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Apoptosis in the Bovine Oocytes by Different *in vitro* Culture Conditions

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Abstract: A total of 88 ovaries in the middle follicular stage were used to detect apoptosis using the modified staining procedure with terminal deoxynucleotide transferase-mediated dUTP nick end-labeling assay (TUNEL), Hematoxylin and Eosin. The analysis observed proportions of cells for apoptosis with several options of culture systems, presenting a control (77.2±7.0), culture 1 (3.8±2.0), culture 2 (1.3±2.0), culture 3 (72.7±6.0), culture 4 (6.7±5.0), culture 5 (80.1±6.0) and culture 6 (80.2±6.0). The apoptotic cells in cultures 1, 2 and 4 were significantly different with other cultures including the control whereas cultures 3, 5 and 6 showed significantly similar apoptotic patterns with the control. The present analysis observed clear differences for relative numbers of internal ovary cells in apoptosis and necrosis. The new circulation method provided from this study with culture media, levels of gas supply (O_2 and CO_2) and incubation temperature for *in vitro* culture systems can prolong the lifetime of cells.

Key words: Apoptosis, ovaries, *in vitro* culture, milk, cows

INTRODUCTION

In vitro fertilization of bovine embryos is one of the most important issues in reproduction research areas due to requirements for the advanced handing techniques for availability of preimplantation bovine embryos (Zeuner *et al*., 2003). In fact *in vitro* techniques influenced many studying areas of oocyte maturation, fertilization and early embryonic development to provide consistently and reliable oocyte maturation. Even though the techniques are dramatically improved many options that have not been exactly defined included types of supplements and cells as well as culturing systems because understanding the embryonic metabolism and growth are complicated matters (Mastromonaco *et al*., 2004).

The diameter of ovaries which were separated from mouse in the period of embryonic stages and cultured for 14-20 days was ranged 46-56 μm and a structural formation showed zona pellucida around the oocyte (Blandau *et al*., 1965). The results are critical with potentials that ovaries can be grown with *in vitro* culture systems focusing on organs and primordial culture procedures for the isolated ovaries from embryonic stages. A study reported that a birth was archived by transplantation of embryos that were isolated from postnatal mouse ovaries cultured *in vitro* at day 0 (Eppig and O'Brien 1996). In addition, a successful *in vitro* culture system has been reported after isolation of mouse oocytes from oocyte-granulosa cell complex by treatments of enzymes in the organ culture (Eppig and Schroeder, 1989). However, the cortex of ovaries in mammals contained lots of primary follicles that ovulate according to the development of sexual maturity with reasonable numbers of follicles per stage (Day, 1991; Braw-Tal and Yossefi, 1997). Depending on the estrous cycle, generation and degeneration of follicles occur periodically in mammalian ovaries and the fully matured follicles will be approximately 0.1% of total follicles. Most follicles go through the process of degeneration (Tsafriri and Braw, 1984; Hirshfield, 1991) after the development of certain period time. The degeneration process of follicles is known as a programmed cell death called apoptosis that is active cell death and different with necrosis (Hughes Jr. and Gorospe, 1991; Hsueh *et al*., 1994). The apoptosis maintained homeostasis and controlled proliferation of particular cells (Host *et al*., 2000). The dominant follicles from the cohort in ovaries can reach ovulation whereas rest of follicles will be degenerated according to the regulation of reproductive

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endocrine systems. Up to date, Follicle Stimulating Hormone (FSH), progesterone, Insulin-like Growth Factor-1 (IGF-1) and Epithelial Growth Factor (EGF) are known as survival factors for preventing apoptosis (Yang and Rajamahendran, 2000; Luciano *et al*., 2000). After induction of multiple ovulations, the levels of apoptosis for granulosa cells in growing follicles are related to degeneration of follicles and it is assumed that embryos derived from IVF (*In vitro* fertilization) follicles with the relatively low apoptosis will have beneficial advantages for pregnancies.

