Three-dimensional hormone-free coculture for the maturation of swine oocytes: an in vitro fertilisation test

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Introduction

Nowadays, *in vitro* maturation (IVM) protocols involve a large use of hormones during the maturation procedures, and there is growing evidence that *in vivo* hormonal stimulation induces a reduction in oocyte quality (Sirard et al., 2006). Therefore recent tendencies in reproductive biotechnologies are oriented towards limited hormonal stimulation in order to increase the yields of high-quality oocytes available for *in vitro* fertilisation (IVF) and embryo transfer (Sirard et al., 2006).
One approach to improving IVM and IVF techniques is to use granulosa cells (GC) in a coculture system: in this case, GC might produce a suitable microenvironment to promote oocyte maturation (Kreeger et al., 2005). A new hormone-free oocyte-GC coculture system has recently been proposed for human oocyte IVM (Vigo et al., 2004). A follicle-mimicking three-dimensional (3D) arrangement enhances maturation yields in a *in vitro* human fertilisation program (Vigo et al., 2005).
The aim of this work is to evaluate the competence of oocytes matured in a hormone-free 3D co-culture and the effect of corona radiata on maturation of competent oocytes.

Materials and methods

Oocyte in vitro maturation and fertilisation

Ovaries were taken from cycling gilts 9-10 months old in a abattoir; follicles were identified on the surface of the ovaries, and follicular fluids containing GC were aspirated, washed, centrifuged and diluted to obtain a cell concentration of $2\text{-}10^6$/ml. The GC pellet was divided into two aliquots, for monolayer hormone-supplemented (**mono + H**) or for hormone-free 3D (**3D**) culture. The 3D culture system was carried out in barium alginate capsules as previously described (Vigo et al., 2004): briefly, a saturated solution of $BaCl_2$ was added to the CG pellet and extruded through a needle into a medium viscosity 0.5% w/v sodium alginate solution. The resultant capsules were collected and suspended in Ham's F10 culture medium.
Seven hundred and seventy-nine cumulus-oocyte complexes (COC) have been isolated from follicular fluids, washed and split in two groups in order to evaluate the effect of the cumulus cells: in the first group oocytes were decumulated (**dec**) by enzymatic treatment with hyaluronidase and gentle aspiration through a "stripper" pipette. In the second group, oocytes were used undecumulated (**undec**).
In 3D culture a single **dec** or **undec** oocyte was injected into each capsule by means of a sterile capillary. Four culture groups were then performed: **3D dec** (n=178): hormone-free 3D co-culture and decumulated oocytes; **3D undec** (n=114)**:** hormone-free 3D co-culture and undecumulated oocytes; **mono dec+H** (n=201): monolayer culture with hormonal treatment (10UI hCG/mL and 10 UI eCG/mL) and decumulated oocytes; **mono undec+H** (n=286) monolayer culture with hormonal treatment and undecumulated oocytes.

IVF was performed by oocyte spermatozoa coincubation at 37°C, 5% CO_2, for 6 hours (Gil et al., 2003). Oocytes were then incubated at 37°C 5% CO_2 for 6 days and then microscopically classified as "cleaved" or "not cleaved". Results were reported as cleavage percentage with respect to the treated oocytes for each group.

Scanning electron microscopy

Samples were submitted to a fixation process with 2.5% glutaraldehyde and then dehydrated with serial ethanol solutions from 50% to 100%, gold sputtered (purity degree 99.9%) with an Edwards S 150A sputter coater and then observed by a scanning electron microscope (Cambridge Stereoscan 250, Cambridge Instruments Ltd., Cambridge, UK) operating at 20 kV.

Statistical Analysis

Cleavage percentages were analysed by chi-square test, followed by Fisher exact test for multiple comparison. The significance level was set at $p<0.05$.

Results and Discussion

Culture of undecumulated in GC monolayer with hormonal supplementation leads to a significant higher cleavage yield with respect to 3D hormone free culture ($p<0.05$) and with respect to decumulated oocyte cultured in monolayer ($p<0.01$) (fig 1).

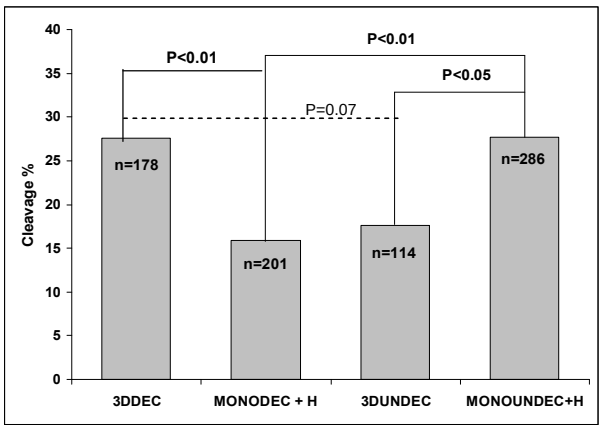

Figure 1 - Fertilization yield and sample size in four treatment groups.

