid production. At the end of each experiment, the monolayers were detached by incubating the wells for 10 min at 38.5°C in a solution of Trypsin-EDTA (0.25% v/v, Sigma) in Ca^{++} and Mg^{++} free PBS. The cells were then washed by centrifugation at 800 × g for 5 min, stained with Trypan blue and counted in a haemocytometer to evaluate their viability. Steroid production was expressed as a function of living cells and all the data were then normalized to 10^4 viable cells/mL.

Pig oocyte preparation

Follicles of 4 to 5 mm in diameter, which had been isolated from the ovaries and treated as described for granulosa cell cultures, were selected on the basis of their translucent appearance, good vascularization, and compactness of their granulosa layer and cumulus mass. Part of the follicles were opened to harvest COCs and to isolate the group of immature oocytes.

For the preparation of fresh immature germ cells (GV oocytes), the selected oocytes were denuded by repeated pipetting using a narrow bore pasteur pipette and immediately used for the preparation of conditioned medium (see below). The procedure was carried out at 18–20°C.

For the preparation of MII oocytes, the process of maturation was obtained in vitro under different culture conditions to reach different degrees of cytoplasmic maturation, that corresponded to different rate of normospermia and ability to remodel male nucleus in pronuclear structure [41,42]. The selected COCs were cultured in the presence of everted follicle walls and hormonal support to obtain properly matured germ cells able to undergo normal fertilization (M+H oocytes). In brief, three healthy follicles of 4–5 mm in diameter were opened in Petri dishes containing about 50 selected COCs in 2 mL of medium TCM 199 added with 10% FCS, 70 mg/L kanamicin, ITS and 1 μg/mL of porcine LH and FSH (USDA-pLH-B-1 and USDA-pFSH-B-1) and turned inside out; the follicle walls were then placed on a stainless steel grid to avoid contact with the Petri dish bottom in this static system. A lower degree of cytoplasmic maturation was obtained by culturing the oocytes in the absence either of hormone support (M-H: COCs matured without hormones, but together with the everted follicle walls) or cell support as denuded oocytes (MD). The cultures of pig oocytes were carried out in at 38.5°C in a humidified incubator with an atmosphere of 5% CO_2, for 44 h. At the end of this period, only fully expanded COCs in the M+H and M-H groups were harvested and denuded in Hepes-TCM 199 with hyaluronidase on a warmed stage at 38.5°C under a stereomicroscope. In order to ensure the same procedure for all the classes of matured oocytes, the MD group, that had already been denuded at the beginning of the culture period, was also submitted to hyaluronidase treatment. Thereafter, only oocytes presenting the first polar body (MII oocytes) under the stereomicroscope were utilized for the following steps.

Preparation of OCM

Following the procedure described by Vanderhyden and colleagues [22], the oocytes belonging to the four classes described above (GV, M+H, M-H, MD) were incubated at the concentration of two oocytes/μL in eppendorf tubes containing TCM 199 supplemented with 10% FCS, 70 mg/L kanamicin, ITS (5 mg/L insulin, 5 mg/L transferrin and 5 μg/L sodium selenite) at 38.5°C in a humidified incubator with an atmosphere of 5% CO_2, in order to obtain OCM that differed from one another exclusively for the type of oocytes used, since the medium was the same for all the groups. To determine the optimal time required for the steroid enabling factor(s) to accumulate in the medium, preliminary experiments were performed maintaining the oocytes in culture for different periods of time (8 to 24 h). Eight hours of co-incubation was finally chosen since the effect of the media obtained after this length of time was not significantly different from that of the media collected after 16 or 24 hours (data not shown) and allowed to obtain OCM from immature oocytes without altering the nuclear status of the cell, that maintained the GV condition for at least 18 hours. At the end of the incubation, the OCM were recovered by centrifuging the eppendorf tubes at 800 × *g* for 5 min, and frozen at -20°C until use; the oocytes used to condition the media were always fixed in 25% (v/v) acetic acid in ethanol at room temperature, stained (Lacmoid, Sigma) and examined under a phase contrast microscope at X400 magnification to confirm nuclear status and check membrane integrity after the procedure of denudation.

