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*Original article*

# Calcium ( $\text{Ca}^{2+}$ ) expression and intensity in cumulus-oocyte complex (COCs) in Kacang goat after vitrification

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## Abstract

The process of vitrification of the cumulus-oocyte complex (COCs) often results in cold shock. When warming, heat shock occurs which can disrupt the balance of intracellular calcium ( $\text{Ca}^{2+}$ ) intensity. Drastic changes in temperature cause Reactive Oxygen Species (ROS), affecting changes on  $\text{Ca}^{2+}$  in COCs. The role of calcium is needed for oocyte activation in the fertilization process. The purpose of this study was to measure the expression of  $\text{Ca}^{2+}$  and the intensity of  $\text{Ca}^{2+}$  in COCs after vitrification. The study was divided into 2 groups, the control group (C) of fresh COCs, and the treatment group (T) of COCs after vitrification. After vitrification for 24 hours, then thawing, the expression of  $\text{Ca}^{2+}$  was examined using the Immunocytochemistry (ICC) method and the intensity of calcium ( $\text{Ca}^{2+}$ ) with a Confocal Laser Scanning Microscope (CLSM). The research data obtained were analyzed statistically by T-Test. The results showed that the expression of  $\text{Ca}^{2+}$  in the control group ( $12.00 \pm 0.00$ ) was different from the treatment group ( $0.35 \pm 0.79$ ). The intensity of  $\text{Ca}^{2+}$  in the control group ( $1059.43 \pm 489.59$ ) was different from the treatment group ( $568.21 \pm 84.31$ ). The conclusion of this study is that cryopreservation affects calcium in COCs; there were differences in the expression and the intensity of  $\text{Ca}^{2+}$  between fresh COCs and COCs after vitrification.  $\text{Ca}^{2+}$  intensity of COCs after vitrification was concentrated in the nucleus, while in fresh COCs it was concentrated in the cytoplasm.

**Key words:** Calcium ( $\text{Ca}^{2+}$ ), confocal laser scanning microscope, cumulus-oocyte complex (COCs), food production, Kacang goat

## Introduction

The Kacang goat is one of Indonesia's germ-plasm, where its body shape is small and may not be profitable economically compared to foreign goats, but it has several advantages, including being easy to adapt because it is native to Indonesia and maintenance costs are not high. The breakthrough technology to produce goat embryos quickly can be done in vitro using the In vitro fertilization (IVF) method, either conventionally by adding mature eggs with spermatozoa outside the body or using the intra-cytoplasmic sperm injection (ICSI) method. The main obstacles to goat embryo production through conventional IVF are low oocyte quality and limited oocyte sources for in vitro goat embryo production (Nasar et al. 2007).

Oocytes as a source of female gamete cells can be stored before maturation as an oocyte bank. The oocyte bank is a breakthrough for providing gamete cells for in vitro fertilization. Storage of oocytes for a long time for oocyte bank purposes can be done by cryopreservation. The best oocyte cryopreservation method is the vitrification method (Rienzi et al. 2017). The vitrification method is often used because there is no crystal ice formation and the processing time is faster than the slow freezing method (Munckand and Vajta 2017). According to Tavukcuoglu et al. (2012), the drawback of the vitrification method is that osmotic stress and toxic content in cryoprotectants can reduce the level of oocyte quality.

Chen and Yang (2009) explained that both the slow-freezing method with increased sucrose concentration and new vitrification techniques significantly improve the results of cryopreservation of human oocytes. The survival of cryopreserved oocytes ranged from 74% to 90% using the slow-freezing method, and from 84% to 99% using the vitrification method. Overall, the survival rate of oocytes from vitrification (95%, 899/948) appeared higher than that of the slow-freezing method (75%, 1,275/1,683). For both protocols, the cells must first be treated with a combination of cell-permeating and non-permeating agents to minimize ice formation which can severely damage the cell. The stark difference between the success of oocyte and embryo cryopreservation has yet to be strictly explained, but differences in cell size and membrane permeability have been proposed (Stachecki and Cohen 2004, Leibo 2008). Previously, high concentrations of cryoprotectant were required for vitrification, but novel containers and submicroliter volumes result in super-fast cooling and subsequent warming rates, so that concentrations closer to those used in slow-freezing can now be used (Mukaida et al. 2002, Kasai and Mukaida 2004).