This result indicates that hormonal maturation response of oocytes is mediated by cumulus GC. Moreover there are significant cleavage differences between the culture of decumulated oocytes (28% in **3D dec** versus 16% in **mono dec+H)**: this indicates that the oocyte supplied with a large number of granulosa cells as physiological support has reached a higher competence maturation, as hypothesized by Sirard et al. (2006). Granulosa cells are in fact necessary for normal oocyte development, and contacts between the oocyte and cumulus are pivotal (Amano et al., 2005). Finally 3D culture seems to be unaffected by the presence of intact corona radiata: this features could be due to the presence of GC that migrate around oocyte and colonize the outer layer of zona pellucida, leading to a pseudo-cumulus structure, as previously reported (Torre et al., in press).

Morphological investigation of 3D culture system (figure 2) confirms that GC have maintained a polyhedral shape and are organised in clumps, adhering to the polymeric support. The barium alginate membrane mimics the *in vivo* environment in which GC are in direct contact with the basement membrane. In these biological-like conditions, GC partially envelope the oocyte even 144 hours after IVF (figures 3-4).

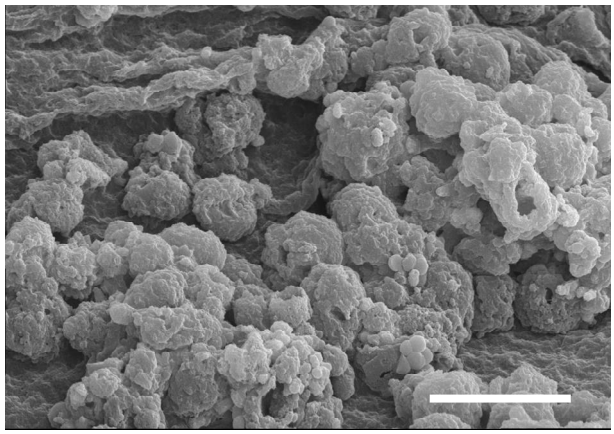

Figure 2 - SEM microphotograph of the inner side of hormone-free 3D co-culture capsule, after 48 hours. GC can be appreciated. Bar: 10μm.

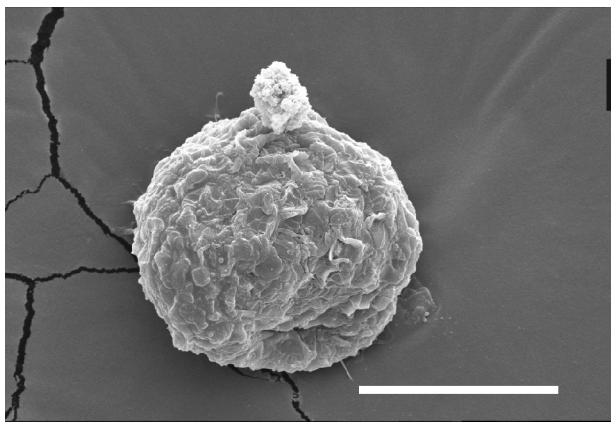

Figure 3 - SEM microphotograph of a cleaved oocyte 48 hours after IVF. The oocyte was matured in 3D hormone-free decumulated system. Bar: 50μm.

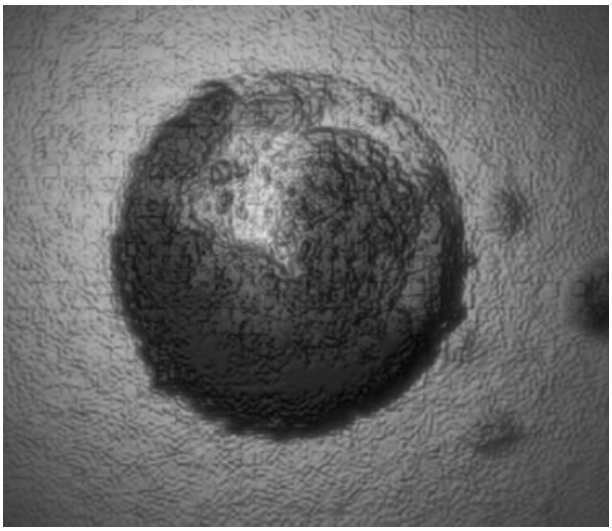

Figure 4 - Stereomicrograph (100X) of a cleaved oocyte 144 hours after IVF. The oocyte was matured in 3D hormone-free decumulated system.

Conclusions

Three-dimensional hormone-free oocyte IVM in barium alginate capsule allows the same cleavage yields as monolayer culture with hormonal supplementation. The new culture technique can increase the yields of high-quality oocytes available for *in vitro* fertilization (IVF) and embryo transfer in mammal species.

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