Apoptosis as a pathological concept can be observed under the optical or electron microscopy. A formation of the apoptotic body by segmentation of cells and chromatins occurs according to the segmentation of nucleosomes with deduction of cell sizes increases of wrinkled membranes and condensation of nuclear or cytoplasmic. A common method to assess DNA degeneration of cells is for attaching enzymes as tags to all DNA fragments that were treated by endonucleases. A highly sensitive procedure, TUNEL (Terminal deoxynucleotide Transferase (TdT), mediated dUTP-biotin and nick end labeling) was used for marking the enzyme TdT to DNA fragments and DNA polymerase can label tags at the end of two strands of restricted DNAs. In addition, TdT can also label tags at the end of one strand of restricted DNAs. Labeled nucleotides can be observed in different ways depending on markers and biotins as a protein marker were combined with the Avidin according to the polyatomic procedure. Four Avidin molecules combine with a biotin molecule amplifying signals. The Avidin combines with enzymes such as Horseradish Peroxidase (HRP) and fluorescent molecules (fluorescein or Texas Red) and emitting fluorescent molecules can be observed by a fluorescence microscopy. According to the detection procedures with enzymes, substrates are added to cells and accumulated around the split points of DNA strands. The 3' 3-Diaminobenzidine (DAB) which is commonly used in HRP, produced brown colors under the optical microscopy. The digoxigenine can be detected with specific anti-digoxigenine antibodies that may not react with mammalian proteins. TUNEL as an antibody can be detected by labeling fluorescent substances and enzymes.

Even though it has proven difficulty to grow and mature follicles *in vitro*, the development of technologies to grow and mature oocytes from the most abundant primordial follicles holds many attractions for clinical practice in animal production areas. One of the beneficial ideas would be finding an optimized recycling procedure for the slaughtered and abolished ovaries that may contain approximately 40,000 primary follicles. At present, the best option should be uses of an extended multistage culture strategy that may provide a complex support system that closely resembles the ovary *in vivo*. Therefore this study was aimed to investigate the best optimized culturing methods for the oocytes grown for extended periods *in vitro* and to evaluate the normality of oocytes.

MATERIALS AND METHODS

Collection of ovaries: The experimental procedures and animals were approved by the ethics and welfare committee of the National Institute of Animal Science (NIAS) in Korea. For cultivation of ovaries, incubators, surgical instruments and a micro catheter for the circulation of media were autoclaved and sterilized. A total of 108 daily cows which were aged average 6 years were used and slaughtered at a packing facility of Nonghyup in Seoul. A thermos lid was slightly opened to minimize the exposing time of ovaries to the air at the slaughter. To remove residual blood in ovaries, approximately 10 mL of heparin was syringed into a substantial portion of ovaries. After immersion of ovaries in a solution (approximately 0.2% of Heparin or saline) at 25°C, samples were transferred to the NIAS laboratories within 1 h. The selection process was based on the sizes of ovaries (approximately 2-3 cm) that did not have ovarian corpus luteum, corpus albicans and follicular cysts. After a total of 88 ovaries were selected, 10 ovaries were assigned in the control and 13 ovaries were randomly placed in each culture option.

Conditions of culture: To circulate a medium, a micro catheter (1.2 mm) was surgically connected to arteries in a clean bench without possible contamination. After connections between the micro catheter and medium tubes, the circulation was confirmed by several times to minimize experimental errors. The culturing device was nearly designed and placed inside of incubators. The medium (TCM-199 (Gibco 300217) or HAMS F-12 (Sigma N8642)) was circulated for 3, 6, 10 and 30 h by an instrument that was placed outside of the incubator and supplied approximately 1.04 mL min^{-1}. *In vitro* implantation was supported with the equipment that supplied ultra-trace amounts of a medium (ISMATEC, MCP motor, Switzerland). The culturing conditions with gases and temperatures were set for each culture system as 5% CO_2 at 25 and 39°C and 5% CO_2+O_2 at 25 and 39°C (Table 1). The circulation of the culture medium has been continually checked to prevent drying the surface of ovaries that were hanged to consider the real positions of ovaries *in vivo*.