During the vitrification process, cryoprotectants are needed to protect oocytes both extracellularly and intracellularly from drastic temperature changes. This temperature change will cause an increase in Reactive Oxygen Species (ROS). Likewise, when warming up, a heat shock will occur. The heat shock occurs when there is a change in temperature from cold to warm. Increased ROS during thawing will cause damage to the mitochondrial function and changes in  $\text{Ca}^{2+}$  ion influx (Favetta et al. 2017).

Changes in temperature due to vitrification and during thawing will highly affect the intensity of  $\text{Ca}^{2+}$  ions. The role of  $\text{Ca}^{2+}$  ions in oocytes is very important and is related to the oocyte maturation process. In the maturation process, there will be maturation of the nucleus and cytoplasm. In the nucleus maturation process, there will be an increase in  $\text{Ca}^{2+}$  influx into the oocyte nucleus, so that protein regulators will run for the nuclear maturation process to occur. Calcium ( $\text{Ca}^{2+}$ ) is necessary for the fertilization process and embryo development. The purpose of this study was to measure the expression of  $\text{Ca}^{2+}$  and the intensity of  $\text{Ca}^{2+}$  in cumulus-oocyte complex.

The immunocytochemistry (ICC) method indicates an increase in  $\text{Ca}^{2+}$  expression. The extracellular  $\text{Ca}^{2+}$  expression can only be observed using the ICC method, while the intracellular  $\text{Ca}^{2+}$  intensity requires the use of the Confocal Laser Scanning Microscope (CLSM) method. The combination of the immunocytochemistry method and the (CLSM) can accurately measure the content and position of  $\text{Ca}^{2+}$  in oocytes, due to the freezing process using the vitrification method.

## Materials and Methods

### Ethical eligibility

The ethical eligibility of this research was obtained from the Faculty of Veterinary Medicine, Universitas Airlangga, ethics number 1.KE.061.04.2019.

### Sample

The sample for this study was an ovary obtained from the waste of a slaughterhouse in Surabaya. Ovarian samples, after being cleaned of tissue and blood, were then washed with PBS. The clean ovary was placed in a bottle containing PBS solution and brought to the laboratory in a warm container at 37°C. The sample unit is cumulus-oocyte complex (COCs) obtained by follicular aspiration with a follicular surface diameter of 8-12 mm. The aspirated COCs are further classified based on the number of layers of cumulus cells surrounding the COCs. Only COCs coated with two or

more layers of cumulus cells were used for this study. From the results of the COCs classification obtained, it is used as a research sample unit. The sample unit was used to test the calcium intensity qualitatively by immunocytochemistry and quantitatively for the calcium intensity by CLSM. In the ICC process, much COCs were lost due to the staining process immediately, so that at the end of the ICC process, the total COCs obtained as a research sample unit was 17 COCs.

### Research site

This study examined the expression of calcium (Ca<sup>2+</sup>) between fresh COCs and COCs after vitrification using an immunocytochemistry method, and also examined the intensity of calcium (Ca<sup>2+</sup>) between fresh COCs and COCs after vitrification using the confocal laser scanning microscope (CLSM) method. The immunocytochemistry examination was carried out at the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga. The CLSM examination was carried out at the Central Laboratory of Biological Sciences, Universitas Brawijaya.

### Experimental design

This study was replicated 5 times, consisting of 3 replicates to the immunocytochemistry examination sample and 2 replicates to the CLSM examination sample. This research was carried out in stages after all the immunocytochemistry examinations were completed, and the research then continued for CLSM examination. In ovarian sampling, it was taken 4-6 ovaries to obtain a number of COCs samples. After aspiration, 40 to 45 COCs were obtained but not all of these COCs qualified as sample units. From the COCs obtained, they were then selected according to the criteria for the sample unit. On average, each collection obtained 24-25 COCs that met the requirements. COCs that met the criteria for the sample unit were then divided into 2 for immunocytochemistry examination of fresh COC samples without being vitrified, and some for vitrification of them being vitrified. 24-25 COCs that met the requirements, the 12 vitrified COCs were divided into 3 straws and 12-13 oocytes for immunocytochemistry examination of fresh non-vitrified COCs. Vitrification was carried out for 24 hours, and the COCs were then thawed and examined using immunocytochemistry. Post thawing and after immunocytochemistry examination, a total of 17 sample units were obtained for each control and treatment group. With immunocytochemistry examination there are always COCs that are lost because the staining and suction process is too strong. At the end of staining only half of the successful oocytes could be observed. The CLSM examination

was repeated twice, and the obtained COCs were divided into 2; 12 were vitrified COCs and 12-13 fresh COCs were not vitrified, and CLSM examinations were immediately performed. For the vitrified COCs, 24 hours later it was thawed and CLSM examination was performed. The total number of eligible ovaries was 20, and COCs as sample units that met the requirements were 104. After immunocytochemistry examination, the total sample units obtained for each group was 17.

