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THE ROLE OF CUMULUS CELLS IN THE *IN VITRO* MATURATION OF COW OOCYTES

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ABSTRACT

The in vitro maturation of oocytes harvested from the ovaries in the form of oocyte-cumulus complexes in order to use them to obtain viable zygotes, is the technique frequently used in bovine reproduction biotechnologies. The identification of a culture medium that determines, after in vitro maturation, the obtaining of oocytes competent for fertilization and the identification of the role of cumulus cells in this process is a topic of continuous research. The study was carried out on cow ovaries, both right and left ovaries were used. The determination of 3 biometric parameters was considered - length, width, thickness, along with weight. Ovarian follicles were identified and numerically quantified, later the oocyte-cumulus complexes were extracted and subjected to in vitro maturation in M199 culture medium. Since the oocyte-cumulus complexes presented a different structure, they were grouped into 3 experimental groups and after the appropriate incubation, the number of mature oocytes was quantified. The mature oocytes were put in contact with the spermatozoa and after proper incubation, the zygotes entering the division process, entering development, were identified. The oocyte-cumulus complexes as several layers of cumulus cells determined after in vitro maturation the obtaining of a high percentage of mature oocytes of 84.61%. Starting from these oocytes maturing in vitro from oocyte-cumulus complexes with several layers of cumulus cells, a rate of zygotes entering development of 89.09% was recorded.

KEY WORDS: *cow, oocyte, in vitro, oocyte-cumulus complexes.*

INTRODUCTION

Reproduction in animals in recent years is based on the use of techniques and methods of reproductive biotechnologies that have determined the increase in animal

productivity. At the present time, a method is being sought that is capable of providing a high production efficiency in terms of animal welfare and finding the most advanced reproductive techniques for inducing pregnancy and improving the genetic background in cattle. Ovarian physiology is a key element that contributes to increasing productivity and cattle herds.

The development, maturation and release of mature oocytes for fertilization, but also the synthesis and secretion of essential hormones for follicular development, the estrous cycle and maintaining the function of the reproductive tract takes place at the level of the ovaries of cows. In cattle, from the heifer stage to reproductive senescence, many follicles are activated to enter the growth phase, which involves both granulosa cell proliferation and oocyte size increase (Gougeon, 2003).

The ovarian follicular population represents the total amount of follicles present in the ovary. Each ovarian follicle contains one oocyte, but a large number of oocytes are known to exist in the ovary. Out of the total number, only a small part of the ovarian enters follicles the ovulation process. As a result, the ovarian follicular reserve represents an important indicator of fertility in cattle, thus influencing the applicability of reproductive biotechniques (Seneda *et al.*, 2021).

The follicular population in the cow's ovaries can vary and is influenced by certain factors: genetics, breed, age, species and hormone levels. Also, non-lactating cows often have a higher number of follicles and oocytes. In the case of cows, it is considered that the number of ovarian follicles is approximately 235.000 (Betteridge *et al.*, 1989; Dalbies-Tran *et al.*, 2020). Primordial follicles represent the starting point in the case of *in vitro* culture for obtaining mature oocytes, their number being much higher compared to that of mature follicles. Thus, starting from these primordial follicles, it would be possible to optimize *in vitro* maturation methods that would lead to obtaining a large number of offspring. In addition, understanding the way in which follicular maturation takes place from primordial follicles would constitute an important element in fertilization techniques (Aerts *et al.*, 2008).

The preantral follicles used in research, in *in vitro* cultures, are obtained from primate ovaries from slaughterhouses after the slaughter of the animal or through ovarian biopsies. Although ovarian biopsy has been established to produce minimal or no changes in ovarian function in cattle, although this technique is more valuable for diagnostic or experimental purposes, obtaining preantral follicles through this technique is avoided (Aerts *et al.*, 2008). In the case of the present experimental study, the first method of obtaining the follicles was used, after the slaughter of the animal. The efficient isolation of the preantral follicles from the ovaries is the key element to obtain competent

mature oocytes, capable of interacting and fertilizing, but more importantly to obtain zygotes capable of developing by entering the division. Thus, *in vitro* immature oocytes from preantral follicles could be used in other assisted reproduction technologies, the most important being the maturation and production of embryos *in vitro*.

In vitro studies have been carried out on the follicle growth system that allows obtaining mature oocytes starting from preantral or early antral follicles. The success of the *in vitro* follicle growth method depends on the initial size of the oocytes as well as the categories of follicles used. In cattle, the success of the process of obtaining tertiary follicles, with a well-defined follicular cavity and a well-developed oocyte, was reported to the use of large, advanced secondary follicles (Gupta *et al.*, 2008).