Evaluation of oocyte developmental competence

In order to verify the developmental competence of pig oocytes matured in vitro under different culture conditions, part of the oocytes (approximately 20 oocytes per group) were submitted to an in vitro fertilization trial parallel to the experiment carried out for the preparation of conditioned media (4 replicates). For the IVF experiments, the oocytes matured under different culture protocols (M+H, M-H, MD), were incubated with capacitated boar sperm at the concentration of 1 × 10^6cells/mL in a 100 μL drop. The fertilization medium consisted of Brackett and Oliphant (B&O) [43] supplemented with 10% FCS, 5 mM calcium lactate, 2 mM caffeine, and 70 mg/L kanamicin. Capacitation of the semen used for the in vitro

fertilization was achieved according to the methods previously described by Barboni et al. [44]. After 2 hours of coincubation, the oocytes were carefully washed and maintained for other 8 hours in a fresh drop of B&O until they were fixed and stained to evaluate their fertilization rate and ability to sustain male pronuclei (PN) formation.

Exposure of cumulus cell monolayers to OCM

After standardization of the bioassay, the effect of oocyte-secreted factors on the steroidogenic secretions of granulosa cell monolayers was studied by challenging the cells with conditioned media obtained only with immature oocytes (GV) or mature oocytes (M+H). In a second set of experiments, coincubation was performed by using MII oocytes characterized by a different cytoplasmic maturation, namely MD and M-H, together with properly matured germ cells (M+H), and the GV oocytes seconding as a control. The wells of monolayers were challenged with 20 μL of the OCM obtained from pig oocytes thawed and diluted 1:1 in TCM 199 containing a double concentration of FCS (20% v/v), ITS (10 mg/L insulin, 10 mg/L transferrin and 10 μg/L sodium selenite), Androstenedione (2.5 μM) and FSH (200 ng/mL). Control media were prepared both with the medium alone (serving as a negative control) and with FSH (positive control). Coincubation was carried out at 38.5°C in a humidified incubator at 5% CO_2 for 12 h. After this period the media were recovered and frozen at -20°C until analyzed, and the cells were detached from the wells to be counted in a haemocytometer and stained with Trypan blue in order to normalize the steroid production for the number of live cells.

Steroid detection by radioimmunoassay

Progesterone and Estradiol 17β levels were determined by RIA using ^{3}H-P_4 and ^{3}H-E_2 (NEN – New England Nuclear). The intra and interassay coefficients of variation were 7.8% and 10.1% for P_4 and 4% and 12% for E_2, respectively. The cross-reactivity of progesterone antiserum was 9.7% with 11α-OH progesterone and 0.3% with 20α-OH progesterone. The cross-reactivity of estradiol 17β antiserum was 0.3% with estrone and estriol and 0.1% with 17α estradiol.

Statistical analysis

The experiments on the baseline production of granulosa cells (n = 10) and those with spent media obtained from the different classes of pig oocytes (n = 5) were performed carrying out at least three replicates for each batch. When comparing multiple treatment groups, data were expressed as mean ± standard deviation; differences between treatment groups were submitted to the Student-t test, while the analyses of variance relative to the time of the culture were carried out by ANOVA one-way. Data for the number of oocytes achieving the MII stage after different culture conditions, for fertilization rate and for the number of oocytes containing at least one male PN following in vitro fertilization were examined by χ^2 analysis. Values of $P < 0.01$ were considered statistically significant.

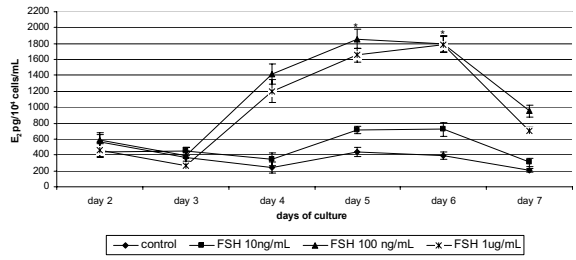

Figure 1
E_2 production by cumulus cell monolayers under chronic FSH stimulation at different concentrations for 7 days. Data are means ± s.d (n = 30). *Results are statistically significant for P < 0.01 both between treatment groups and in the same group depending on the day of culture (ANOVA one way-analysis of variance).