### Cumulus-oocyte complex collection

The ovaries of goat were cleaned of ligaments and fatty tissue and washed with 0.9% physiological NaCl, which was added to 50 l of gentamicin sulfate. They were then put in a sterile tube and heated in a water bath at a temperature of 30-35°C. COCs are collected by follicular aspiration with a follicular surface size of 8-12 mm in diameter. Collected using a 10 ml disposable syringe with an 18 G needle previously filled with MEM media. The COC that had been collected was washed with MEM media (Widjiati et al. 2012). From 20 eligible ovaries with good ovarian condition, 34 COCs met the criteria as sample unit (the immunocytochemistry process obtained 17 COCs and CLSM as many as 17 COCs).

### Cumulus-oocyte complex vitrification

The COC samples were immersed in the equilibration medium for 18-20 minutes then immersed in the vitrification medium 1 for 30 seconds and the vitrification medium 2 for 30 seconds. 30 seconds in vitrification medium 1 and 2 is the optimum time for the entry of intra and extra cellular cryoprotectants into the COC, and the COC form has returned to its initial form and does not shrink. The soaked COC was placed at the end of the hemistraw as a place for COCs during vitrification. Furthermore, the hemistraw which already contained COC was evaporated over liquid N<sub>2</sub>, then put into liquid N<sub>2</sub>, then put in a canister and stored in a liquid N<sub>2</sub> container for 24 hours. After 24 hours, they were thawed for immunocytochemistry and CLSM examination. Post thawing, if there is a degenerated COC, it is not used as a research sample unit.

### Cumulus-oocyte complex warming

Warming of COCs was carried out at room temperature. COCs removed from the hemistraw were immersed in preheated thawing medium. The warming medium contained sucrose solution with a gradual concentration of 0.5 Mol and 1 Mol each for 1-3 minutes until the COCs returned to their original shape, then



Table 1. Assessment of immunocytochemistry examination scoring according to Novack et al (2007) semi-quantitatively based on the IRS value (AxB), which is the result of multiplying the percentage score of positive cells (A) with the color reaction intensity score (B).

A	B
Score 0: no positive cell	Score 0: no color reaction
Score 1: Positive cells less than 10%	Score 1: low color intensity
Score 2: Positive cells between 11% - 50%	Score 2: medium color intensity
Score 3: Positive cells between 51% - 80%	Score 3: strong color intensity
Score 4: Positive cells more than 80%	

Table 2. Qualitative examination of calcium ( $\text{Ca}^{2+}$ ) intensity by immunocytochemistry between fresh cumulus-oocyte complexes (COC) without vitrification and COC after vitrification.

Group	Calcium Expression					
	Total COCs	Mean	Standard Deviation	Minimum	Maximum	P
Control	17	0.351	0.79	0	2	0.000*
Treatment	17	12	00.00	12	12	

\* significant at  $p < 0.05$ ; C: fresh COCs without vitrification; T: COCs after vitrification.

Description: C: Fresh COCs without vitrification, T: COCs after vitrification.

transferred to the culture medium and ready for the stage of immunocytochemistry and CLSM examination.

#### Examination of $\text{Ca}^{2+}$ expression by immunocytochemistry

The fresh COCs was fixed on a glass slide, then rehydrated with graded alcohol, then washed with PBS, then soaked in 3% hydrogen peroxide  $\text{H}_2\text{O}_2$  (in DI water) for 20 minutes, 1% BSA in PBS for 30 minutes at room temperature, primary antibody  $\text{Ca}^{2+}$  1:1000 overnight, cold temperature  $4^\circ\text{C}$ , Biotin-labeled secondary antibody (Anti Rat IgG Biotin Labelled) and  $\text{Ca}^{2+}$  primary antibody, 1 hour at room temperature, SA-HRP (Streptavidin-Horseradish Peroxidase), 60 minutes, room temperature, Chromogen DAB (3,3-diaminobenzidine tetrahydrochloride), 20 minutes, room temperature, Counterstain (Aceto orcein), 3 minutes, room temperature then examined under a microscope (Widjiati et al. 2017). Semi-quantitative observation of the expression of  $\text{Ca}^{2+}$  was carried out according to the modified Remmele method (Novak et al. 2007). The Remmele scale index (IRS) is the result of multiplying the percentage score of immunoreactive cells with the color intensity score produced in the cells (Table 1).