Table 1: The conditions for each culture systems regarding medium, proportions of gases, temperature and incubation times

		Percentage			
Culture No.	Medium	O_2	CO_2	Temperature (°C)	Incubation (h)
1	TCM-199 Gibco 300217	-	5	39	10
2	TCM-199 Gibco 300217	-	5	39	30
3	TCM-199 Gibco 300217	5	5	39	3
4	TCM-199 Gibco 300217	5	5	39	6
5	HAMS F-12 Sigma N8642	5	5	25	3
6	HAMS F-12 Sigma N8642	5	5	25	6

Circulation performed with an equipment that supply ultra-trace amounts of medium (ISMATEC, MCP motor, Switzerland) and a culturing device with conditions for circulation rate of 1.04 mL min^{-1}, fixation with 10% formalin for 24 h and staining by the TUNEL procedure

Culturing process: Normal ovaries which were prepared with injecting approximately 5 mL of saline into ovaries using a syringe to remove blood were set for controls. After removing accessed blood in ovaries, some residual blood was eliminated by inserting a micro catheter containing saline into the artery of ovaries. The tissue was entirely fixed with 10% of formalin for 24 h and the staining process was followed by the TUNEL procedure. For the treatment of culture options, the medium (TCM-199 Gibco, 300217 or HAMS F-12 Sigma N8642) was circulated at a rate of 1.4 mL min^{-1} with several combined options with temperature (25 and 39°C), gases (5% of O_2 and CO_2) and incubation periods (3-30 h). The detailed conditions for each culture were described in Table 1. At the end of cultures, ovaries were fixed with 10% formalin for 24 h followed by the TUNEL procedure to stain tissues.

Preparing of ovaries: After collection of ovaries from each treatment, the cultured ovarian tissue was minimized for the external exposure to prevent contamination. Tissues were fixed in micro cassettes (Microm Turboflow 1500 tissue cassettes) and dissected by 3 mm thickness in a solution (10% Formalin). After 24 h fixation, the tissues were dehydrated with ethanol and cleaned with xylene. The paraffin embedding was carried out and steps of the dehydration were conducted with the automated equipment (Microm, STP-120, Germany) for 18 h. The embedded tissue sections were placed on ice plates or put in a freezer to condense paraffin. The paraffin block was stored at 4°C until the tissue dissection was done (Microm, HM-340E, Germany) and then saved at room temperature.

Staining of tissues: To stain nucleus (Hematoxylin, H) and cytoplasm (Eosin, E) the embedded blocks were mounted. Before cutting with a very sharp knife, the slide was equilibrated with 40% ethanol for 2 sec. After adjusting water temperature for 45°C in a water bath, tissue sections were attached to the slide glasses. The TUNEL staining which is used for determining the fate of cells in cultured ovaries was performed according to the manufacture's guidelines. Procedures applied Hematoxylin (H) and Eosin (E) staining for analyzing structural changes of surrounding areas for oocytes in ovaries.

Statistical analysis: The same pictured areas of ovaries (X1000) were divided into 9 sections (50 μm $section^{-1}$) and cells were counted at 4 locations (directions of 3, 6, 9 and 12) to analyze apoptosis and necrosis. Proportions of cells for each treatment showed mean values with standard deviations and statistical differences for each culture procedure were tested using the Mann-Whitney U procedure with a comparison error rate of 0.05.

RESULTS

Structural analysis of cells: Uncultured ovaries as a control were used for the reference image that can compare ovaries from several culture options. Figure 1 presented the bovine ovaries that have been collected from the slaughter within 1 h and H&E and TUNEL Staining Methods were carried out to compare structural changes of tissues by different culturing options. The structural analysis found the red and tiny blood vessel (numbered 10 in Fig. 1) around substrate areas and the result may be caused by cycles of oocytes for development, maturation and ovulation. The surrounding areas of oocytes in normal ovaries showed very dense and complex structures for generation, development and maturation of cumulus cells (numbered 4). The medium was circulated along with blood vessels of ovaries to investigate maturation levels of oocytes and extension ability of cells.

Cellular changes according to culturing options: Figure 2 illustrated apoptosis and necrosis around the immature oocytes by counting numbers of cells according to the different experimental batches with options of incubation time, medium and culture conditions. Clear images for each culture using specified staining procedures showed that the blue and red colors were described as apoptosis and necrosis, respectively.