Ovarian follicular development and oocyte growth depends on the bidirectional communication established between oocytes and somatic cells. Oocytes have an essential role in controlling granulosa cell proliferation and differentiation during follicular development. The tight connection between the oocyte and the cells of the cumulus is achieved through the jap-type junctions (Gutierrez *et al.*, 2000). The ability to support *in vitro* growth of the preantral follicle to support the acquisition of oocyte competence represented a breakthrough in the field of reproduction, as this source of oocytes could be used in assisted reproduction technologies. In addition, the studies carried out under *in vitro* conditions aimed at understanding the interactions between somatic cells and the oocyte in animal species with prolonged follicular growth, the most obvious example in cattle, would represent an advantage for human reproduction as well (McLaughlin *et al.*, 2010). Since the majority of ovarian follicles in cows during the growth phase in vivo enter the state of atresia, it is necessary to identify and develop a culture system that is able to maintain follicular growth and thus avoid the loss of follicles. The purpose of the work was to perform biometric determinations on cow ovaries and to assess the effect of the number of layers of cumulative cells on the *in vitro* maturation process of oocytes.

MATERIALS AND METHODS

Harvesting the ovaries. The reproductive tract of cows was harvested in a fresh state from different profile micro-farms in Timis County, in 2022. After the slaughter of the animal and its slicing, the female genital apparatus was highlighted and the ovaries were detached. After excision, the ovaries were placed in a sterile container with 9% saline solution at a temperature of 30°C, the temperature was maintained constant during the transport to the laboratory. The saline solution was prepared in advance in the

laboratory from 0.9 g/ L NaCl in sterile distilled water. In total, 16 cow reproductive tracts were sampled, from which: 16 right ovaries and 16 left ovaries were isolated.

Examination of the ovaries. The ovaries were brought to the laboratory, weighed analytically (Kern model ALJ 220-4NM, Denver Instrument Gmbh, Göttingen, Germany). The ovaries were measured using slide calipers to determine the length, width and thickness of each ovary. Later, the ovarian follicles were highlighted and numerically quantified.

Harvesting cumulus oocyte complexes. The oocyte-cumulus complexes were collected by puncturing the ovarian follicle with the help of a 10 ml syringe with an 18G needle. The oocyte-cumulus complexes collected from both right or left ovaries were collected together and later, 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 ovaries and grouped into the 3 batches were subjected to the maturation process in the M199 culture medium, enriched with 20% fetal bovine serum, for a period of 48 hours, in the cell culture incubator. The incubation conditions assumed compliance with the values of the following parameters: temperature of 37° C, CO2 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 in the same conditions mentioned, and after 24 hours the plates were examined to identify any zygotes formed and the zygotes that underwent the first division. The microscopic examination was performed with an inverted microscope -Olympus CX41 microscope.

Statistical analysis. The values of the biometric parameters and the maturation rate of the ovaries were statistically analyzed with the non-parametric Mann-Whitney test. Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

The weight of the ovaries. The right and left ovaries that arrived in the laboratory were subjected to weighing. The values for the weight parameter were between 3.60 g and 4.75 g, the average weight values are given as mean ±DVS, respectively the total weight value was calculated for the cow ovaries taken in the study (table 1).

The sizes of the analyzed ovaries. The right and left ovaries were subjected to the determination of 3 biometric parameters: length, width, respectively thickness in

order to identify the differences between the 2 groups of ovaries. The statistical analysis did not identify any significant differences between the 2 groups of ovaries, right ovary-left ovary, although the values are lower in the case of the biometric parameters at the level of the left ovary (table 2).

TABLE 1. Weight of analyzed ovaries (*p<0.05)

Type of Ovary	Number of ovaries	Medium weight (g)	Total weight (g)	Weight limits (g)	
				Minimal value	Maximal value
Number of right ovaries	16	4.57±0.56	441.75	3.95	4.75
Number of left ovaries	16	4.23±0.13	410.26	3.60	4.33

TABLE 2. Ovary size (*p<0.05)

Type of ovary	Number of ovaries	Medium length (cm)	Medium width (cm)	Medium thickness (cm)
Number of right ovaries	16	2.87±0.36	1.95±0,03	1.75±0.06
Number of left ovaries	16	2.46±0.10	1.76±0.07	1.69±0.05

The values of the biometric parameters obtained were compared with those obtained in other studies carried out on ovaries taken from cows, the values recorded by us were slightly higher. The values recorded by us were attributed to the breed from which the ovaries came, this aspect seems to influence the values of the analyzed biometric parameters. The results of the study on ovaries from Holstein Friesian cows showed the following values for the right ovary: length 2.52±0.05 cm, width 1.88±0.06 cm, average thickness 1.58±0.06 cm, respectively for the left ovary length 2.41±0.05 cm, width 1.80±0.06 cm, thickness 1.59±0.05 cm. The weight of the right ovary was 3.81±0.11 g, and the left one was 3.87±0.12 g (Islam *et al.*, 2018).