#### Calcium ( $\text{Ca}^{2+}$ ) intensity examination with confocal laser scanning microscope (CLSM)

The oocytes were added to 10 l Fluo-3 40 M and incubated for 30 minutes, immersed in 100 l PBS (Ca-Mg) for 30 minutes, then coverslipped and observed with a confocal microscope.

#### Data Analysis

Expression and intensity of calcium ( $\text{Ca}^{2+}$ ) data were analyzed non-parametrically using the T-Test.

#### Results

##### Examination of calcium<sup>2+</sup> cumulus-oocyte complex expression with immunocytochemistry

Calcium<sup>2+</sup> intensity data were qualitatively examined by immunocytochemistry staining to investigate the effect of cold shock and heat shock on indications of changes in calcium ions. The total sample of calcium intensity, qualitatively, can be seen in Table 2.

The data obtained in Table 2 showed the average qualitative examination of calcium ( $\text{Ca}^{2+}$ ) intensity of Fresh COCs without vitrification and COCs after vitrification.

Based on the results of the different test, the T-Test showed that there was a significant difference between the COCs of the Fresh COCs group without vitrification and the COCs after vitrification group ( $p < 0.05$ ).

##### Examination of calcium intensity ( $\text{Ca}^{2+}$ ) in cumulus-oocytes complex with the confocal laser scanning microscope (CLSM) method

To visualize the intensity of calcium ions, the COCs examination was performed using the confocal laser scanning microscope method.

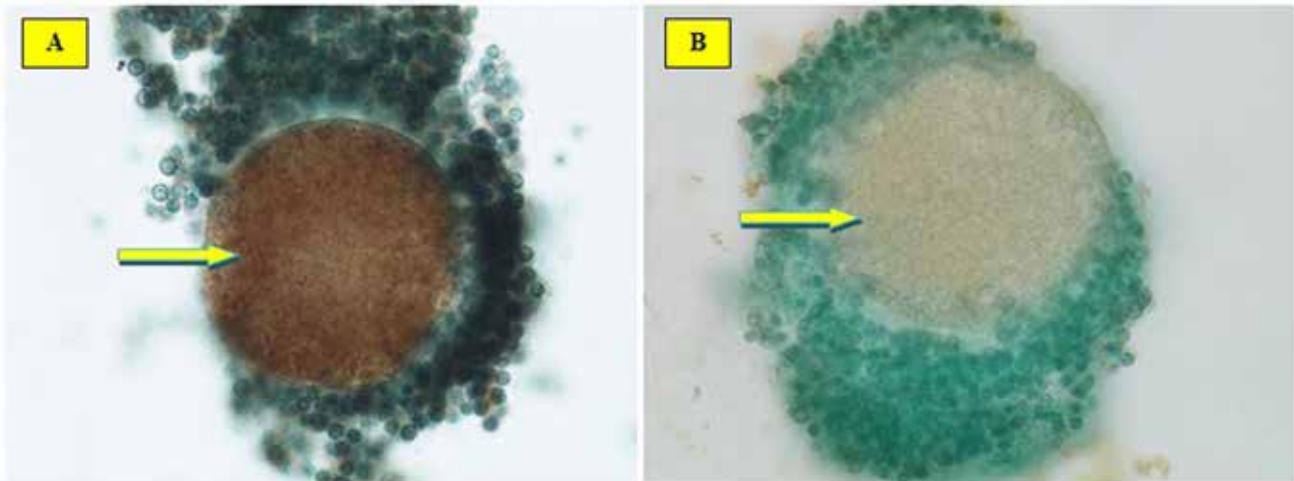


Fig. 1. Qualitative examination of calcium ( $\text{Ca}^{2+}$ ) intensity indicated by a very strong brown color (arrow) in COCs (arrow). On slide (A) the control group of fresh COCs without vitrification, showed a number of immunoreactive cells  $>80\%$  (score 4) with very strong color intensity (score 3), In (B) the group of COCs after vitrification showed a low expression with Score 1 (immunocytochemistry staining; 400x magnification; Olympus BX-50. Pentax optio 230; 2.0 megapixel Digital Camera).

