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THE INFLUENCE OF THE NUMBER OF CUMULUS CELL LAYERS ON THE *IN VITRO* MATURATION OF SWINE OOCYTES

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ABSTRACT

The in vitro maturation process of swine oocytes represents a challenge for many groups of researchers. In the present study, the quantitative and qualitative evaluation of the ovarian follicles and the detailed analysis of the oocyte-cumulus complexes in terms of structure and effect on the oocyte maturation process were followed. In the present study, 16 pig ovaries were used, at the level of which 261 ovarian follicles were identified and isolated, from which 234 oocyte-cumulus complexes with different organization were extracted. The oocyte-cumulus complexes were subjected to in vitro maturation in the TCM-199 culture medium. The maturation process of the oocytes in these complexes was realized differently, the maturation rates being influenced by the number of layers of cumulus cells, the highest maturation rate of 92.12% was recorded at the level of oocyte-cumulus complexes with several layers of cumulative cells. However, the process of fertilization and embryonic development was relatively low, the rate of fertilization and development of zygotes being 10.61% and it was recorded from oocytes matured from those oocyte-cumulus complexes with several layers of cumulus cells.

KEY WORDS: *oocyte, maturation, in vitro, cumulus cells.*

INTRODUCTION

Assisted animal reproduction technologies are based on ovarian super-stimulation, which leads to obtaining immature oocytes that will have to be subjected to the *in vitro* maturation process, which often leads to obtaining low-quality oocytes. The in vitro maturation of oocytes is influenced by several factors: the size of the ovarian follicles, the structure of the oocyte-cumulus complex, the protein composition of the structure of this complex, etc. The quality of the oocytes obtained through *in vitro* maturation further affects the fertilization process, the development and early survival

of the embryo, the development and maintenance of the pregnancy, the development of the fetus and even the appearance of various types of diseases in the adult animal. Oocytes obtained from preantral follicles and cultured *in vitro* have the ability to resume the process of meiosis, but show a relatively low development capacity up to the blastocyst stage. It should be noted that the metabolic activity has a very important role in ensuring the quality of the oocytes, since the glycolytic activity at the level of the mature oocytes is positively correlated with the embryonic growth and development. At the level of the oocyte-cumulus complexes, a communication between the oocyte and the cumulus cells has been identified, a communication that influences the multitude of processes that take place at the level of the cytoplasm and nucleus of the oocyte and which are responsible for ensuring in vitro maturation of the oocytes and obtaining qualitatively appropriate oocytes (Kikuchi *et al.*, 1993; Krisher, 2004).

The *in vitro* development and maturation of swine oocytes is difficult to achieve, the rate of obtaining qualitative oocytes is relatively reduced by 20-30%, since the oocytes remain immature and closed in the cumulus. The methods of ensuring the in vitro maturation of oocytes in pigs require improvements in this regard, as this process depends on obtaining a greater number of embryos, but also of better quality embryos capable of developing without anomalies (Appeltant *et al.*, 2015).

Cumulus oophorus represents a group of granular cells that are closely associated and surround the oocyte at the level of the antral follicle, being also known as cumulus cells. The fully developed cumulus oophorus provides 3 important functions in relation to ensuring ovulation: it helps the oocytes in the maturation process (before ovulation), orients the oocyte in the oviduct (during ovulation), participates in the complex mechanisms that control the interaction of the spermatozoa with the oocyte (shortly after ovulation). Although there are data on the functions of cumulus oophorus in the processes of oocyte maturation and ovulation, the role of cumulus oophorus in the fertilization process in mammals is not clear. Thus, in porcine, the removal of cumulus cells before *in vitro* fertilization decreases the ability of spermatozoa to penetrate and the formation of a normal pronucleus. This finding supports the role and direct involvement of cumulus cells in influencing the processes of oocyte maturation and ovulation induction (Tanghe *et al.*, 2002).

The hormonal content also has a role in ensuring the maturation process of the oocyte, so the somatic cells have the ability to detect the signal emitted by the release of LH and in turn emit a signal to the oocyte. LH acts either indirectly on the follicular wall, causing the granulosa cells to emit a signal to the follicular fluid, or directly on the cumulus cells that communicate with the oocyte through gap junctions. After the release

of LH, there is an increase in cAMP in the oocyte, so cAMP can no longer be considered responsible for inhibiting the maturation process, and its regulation intervention is based on its concentration in the oocyte and cumulus cells (Mattioli and Barboni, 2000). After exposure to LH, cumulus cells depolarize and due to their coupling with the oocyte lead to depolarization of the oocyte. The oocyte-cumulus complex thus undergoes changes during the maturation process, the gap junctions between the cumulus cells and the oocyte are lost, as a result the transmission of inhibitory signals from the cumulus cells to the oocyte entering meiosis is blocked. In the second part of the maturation process, the connection between the oocyte and the somatic cells is limited to the radiated crown, cells from the outer layers detach (Mattioli and Barboni, 2000).