Results

Steroid production by cumulus cell monolayers

To identify which culture conditions were able to determine a stable steroidogenic secretion on cumulus cell monolayers, the primary cultures of cumulus cells were exposed to different FSH doses and the levels of E_2 within the culture were analyzed during the first seven days of culture. Monolayers obtained by plating cumulus cells initially displayed less responsiveness to FSH stimulation at all the doses used, since E_2 production remained at a mean level of approximately 431.0 pg/10^4cells/mL in all the FSH treatments analyzed as compared to 464.5 pg/10^4cells/mL in the control group. Responsiveness to FSH stimulation began on culture day 4 with a steroidogenic secretion that was directly related to the gonadotropin concentration in the system, as shown in figure 1. The production of E_2 was observed to increase in a dose-dependent fashion in that 10 ng/mL of FSH were only able to sustain a production 1.5 times higher than the baseline estradiol synthesis (345.0 ± 43.9 vs 243.0 ± 59.7 pg/10^4cells/mL), while 100 ng/mL of FSH drove the cumulus cell secretion up to 1415.3 ± 116.9 pg/10^4cells/mL, reaching levels that were significantly higher ($P < 0.01$) than the ones recorded in the control wells. One hundred ng/mL of FSH proved to be a saturating dose of gonadotropin, since higher doses of FSH (1 μg/mL) did not increase steroid production (1202.4 ± 88.3 pg/10^4cells/mL). After 4 days of culture, the steroidogenic activity of the cells stabilized,

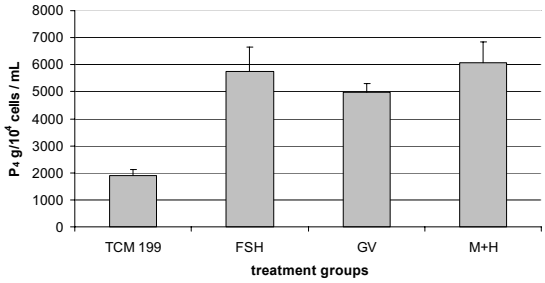

Figure 2
Mean progesterone production (± sd) by cumulus cell monolayers coincubated with spent media from immature (GV) or properly matured oocytes (M+H). Control wells were challenged with medium alone (for baseline P_4 production) or medium containing FSH (positive control).

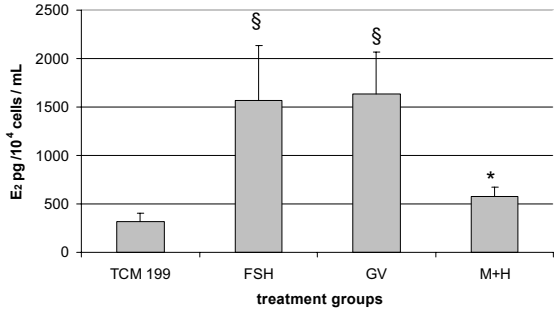

Figure 3
Mean estradiol 17β (± sd) production by cumulus cell monolayers coincubated with spent media from immature (GV) or properly matured pig oocytes (M+H). Control wells were challenged with medium alone (for baseline E_2 production, TCM 199) or medium containing FSH (positive control, for gonadotropin-stimulated production). *P < 0.01 (Student t-test).

and the levels of E_2 synthesis remained practically unchanged as of day 5, and declined the last two days of culture. On the basis of these results, all the following experiments were carried out by exposing the cumulus cell monolayers to the oocyte-conditioned media on the 5th day of culture and maintaining gonadotropin stimulation at a concentration of 100 ng/mL FSH during the culture.

Role of maturation on the production of oocyte-produced soluble factor(s)

Preliminary experiments were performed to determine the influence of the stage of maturation of the oocytes on P_4 and E_2 secretion by the cumulus cells. The cumulus cell monolayers were challenged with OCM obtained from GV oocytes and matured oocytes cultured as everted follicles in the presence of adequate hormonal support, such as 1 μg/mL of FSH and LH (group M+H).

The baseline P_4 production (figure 2) was slightly depressed when the cumulus cell monolayers were exposed to GV conditioned media and dropped from 5764.66 ± 875.9 in the FSH treatment group to 4980.0 ± 315.0 ng/10^4 cells/mL ($P > 0.05$). The matured oocytes, instead, were totally unable to influence P_4 synthesis (6086.66 ± 770.0 ng/10^4 cells/mL) and provided P_4 levels similar to those recorded in the control monolayer wells (FSH treated group).