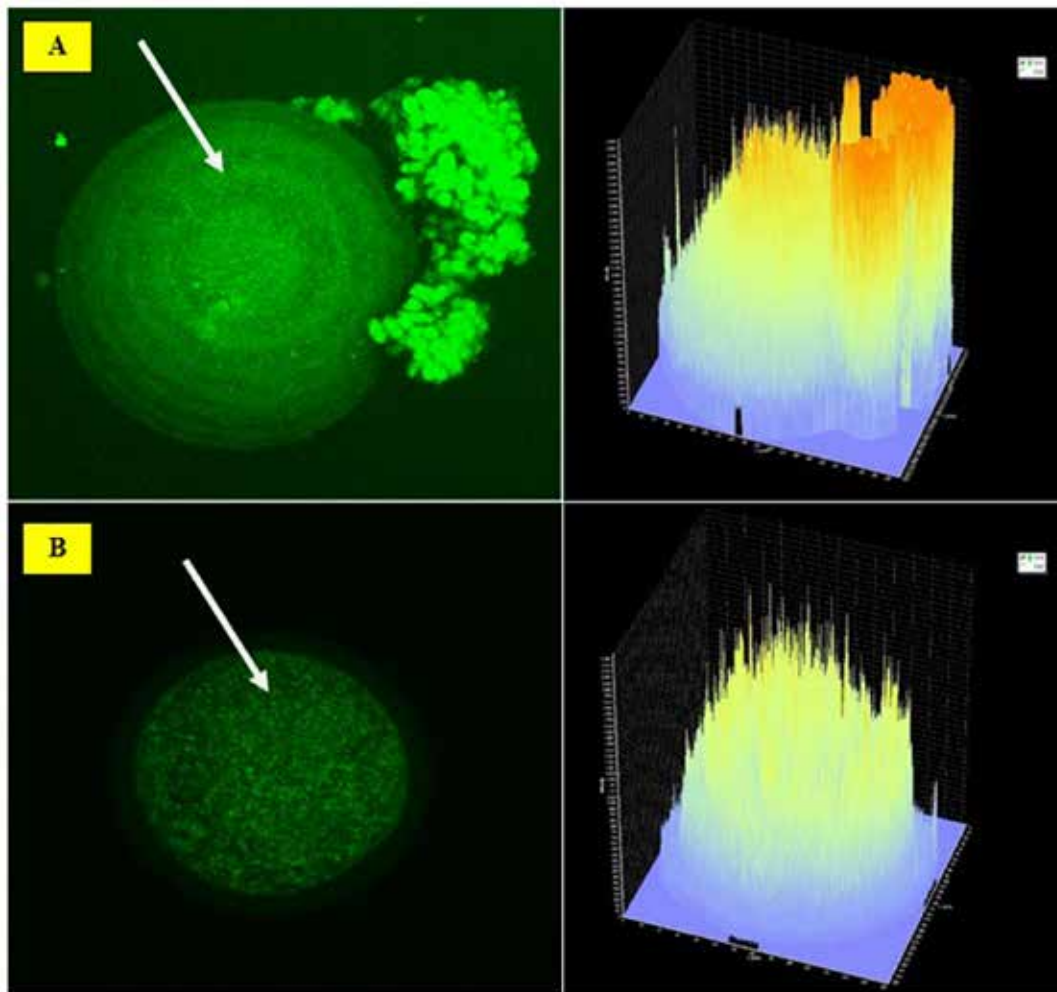


Fig. 2. Calcium intensity ( $\text{Ca}^{2+}$ ) in COC after vitrification was concentrated in the nucleolus (B), in fresh COC without vitrification it was concentrated in the cytoplasm (A); White arrows: COCs are in good condition, and arrows show fluorescent luminescence in the nucleus and cytoplasm (Olympus Microscope, Japan Type FV1000, Olympus Software FLUoview version 4.2a; x100).

Table 3. Quantitative examination of calcium ( $\text{Ca}^{2+}$ ) intensity by confocal laser scanning microscope between fresh COC without vitrification and COCs after vitrification.