The results of the pig studies regarding the oocyte maturation process are contradictory, in a more recent study it was shown that the cumulus-oocyte connections are disconnected between the metaphase -I and -II stages as a result of the expansion of the cumulus cells, while the cumulus - cumulus remain intact in these stages of division (Suzuki *et al.*, 2000). The studies carried out are contradictory and are difficult to resolve, because the selective closing of a part of the gap junctions cannot be achieved by using junction inhibitors (heptanol), or uncoupling the gap junctions (glyceretinic acid) (Mori *et al*., 2000).

Obtaining quality oocytes is influenced by several factors, which also affect follicular development: (i) the origin of the oocyte (oocytes derived from large follicles are more competent than those derived from small follicles), (ii) the state of health of the follicle (dominance of the follicle and atresia are associated with oocyte developmental capacity), (iii) hormonal stimulation of follicle development clearly improves oocyte quality (Sirard *et al*., 2006), (iii) communication between oocyte and cumulus cells is a necessity to ensure o development of a mature and quality oocyte (Krisher, 2004). In vitro matured oocytes are known to have a lower developmental competence compared to in vivo matured oocytes, a fact due to inadequate culture conditions involving the culture medium used in vitro to ensure complete oocyte maturation (Sutton *et al*., 2003).

The purpose of the study was to highlight the role of cumulus cells in the structure of the oocyte-cumulus complex on the oocyte maturation process in swine in close connection with the culture medium used.

MATERIALS AND METHODS

Harvesting the ovaries

Ovaries were harvested on the slaughter and transection line in a slaughterhouse specialized in animal slaughter. After cutting the animal and highlighting the female genital apparatus, the ovaries were excised with the help of scissors. After excision, the ovaries were placed in a sterile container with 9% saline at 30°C. The saline solution was previously prepared in the laboratory from 0.9 g/L NaCl in sterile distilled water. In total, 16 swine ovaries were sampled and brought to the laboratory for the tests.

Examination of the ovaries

The ovaries were brought to the laboratory, weighed individually using an analytical balance (Kern model ALJ 220-4NM, Denver Instrument Gmbh, Göttingen, Germany). Later, after sectioning, the ovarian follicles were identified and counted.

Harvesting cumulus oocyte complexes

Harvesting oocyte-cumulus complexes can be done by several methods, the method used in this study was one that allows us to harvest the oocyte-cumulus complex both from follicles larger than 6 mm and from follicles smaller than 2 mm. The collection of the oocyte-cumulus complex consists in the puncture of the ovarian follicle with the help of a 10 ml syringe with an 18G needle. The harvested oocyte-cumulus complexes were grouped into 3 groups:

Group 1. oocyte without cumulus cells around,

Group 2. oocytes with a layer of cumulus cells,

Group 3. oocytes with several layers of cumulus cells.

The oocyte-cumulus complexes extracted from the ovary and divided into the 3 groups were subjected to the maturation process in the M199 culture medium added with 20% FBS, for 48 hours, in the cell culture incubator respecting the following incubation conditions: temperature of 37° C, CO_2 concentration 5 %, humidity 99%. After the 48 hours of incubation, the oocytes were put in contact with the spermatozoa to carry out the fertilization process. The plates were subjected to incubation under the same mentioned conditions, and after 24 hours the plates were examined to identify possible zygotes, respectively the zygotes that underwent the first division.

All microscopy examinations were performed with an Olympus CX 41 microscope.

Statistical analysis

The data were statistically analyzed with the Mann-Whitney parametric test. Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

The weight of the ovaries

The ovaries that arrived in the laboratory were weighed individually. The values for the weight parameter were between 10.21 g and 15.53 g, the average weight values are given as mean ±SD, respectively the total weight value was calculated for the ovaries taken in the study (table 1).

TABLE 1. Weight of analyzed ovaries

Number of ovaries	Medium weight (g)	Total weight (g)	Weight limits (g)	
			Minimum value	Maximum value
16	12,87±5.56	125,30	10.21	15.53

Analyzed ovarian follicles

After sectioning, the ovarian follicles were identified and counted, the maximum number of ovarian follicles / ovary was 43 and the minimum number of ovarian follicles / ovary was 7 (table 2).

TABLE 2. Isolated ovarian follicles

Number of ovaries	Total number of ovarian follicles	Ovarian follicles average	Ovarian follicles limits	
			Minimum value	Maximum value
16	261	25,30 ± 8,37	7	43

Oocyte-cumulus complexes collected from sow ovaries

From the total number of 261 ovarian follicles identified, a number of 234 oocyte-cumulus complexes were extracted and grouped into the 3 groups. The maximum number of oocyte-cumulus complexes / ovary was 45 and the minimum number of oocyte-cumulus complexes / ovary was 2.