As shown in figure 3, estradiol secretion of cumulus cell monolayers was totally unaffected by conditioned media obtained with GV oocytes, while it was significantly influenced by the soluble factors secreted by mature oocytes whose conditioned media always caused a strong and significant ($P < 0.01$) inhibition of estrogen synthesis (1570.28 ± 560.3 vs 1632.87 ± 430.5 vs 580.25 ± 89.7 pg/10^4 cells/mL for FSH stimulated vs GV vs MII oocytes respectively).

In order to know whether attaining an adequate cytoplasmic maturation was important for the production of soluble factor(s) by the oocytes, the cumulus cell monolayers were challenged with conditioned media obtained from MII oocytes. The MII oocytes belonged to the classes of cells that had matured in poor conditions, i.e. matured denuded oocytes (MD), or cells cultured as COCs without hormone support (M-H), or with a complete cell and gonadotropin support (M+H). The results of this challenge (figure 4) demonstrate that oocytes matured in the absence of cellular or hormonal support were unable to inhibit E_2 production by cumulus cell monolayers while they confirm ($P < 0.01$) the inhibitory role of media conditioned with M+H oocytes (600.57 ± 287.9 pg/10^4 cells/mL vs 1730.33 ± 205.4 pg/10^4 cells/mL for FSH treated group).

No class of MII oocytes, whatever the degree of competence achieved, exerted any effect on P_4 production by cumulus cell monolayers (figure 5).

Effect of culture conditions on the developmental competence of pig oocytes matured in vitro

At the end of the culture period (44 h), the percentage of oocytes presenting an extruded first polar body (MII

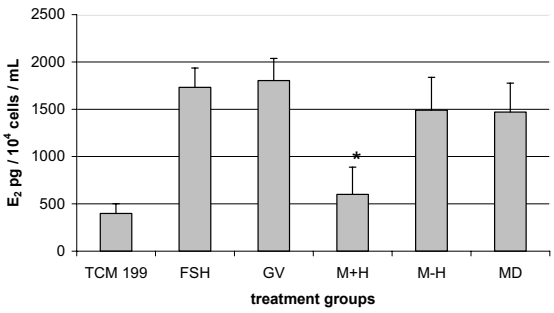

Figure 4
Challenge of cumulus cell monolayers with conditioned media from pig oocytes cultured in vitro under different, unfavorable conditions, namely COCs without hormonal support (M-H), and matured denuded oocytes (MD), compared to immature (GV) and properly matured cells (M+H). Histograms represent the mean E_2 production ± sd. Control wells were treated as in fig 2 and 3. * $P < 0.01$ (Student t-test).

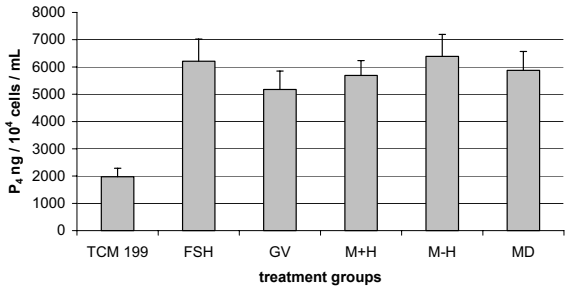

Figure 5
Mean P4 production (± sd) by cumulus cell monolayers challenged with conditioned media from different classes of pig oocytes matured in vitro (matured without hormones, M-H; matured denuded, MD; matured with hormones, M+H) vs immature (GV) cells, compared to baseline and FSH-stimulated production.

oocytes) for each treatment group in the five replicates did not differ significantly (see table 1). The fertilization rate and the ability of the cells to promote decondensation of sperm heads and formation of male PN were used as parameters of developmental competence. As shown in table 1, the fertilization rate was not significantly affected ($P > 0.05$) when the oocytes received cell support at least during the culture period (78.0%), while sperm penetration was significantly reduced in the denuded oocytes (56.3%; $P < 0.01$). The percentage of oocytes capable of sustaining the formation of male PN was significantly higher ($P < 0.01$) in those that had matured with adequate cell and gonadotropin stimulation (66.0%), while a progressive and significant reduction in PN formation was observed both in the oocytes that had matured in the absence of (30.7%) and in the denuded oocytes (6.0%).