Group	Total COCs	Mean	Std. Deviation	Sig.
Control	17	1059.43 <sup>a</sup>	489.59	0.006
Treatment	17	568.21 <sup>b</sup>	484.31	

Description: Different superscripts in the same column show significant differences ( $p < 0.05$ ).

C: Fresh COCs without vitrification, T: COCs after vitrification.

## Discussion

The results obtained qualitatively indicated that the intensity of calcium in fresh COCs without vitrification was higher than in vitrified COCs. Likewise, the quantitative examination of calcium intensity in fresh COCs without vitrification was higher than that of vitrified COCs. The results of qualitative examination of calcium intensity showed brown color expression in COCs in the group of fresh oocytes without vitrification (a number of immunoreactive cells with very strong color intensity and COCs after vitrification exhibited a low expression shown in Fig. 1 above. Fig. 2 shows that calcium intensity ( $\text{Ca}^{2+}$ ) in fresh COC without vitrification was concentrated in the cytoplasm (A) and in COC after vitrification was concentrated in the nucleus (B).

The results of statistical analysis showed that the average calcium intensity qualitatively in fresh oocytes without vitrification was significantly different from vitrified COCs ( $p < 0.05$ ). Expression of calcium ( $\text{Ca}^{2+}$ ) was higher in the fresh oocyte group compared to COCs after vitrification (Table 3). This is in accordance with the research of Bonte et al. (2020), who stated that rat oocytes after vitrification-thawing showed decreased  $\text{Ca}^{2+}$  potential during oocyte activation induced by cryoprotectant exposure.

From the research data that has been obtained on vitrified COCs there is a qualitative decrease in calcium intensity. The results of this study are consistent with Sanaei et al. (2018), who state that calcium is needed to maintain oocyte quality. Likewise, according to the results of Marques et al. (2018), the use of vitrified solution with calcium chelator can increase the ability of COCs after vitrification to maintain mitochondrial distribution and function as well as chromosomal segregation. The release of  $\text{Ca}^{2+}$  ion concentration in vitrified media is the most effective protocol for oocyte cryopreservation (Marques et al. 2018).

The decrease in calcium intensity qualitatively by immunocytochemistry examination in this study was influenced by many factors, among others, heat shock when the COCs were thawing. According to Jang et al. (2017) and Orief et al. (2005), post-thawing greatly affects the quality of COCs. Many factors affect the

quality of post-thawing COCs; including the length of exposure to the cryoprotectant, the type of cryoprotectant used, temperature, and viscosity. At the time of thawing there will be a change in temperature from frozen to melting, and this condition will affect the release of free radicals. The release of free radicals will affect changes in calcium oscillations and cause disturbances in mitochondrial function (Roth 2018).

The success rate of cryopreservation is influenced by lipid content in the cytoplasm. For example, centrifugation to remove lipids can increase the survivability of post-freezing embryos. The specific mechanism of lipid accumulation in vitro is still not fully understood, but the most probable mechanism is lipid peroxidation, which is amplified in the cryopreservation process due to excessive production of free radicals, and the inhibition of ROS production in the cytoplasm increases viability (Barceló-Fimbres and 2007).

According to Barceló-Fimbres and (2007), unsaturated fatty acids such as albumin-bound linoleic are a useful additive during culture, although they do not affect embryonic development. Unsaturated fatty acids increase post-vitrification viability. Unsaturated fatty acids can increase the fluidity of the membrane.

In this study, COCs were not matured first. Vitrification of COCs was at the germinal vesicle stage. Germ-stage vesicle COCs were more tolerant of the effects of stress during vitrification. The genetic material is still encased in the nuclear membrane (Daddangadi et al. 2020).

The results obtained from quantitative examination of calcium intensity with CLSM showed that the intensity in the fresh COCs group without vitrification was higher than in the vitrified COCs group ( $p < 0.05$ ).

In this study, using immunocytochemistry examination,  $\text{Ca}^{2+}$  expression was seen only in COCs, but not in granulosa cells. Calcium ( $\text{Ca}^{2+}$ ) present in COCs regulates COCs membrane balance and plays a role in the COC maturation process. The results of this study are in accordance with the theory that intracellular  $\text{Ca}^{2+}$  plays a role in ATP regulation and the regulation, of cytokines present in the oocyte nucleus is related to the maturation process in the oocyte nucleus and cytoplasm.