The differences recorded for the parameters studied are due to race, age, parity, body weight, body condition score and managing the variation of environmental factors. However, in most studies it was concluded that the left ovary is shorter in length and width compared to the right one. Also, the weight of the right ovary is greater compared to the left one. The determined values proved to be influenced mainly by 3 factors - genotype, age and body weight (Bello *et al.*, 2012; Ibrahim *et al.*, 2012; Jaji *et al.*, 2012; Leal *et al.*, 2013; Islam *et al.*, 2018). In other studies, no differences were recorded between the biometric parameters in the right and left ovaries in terms of length, width and thickness, and in terms of the weights recorded for the right and left ovaries, the differences were not statistically significant. Variations in biometric parameters (weight, length, width, thickness, etc.) can be attributed to geographic location, breed, season and type of nutrition, etc. (Ibrahim *et al.*, 2012; Ramsingh *et al.*, 2013; Leal *et al.*, 2013).

Ovarian follicles. After determining the 3 biometric parameters at the level of the ovaries, the ovarian follicles were identified and numerically quantified, the maximum number of ovarian follicles / right ovary was 13, and the minimum number of ovarian follicles / right ovary was 5. At the level of the left ovary, the number the maximum number of ovarian follicles / ovary was 10, and the minimum number of ovarian follicles / right ovary was 1 (table 3).

TABLE 3. Ovarian follicles (*p<0.05)

Type of ovary	Number of ovaries	Total number of ovarian follicles	Average of ovarian follicles	Limits of ovarian follicles	
				Minimal value	Maximal value
Number of right ovaries	16	143	10±2.33	5	13
Number of left ovaries	16		7±1.40	1	10

Oocyte-cumulus complexes collected from cow ovaries. From the total number of 143 ovarian follicles identified, a number of 136 oocyte-cumulus complexes were extracted and grouped into the 3 groups. The maximum number of oocyte-cumulus/ovary complexes was 13, and the minimum number of oocyte-cumulus/ovary complexes was one. The oocyte-cumulus complexes were provided with specific maturation conditions, the values recorded regarding the maturation rate are shown in Table 4. From the total of 65 cumulus oocyte complexes that presented several layers of cumulus cells, 55 oocytes were matured *in vitro*, indicating the highest maturation rate. The presence of cumulative cells in several layers positively influences the degree of maturation of the oocytes *in vitro*, 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 of 76 mature oocytes, a relatively high number of 54 zygotes were identified, of which 49 zygotes came from mature oocytes originating from oocyte-cumulus complexes with a higher number of cell layers (table 4). The population of ovarian follicles in cows consists of thousands of follicles, which can be preantral and antral, at the level of which the oocytes are included. During fetal life, the first follicles produced are preantral, as they go through the process of development, they reach the final stage of antral follicles (those that develop a cavity or antrum). This entire phase of follicle growth is called folliculogenesis, and not all follicles reach the preovulatory phase to be fertilized. As a result, the intervention of reproductive biotechniques is necessary to increase the fertility of cows (Seneda *et al.*, 2021).

TABLE 4. Influence of cumulus cells on *in vitro* maturation of oocytes (*p<0.05)

Experimental group	Oocyte - cumulus complexes	Cow oocytes			
		Mature oocytes		Fertilized mature oocytes entering development	
		Number	%	Number	%
Oocytes without cumulus cells	24	1	4.17	-	-
Oocytes with one single layer of cumulus cells	47	20*	42.55	5	25.00
Oocytes with more layers of cumulus cells	65	55*	84.61	49*	89.09

The *in vitro* maturation of cow oocytes is a complex phenomenon that includes a sequence of steps that takes place both in the nucleus and in the cytoplasm of the oocyte, finally leading to the resumption of meiosis (Krisher, 2004; Kimura *et al.*, 2007). Adequate oocyte maturation is an essential step in the *in vitro* fertilization process, as it ensures the obtaining of mature oocytes capable of participating in fertilization (Lonergan *et al.*, 2003).

A fundamental role in the process of in vivo and *in vitro* maturation of oocytes is played by the somatic cells around the oocyte, known as cumulus cells (Tanghe *et al.*, 2002). Cumulative cells are in direct contact with the oocyte through functional gap junctions and through the adjacent zona pellucida (Wigglesworth *et al.*, 2013). Oocyte maturation and blastocyst development after *in vitro* fertilization in cattle is influenced by premature loss of cumulus-oocyte communication (Thompson *et al.*, 2007). While follicular fluid has a metabolic and ionic composition similar to that of plasma, the cumulus cells can be considered a barrier between the oocyte cytosol and this follicular fluid (Aardema *et al.*, 2013). Cumulus cells regulate the metabolism of oocytes through gap junctions, providing them with a microenvironment, biochemical microclimate favorable for development (Gilchrist *et al.*, 2008).

CONCLUSIONS

The *in vitro* cultivation method used allowed the entry into the maturation process of the oocytes originating from the oocyte-cumulus complexes with several layers of cumulus cells. So the presence of several cumulus cell starts within the oocyte-cumulus complex determined the highest oocyte maturation rate of 84.61%. From the total of oocytes matured *in vitro* from oocyte-cumulus complexes with several layers of cumulus cells, a percentage of 89.09% responded positively to the fertilization process and led to the formation of zygotes that were able to enter development.

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