Figure 3 the analysis of uncultured ovaries fixed for 1 h after slaughter revealed 77.2% of apoptosis and

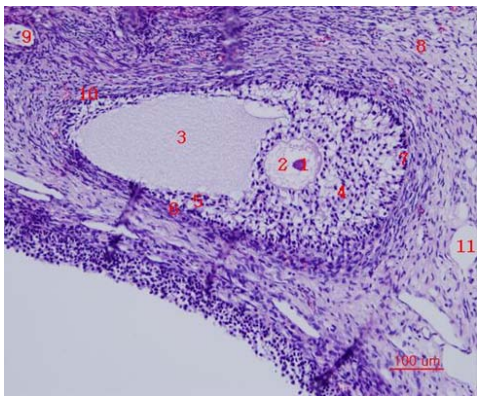

Fig. 1: The structural analysis for surrounding areas of ovaries under the microscope (X200). The numbers on the image were referred as 1 (Nucleus), 2 (Cytoplasm), 3 (Cavity), 4 (Cumulus cells), 5 (Theca interna), 6 (Theca externa), 7 (Granule membrane), 8 (Interstitial cell), 9 (Primordial follicles), 10 (Blood vessel) and 11 (Cavity)

10.9% of necrosis in cells, implying that most of the cells in ovaries were not alive. Based on the basic feature of the uncultured ovaries this experiment performed *in vitro* cultures to develop how to extend the retention periods of functional ability for cells that were placed *in vitro* using various culturing options. Cultures 1 (39°C, 5% CO_2, TCM-199 medium and 10 h incubation) and 2 (39°C, 5% CO_2, TCM-199 medium and 30 h incubation) presented *in vitro* cultures with specified conditions that were differentiated by incubation times and the results revealed no significant differences between options meaning that incubation periods may not be major factors in these culturing options. Cell morphologies were observed a high proportion of necrosis in both cultures 1 (81.4%) and 2 (86.7%) using the TUNEL staining whereas apoptosis was nearly found.

Cultures 3 (39°C, 5% of O_2 and CO_2, TCM-199 medium, 3 h incubation) and 4 (39°C, 5% of O_2 and CO_2, TCM-199 medium, 6 h incubation) presented cell morphologies and the analysis found 72.7% of apoptosis in culture 3, showing approximate the same levels of

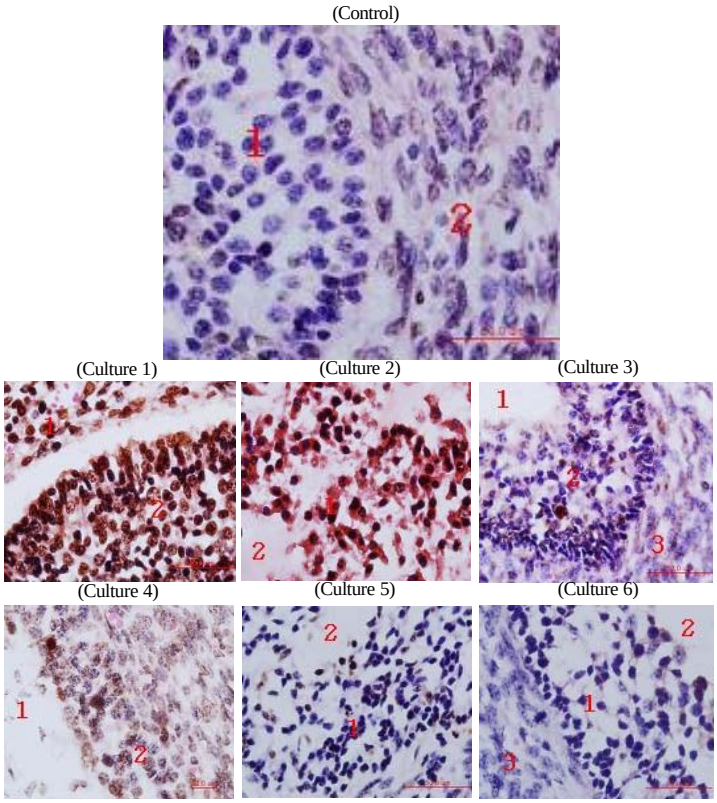

Fig. 2: Observation of ovaries treated differently by incubation times, medium and temperatures and images for occurring rates of Apoptosis (blue color) and Necrosis (red color) were observed after staining. Control (1: Granulosa cell and 2: Theca interna). Culture 1 (1: Cumulus oospores and 2: Cumulus cell); culture 2 (1: Cumulus cell and 2: Antrim); culture 3 (1: Ntrim, 2: Cumulus cell and 3: Theca interna); clture 4 (1: Antrim and 2: Cumulus cell); culture 5 (1: Cumulus cell and 2 Antrim) and culture 6 (1: Cumulus cell, 2: Antrim and 3: Theca interna)