The immunocytochemistry method has not been

able to fully describe the intensity of Ca<sup>2+</sup> levels in oocytes. Calcium (Ca<sup>2+</sup>) expression is only expressed descriptively on the surface of oocytes, while oocytes are 3-dimensional cells. For this reason, it is necessary to measure the intensity of Ca<sup>2+</sup> using the confocal laser scanning microscope (CLSM) method, so that the intensity of Ca<sup>2+</sup> can be measured in the intracellular part of the oocyte.

Intracellular Ca<sup>2+</sup> intensity in fresh COCs without vitrification and vitrified COCs can be detected using a confocal laser scanning microscope (CLSM), using a 40 M Fluo 3 calcium probe in Phosphate Buffer Saline (-Ca, -Mg) medium. Calcium (Ca<sup>2+</sup>) was detected using a 488 nm argon laser. The intensity value is determined by determining the cut-off, by adjusting the wavelength of the instrument used. The cut-off value is the intensity value of the reflected light. Calcium (Ca<sup>2+</sup>) oscillation in oocytes is an important factor because it determines the success of fertilization and subsequent embryonic development. In the process of fertilization, spermatozoa will release Phospholipase C-Zeta (PLC $\zeta$ ) to induce the release of Ca<sup>2+</sup> in the ooplasm before the occurrence of fertilization (Ramdan et al. 2012, Chithiwala et al. 2015). The increase in intracellular Ca<sup>2+</sup> is a signal that indicates the occurrence of the meiotic stage, and an increase in Ca<sup>2+</sup> allows interactions between oocytes and spermatozoa in the fertilization process (Borgers et al. 2009, Kang et al. 2015, Karabulut et al. 2018). The combination of the immunocytochemistry method to visualize the expression of Ca<sup>2+</sup> and the CLSM method to measure the intensity of Ca<sup>2+</sup> is an appropriate method for accurately determining the increase in Ca<sup>2+</sup> levels in oocytes.

In thawing COCs after vitrification, there will be an increase in HSP 70 to protect the oocyte from changes in cold temperatures to warm temperatures, so that the cells do not experience heat shock. If HSP 70 is not able to protect cells from temperature changes, there will be a decrease in Ca<sup>2+</sup> which will cause a change in the ratio of Ca<sup>2+</sup> and Sodium (Na). Changes in Ca<sup>2+</sup> will affect the quality of the COCs.

Calcium (Ca<sup>2+</sup>) is an important factor in the process of oocyte development. Changes in Ca<sup>2+</sup> will affect ATP regulation and impaired Ca<sup>2+</sup> stabilization intracellularly (Feng et al. 2018). The decrease in the intensity of intracellular Ca<sup>2+</sup> in post-warming oocytes will cause changes in proteins in the oocyte nucleus and will activate the pro-apoptotic cascade.

The biochemical and structural changes that occur during the COCs maturation process affect the oocyte's ability to increase the calcium concentration in the cytoplasm. The structure of the Endoplasmic Reticulum will change during the freezing and warming process and will affect its ability to release calcium. Increasing

the concentration of calcium is important in maintaining oocyte quality (Fraser 1982, Wang and Machaty 2013).

Calcium (Ca<sup>2+</sup>) is important in the regulation of cytokine signaling in oocytes for the COCs maturation process. Calcium (Ca<sup>2+</sup>) oscillation is important in the preparation of the COCs maturation process, which will activate MPF and MAPK. When the COCs are mature and have released polar body II, the concentration of Ca<sup>2+</sup> is high which indicates good COCs quality, whereas, in poor COCs quality, the level and intensity of Ca<sup>2+</sup> will decrease. High concentrations of Ca<sup>2+</sup> in the intracellular will support successful fertilization and will indirectly improve the quality of embryo development (Fernandez et al. 2012, Cheon et al. 2013).

## Conclusion

There was a qualitative and quantitative difference in Ca<sup>2+</sup> intensity between fresh non-vitrified COC and vitrified COC. The intensity of calcium (Ca<sup>2+</sup>) was qualitatively higher in fresh COCs without vitrification. The intensity of calcium (Ca<sup>2+</sup>) in COCs vitrification was concentrated in the nucleus and fresh COCs without vitrification were concentrated in the cytoplasm.

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