Discussion

A correct nuclear and cytoplasmic maturation of the oocytes is essential for normal fertilization and male nucleus decondensation to occur, and thus permit subsequent embryo development. In 1989 Mattioli et al. demonstrated that coculturing pig oocytes in the presence of everted follicle walls and hormones in a non-static system yielded mature germ cells able to undergo normal embryonic development after in vitro fertilization; this was further shown by the number of blastocysts and newborns obtained from the transferred embryos [5]. The importance of follicle cells and their effect on in vitro fertilization has also emerged in this investigation with results that are in agreement with those of several authors [42,45,46]. Correct hormonal support is fundamental to achieve adequate developmental competence by increasing the number and permeability of gap junctions between the oocyte and cumulus cells, thus leading to a better interaction between the germinal and somatic compartment of the follicle [42,47]. In short, when IVM techniques do not provide the correct environment for the oocytes, although they may ensure nuclear maturation and some degree of fertilization after IVF, the end result is a low rate of poor quality blastocysts. Obtaining healthy and fully competent oocytes by in vitro or in vivo cultures is one of the major problems of reproductive techniques (reviewed in [46]). Indeed, nuclear maturation, i.e. the ability of the germ cell to resume meiosis, is easily detectable at the end of the culture period with the aid of a microscope, while cytoplasmic maturation cannot be measured with conventional, non-invasive tools. Cytoplasmic maturation, however, is as important as nuclear maturation in determining whether a germ cell is developmentally competent. There is a relatively large array of useful techniques by which several features of cytoplasmic maturation may be demonstrated by studying specific changes in the ooplasm during maturation, but most of them are very aggressive and lead to the death of the cell analyzed. The best approach will necessarily provide an integration of the different means of evaluation in order to gather as much information as possible.

Table 1: Fertilization rate and percentage of penetrated oocytes forming male pronuclei in pig oocytes cultured under different conditions and fertilized with capacitated boar sperm.

Treatment	MII oocytes	Fertilization rate	Oocytes forming male PN
M+H (n = 83)	90.2%[a] (n = 75)	84.0%[a] (n = 70)	66.0%[a] (n = 55)
M-H (n = 81)	70.3%[a] (n = 57)	78%[a] (n = 63)	30,7%[b] (n = 25)
MD (n = 80)	85.9[a] (n = 69)	56,3%[b] (n = 45)	6.0%[c] (n = 5)

The cultures were carried out in the presence of gonadotropins and everted follicle walls (M+H) or in the absence of hormonal (M-H) or of both hormonal and cellular support (MD: matured denuded). Columns with different superscripts differ $P < 0.01$ (υ^2 test)