The oocyte-cumulus complexes were provided with specific maturation conditions, the values recorded regarding the maturation rate are shown in table 3. From the total number of 127 cumulus oocyte complexes that presented several layers of cumulus cells, 113 oocytes matured, indicating the highest rate of maturation. The presence of cumulative cells in several layers positively influences the degree of maturation of the oocytes, the recorded values being statistically significant when compared to the other 2 groups ($p<0.05$).

The matured oocytes were put in contact with the spermatozoa, the calculation of the percentage of fertilized mature oocytes entering development was done by referring to the number of mature oocytes. From the total number of 132 mature oocytes,

only a number of 13 zygotes were identified, of which 12 zygotes came from mature oocytes originating from oocyte-cumulus complexes with a greater number of cell layers.

TABLE 3. Influence of cumulus cells on oocyte maturation (*p<0.05)

Experimental group		**Oocyte - cumulus complexes**	**Swine oocytes**			
			Mature oocytes		**Fertilized mature oocytes entering development**	
			Number	**%**	**Number**	**%**
Group 1	Oocytes without cumulus cells	73	3	4.10	-	-
Group 2	Oocytes with a single layer of cumulus cells	34	16*	47.06	1	6.25
Group 3	Oocytes with several layers of cumulus cells	127	113*	92.12	12*	10.61

From the analysis of the data presented above, it was found that cumulus cells play a decisive role in the oocyte maturation process. Since only 3 oocytes matured from oocyte-cumulus complexes that did not present cumulus cell layers during culture, compared to the 16 oocytes obtained from oocyte-cumulus complexes with only 1 cumulus cell layer, respectively 113 oocytes that came from oocyte-cumulus complexes with several layers of cumulus cells, represent clear evidence that cumulus cells are indispensable for the process of oocyte maturation.

The calculation of the percentage of oocytes that were able to enter the fertilization process was done by dividing the number of oocytes that matured. The inability to achieve fertilization is found for mature oocytes obtained from oocyte-cumulus complexes without layers of cumulus cells, respectively a very small percentage of 6.25% of oocytes that were fertilized, oocytes obtained from oocyte-cumulus complexes with a single layer of cumulative cells (table 3).

The relatively small percentage of mature oocytes that fertilized and led to the obtaining of zygotes that entered the first cell division, indicates the need to continue studies on the *in vitro* maturation of oocytes and to obtain specific culture media that ensure obtaining oocytes of high quality.

Since the mechanism of interaction between oocytes and cumulus cells is not clarified, studies were carried out in this regard (Krisher, 2004; Appeltant *et al.*, 2015). In one of the studies, an attempt was made to clarify the interactions between oocytes and cumulus cells in swine, which targeted the expansions made by oocytes and cumulus cells, but also the *in vitro* maturation of oocytes by using a specific culture medium that allows the individual tracking of the development of each oocyte. Thus, swine oocytes influence the expansion of cumulus cells (a fact probably due to junctional communications or factors secreted by the oocyte), but this process does not seem to require the prior presence of oocytes. The cumulus cells instead influence the oocyte maturation process, keep them inside the cumulus and block their cell division, entry into meiosis (Appeltant *et al*., 2015).

Understanding the interactions that are established between the oocyte and the cumulative cells are very important to be able to bring improvements to the cultivation and maturation systems *in vitro*, with the aim of obtaining quality oocytes. The possible research directions are directed towards the analysis of the bidirectional communication that is established between the oocytes with the granulosa cells and the cumulus cells, the analysis of the secretory products released by the oocytes (it is assumed that they are paracrine and that they have an essential role in the regulation of the folliculogenesis process). In swine, the studies focused on the analysis of the effects of oocytes and their secretory products on the expansion of the cumulus, the maturation of other oocytes and their development capacity. According to some authors, it is considered that the oocyte determines the expansion of the cumulus, according to others, in the case of oocyte maturation *in vitro*, their removal from the level of the oocyte-cumulus complex does not affect the expansion (Appeltant *et al.*, 2015). The addition of culture media with oocyte secretion products and the effects of oocyte secretion products for their own maturation remain unknown and further studies are not required (Hussein *et al.*, 2006).

CONCLUSIONS

The method used allows or not, depending on the structure of the oocyte-cumulus complex, to enter the oocyte maturation process. The presence of several cumulus cell starts within the oocyte-cumulus complex determined the highest oocyte maturation rate of 92.12%. From the total of matured oocytes from the cumulus oocyte complexes with several layers of cumulus cells, a percentage of 10.61% were fertilized and led to the formation of zygotes capable of entering development.

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