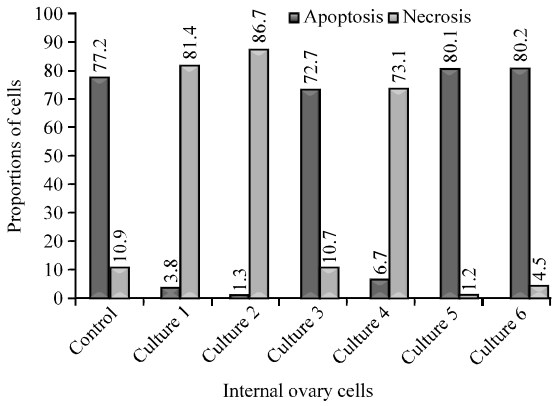

Fig. 3: Relative measurements of internal ovary cells in apoptosis and necrosis for the active proliferation around follicles by different culturing conditions. Control: Unclutured; culture 1: TCM-199 Gibco 300217, 5% CO_2, 39°C and 10 h incubation; culture 2: TCM-199 Gibco 300217, 5% CO_2, 39°C and 30 h incubation; culture 3: TCM-199 Gibco 300217, 5% O_2 and CO_2, 39°C and 3 h incubation; cluture 4: TCM-199 Gibco 300217, 5% O_2 and CO_2, 39°C and 6 h incubation; culture 5: HAMS F-12 Sigma N8642, 5% O_2 and CO_2, 25°C and 3 h incubation; culture 6: HAMS F-12 Sigma N8642, 5% O_2 and CO_2, 25°C and 6 h incubation

apoptosis compared with the control (77.2%). On the other hand, culture 4 revealed 73.1% of necrosis with 6 h incubation that did not have advantages for maintaining the ability of cells even though the same culture conditions were preceded with culture 3 except the incubation time. Therefore, it may assume that 3 h incubation seems to have better for maintaining cells with 5% of oxygen and carbon dioxide.

Cultures 5 (25°C, 5% of O_2 and CO_2, HAMS F-12 medium, 3 h incubation) and 6 (25°C, 5% of O_2 and CO_2, HAMS F-12 medium, 6 h incubation) presented a little high proportion of apoptosis compared with the control showing no statistically significant increases of apoptosis, whereas proportions of necrosis (1.2-4.5%) was lower than the control (10.9%). The circulation of culture media (HAMS F-12) showed a beneficial statute to extend viability of cells in ovaries with 5% of oxygen and carbon dioxide even though different incubation times were proceeded.

Although, the same medium (TCM-199) and temperature (39°C) were applied with different incubation time in cultures 1 and 2, the analysis found nearly similar apoptosis between options as 3.8 and 1.3%, respectively and the results showed a clear opposite pattern against the control. Overall results found that cell morphologies from cultures 1 and 2 presented higher effects of extinction rather than maintaining of cell functions, depending on the medium circulation time of media rather than the incubation temperature (39°C) that was a generally known important factor. However, changes of cell numbers showing necrosis (~73.1%) in culture 4 (39°C, TCM-199 medium and 6 h incubation) may be caused by a long period of incubation that leads a rapid decline of cell functions in ovaries. Therefore, it may assume that over the 3 h incubation time influences unfavorable effects on cultures. To identify favorable conditions for the extension of cell functions *in vitro*, the temperature in cultures 5 and 6 was set to 25°C that was similar to the delivery conditions of ovaries from a slaughter house and a medium was replaced with HAMS F-12 to reduce environmental impacts. The results revealed no significant differences of cell morphologies compared with uncultured ovaries, implying that rates of extension ability of cell functions in ovaries with cultures 5 and 6 may have benefits for the bovine organ cultures.

DISCUSSION

Mechanisms of reproduction, development, maturation and ovulation of ovaries are important process in mammals. The present study analyzed adjustable factors that influence the extension ability of cell functions in ovaries to understand what the biochemical changes were regarded as environmental factors. Several major elements for culturing systems in laboratories such as incubation temperature and time and types of a medium were tested using cells of follicles in mammal ovaries. Maintaining functions of cells that are very similar to *in vivo* conditions of the bovine ovary should be important *in vitro* conditions and therefore a comprehensive controlling system may be established. This analysis controlled factors artificially such as delivering conditions of ovaries, supplying a medium, incubation temperature and time and levels of gases. Moving conditions of the isolated ovaries from the body and incubation temperatures showed limited levels to maintain functions of cells in ovaries when temperature was 25°C. The functions of cells were similar to uncultured ovaries until 6 h incubation was applied and the result tended to agree with previous reports by Aoyama *et al*. (1995). They also reported that controlling humidity on the surface of ovaries with adding the HAMS F-12 medium showed some beneficial aspects than procedures of putting ovaries on shelf or immersing ovaries in a medium. A comparative analysis revealed that supplying a gas mixture with 5% of O_2 and CO_2 showed a beneficial statue to observe apoptosis and necrosis of