In agreement with several authors [22,35], the findings of this investigation once again demonstrated the ability of porcine oocytes to affect cumulus cell steroidogenesis through the production of soluble factor(s). To test the biological activity of oocyte-secreted soluble factors, porcine cumulus cells were maintained in culture conditions that steadily produced steroidal hormones in four days, yielding detectable estrogens concentrations and responding to gonadotropin stimulation in a dose-dependent fashion. In the antral follicle there are two types of GC: those adjacent to the oocytes, called cumulus cells, and those lining the antrum, called mural granulosa cells. These cells have distinct functions and are phenotypically different, the latter showing more steroidogenic activity than the former [28]. In spite of the unquestionable evidence of such activity, our attempt to produce a stable bioassay by using plated mural GC failed since these MGC monolayers were absolutely irresponsive to gonadotropin stimulation while the cumulus cell monolayers were not (unpublished data). Unlike other authors that prefer intact cumuli to overcome problems such as gap junction disruption and changes in cytoskeleton organization [22], we decided to utilize the monolayer technique because it offered several practical advantages. In fact, when attempting to develop a repeatable bioassay, primary cultures are the only technique in which the number of plated cells may be known and differences between trials minimized. By working on this monolayer model, we demonstrated that pig oocytes can modulate cumulus cell steroidogenesis through the production of soluble factors and that this ability is strictly dependent on both the nuclear and cytoplasmic maturation achieved by the germ cell. Indeed, the pig oocytes prove to be capable of directing the functional production of cumulus cells by changing their signals on the basis of their own biological conditions. Immature oocytes (GV stage) are mainly capable of depressing P_4 production through an antiluteal function that is believed to be fundamental in coordinating the oocyte with the follicle, when the GC have to supply estrogens while, after the LH surge, the signals produced by the germ cell change. We were able to demonstrate that properly matured oocytes no longer affect progesterone production but acquire the ability to inhibit estrogens production. This result seems to agree with that of Glister and colleagues (48); working on MGC in the cow, although by using different methodological approach, they have shown that the oocyte, in the late stage of folliculogenesis, can suppress E_2 production and promote corpus luteum formation, probably through TGFα action. Our data, on the other hand, become extremely interesting since they demonstrate, for the first time, that the ability to modulate steroidogenesis of GC depends not only on the status of meiotic progression, but also on the degree of cytoplasmic maturation achieved by pig oocytes. Thus, properly matured oocytes not only control the activity of cumulus cells but also modify their messengers in order to favour the functional luteinization of GC after the gonadotropin surge. Spent media from oocytes possessing a poorly matured cytoplasm has very little effect on E_2 production by cumulus cells. By differentiating the secretion of estradiol when challenged with different classes of MII oocytes, our bioassay always responded in a sensitive, stable and coherent way, by sending signals in advance and properly differentiating competent cells from those unable to sustain normal embryo development [6,7,42].

In this study we demonstrated that only the class of oocytes matured in optimal conditions and thus more effective in transforming the male nuclei, significantly affected steroidogenesis by the cumulus cells. By contrast, no effect on E_2 production was exerted by oocytes whose nuclear maturation had not been accompanied by a correct cytoplasmic environment resulting in their inability to physiologically develop male PN. The worst culture conditions (absence of hormonal and cellular support) impaired the oocytes at the first step of fertilization by drastically reducing the percentage of spermatozoa able to enter the cells that were also completely unable to decondense.

Conclusions

Our findings showed that the use of pig cumulus cells cultured as monolayers is a practical and non-invasive method given that the oocytes can be kept in the same medium for a few hours and the exact conditions of the

cells are known in real time, well before the transfer of any embryo.

The possibility of using cumulus cell monolayers as a bioassay by which to evaluate the quality of maturation in vitro or in vivo is obviously of great advantage for both veterinary and human medicine, where maturation protocols are still far from reproducing the process of normal cycling that physiologically occurs in vivo. Together with the assessment of MII stage and cumulus expansion, the detection of oocyte-produced factor(s) or of their effect on a bioassay may constitute one of the most sensitive and non-invasive parameters for predicting the quality of maturation obtained in the laboratory. In the case of veterinary medicine, this assay could be extremely advantageous in helping biotechnologists reduce failures in reproductive techniques in animals of high genetic value, or those belonging to rare breeds or endangered species. In such cases, assessment of the ability of the matured oocytes to suppress E2 synthesis may be used together with the morphological scores of the embryos produced to avoid transferring embryos that are unable to develop properly.

The question remains as to whether the system should be brought to the point of detecting the soluble factor(s) produced by a single oocyte. Our current operating conditions allowed us to work with volumes of 10 μL; below that threshold no effect of the factor(s) could be detected (data not shown). There are two possible options that may be considered to counter this limit: i) the incubation time may be extended with a blind use of the fertilized ova; ii) cells in suspension and very small volumes may be used in the attempt to recreate the same conditions – in terms of stability and repeatability – as obtained in the monolayer system. Further investigations are certainly needed regarding both the chemical nature of the factor(s) and the specific role of their presence or absence in the conditioned media, and studies will have to focus on whether the evaluation of the capability of oocytes to affect cumulus cell steroidogenesis can be used as a definitive approach to predict the fate of the fertilized egg or of the blastocysts derived from those germ cells.

Authors' contributions

LP, BB and MM conceived, and designed the study. LP carried out and coordinated the experimental procedures. BN performed the statistical analysis. TM carried out the immunoassay. All the Authors read and approved the final manuscript.

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