cells in ovaries. However, future research efforts may be required to verify a prolonged maintenance ability of cell functions and *in vitro* developmental capacity of oocyte because *in vitro* culture systems forlive organisms in order to make the similar statue like *in vivo* mechanisms are the biggest challenge for many studies.

Very limited numbers of follicles in ovaries can be developed and ovulated whereas rest of follicles will be destroyed according to the mechanisms of degeneration or atresia (Fortune, 1994). The growth of follicles occurs with follicular cells and the primary oocyte and laden substrate are also growing. Follicular cells in the primary follicle which can be grown with primary oocytes as well as surrounded substrates, grow actively by mitosis, formed granulosa cells. At this point, sizes and numbers of granulosa cells are increased and zona pellucida is formed by composing of glycoproteins secreted from granulosa cells and oocytes. The follicular fluid will be filled-in secondary follicles forming a follicular cavity. In the degenerating process caused by changes of endocrine environment in follicles with several factors most of the follicles were destroyed except for a few follicles (Tsafriri and Braw, 1984; Peter and McNatty, 1980). Follicular degeneration occurs in periods of non-ovulation (prenatal, minor maturity, pregnancy and lactation), menopause and normal ovarian cycles (Koering *et al*., 1982). Several studies reported that follicular degeneration occurred throughout the whole life in human (Tsafriri and Braw, 1984; Turnbull *et al*., 1977). In this study, degeneration of follicles in the tissue of normal ovaries was observed with a moderately atrophied shape of follicles and condensing nuclei of granulosa cells. In addition, a reticulum fiber of follicular membranes was slightly released and the nuclei of granulosa cells were heavily stained in contrast with nuclei of normal growing follicles. Surrounded areas of substrate in the ovaries showing the development of red capillaries were not a problem for occurrence, development, maturation and ovulation of oocytes. The analysis revealed that the number of cumulus cells in the surrounded area of oocyte in the ovaries was very elaborate and complex structures for generation, development and maturity.

The present study confirmed that circulations of a medium should be considered as the first important factor regarding extension of functions *in vitro* for substrate tissues in ovaries and development of oocytes. As the present study found that uncultured cells in the ovary showed approximately 77.2 and 10.9% of the apoptosis and necrosis, respectively the status of most cells in the ovary was not in alive and therefore, the extension of cell functions should be considered for future studies based on the results in this analysis. An improper management of temperature for *in vitro* cultures of ovarian tissues influenced a loss of cell functions and this study showed important results that the incubation temperature (25°C) presented benefits. Culture conditions in this experiment obtained similar results of cell morphologies with uncultured ovaries <6 h incubation and a prolonged incubation condition may be required for future studies. Culture procedures from 1-6 showed changes of morphologies of cell formations and nuclei shapes by comparison with granulosa cells from different cultures and the nuclear fragmentation and debris were observed in some cells showing scatter patterns, horse hooves and condense. The results were strongly agreed with previous reports (Dobson and Cooper, 1974; Kapoor *et al*., 1985; Satow *et al*., 1989) that condensation and fragmentation of nuclei were very clear when incubation time was increased.

CONCLUSION

The present analysis ascertained that the incubation time is a key factor *in vitro*, concerning other many factors that influenced cellular functions. The circulations of a medium regarding extension of functions *in vitro* for substrate tissues in ovaries and development of oocytes should be regarded as the first important factor. In addition, the tested options for incubation temperature, time and types of medium using follicles in cattle ovaries in this analysis will be a useful reference for construction of a well optimized culturing system. As morphologies of granulosa cells in cultured ovaries showed characteristics of apoptosis, further studies may need to investigate the best optimized conditions that extend cellular abilities with *in vitro* cultures for mammalians.

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