# The Role of Cumulus in the in vitro Maturation Process towards the Maturation Level of Kacang Goats (*Capra Aegagrushircus*)

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**Abstract**

Kacang goat is a local specialty in Indonesia. The level of oocyte maturation of Kacang goat after in vitro maturation will determine the quality of the embryo. Cumulus cells have an important role during the process of maturation for nutrient transportation. The purpose of this study was to determine the role of cumulus on the level of maturation of Kacang goat oocyte. Oocytes collected by aspiration method. The study was divided into 2 groups: the group I oocytes surrounded by more than 3 layers of cumulus. The group II was oocytes around only 1 layer of cumulus. All oocytes were matured in EBSS medium which was hormone supplemented and incubated in a 5% CO2 incubator at 38.5oC for 22 hours. Microscopic examination of oocyte maturity level was based on cumulus expansion and the presence of Polar body I. The results showed that the maturation rate in the group I was 59.26% and immature oocyte 40.74%, in group II the number of mature oocytes was 49.37% and immature oocytes 50.63 %. The conclusion of the study was that the level of oocyte maturation with thick cumulus was better than bald oocytes. Cumulus has a very important role in the maturation process of Kacang goat oocytes. The level of oocyte maturity can support embryonic development after IVF.

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## Introduction

Kacang goat is a local goat in Indonesia which has a very varied level of productivity and reproducibility in each region. Kacang goat population in Indonesia decreases every year. It is feared that someday the goat will become extinct if no conservation efforts are carried out again. Efforts to conserve goats are closely related to the use of genetic quality improvement strategy technologies [1].

The local goat is one of Indonesia's germplasm, its body size is small and economically disadvantageous compared to import goats but it has several advantages including adaptability because it is native to Indonesia and the cost of treatment is not expensive. This local goat has a kind of existence because of the introduction of Artificial Insemination (AI) technology and other big imports goat such as Boer goats from Australia. The large number of imported goats has caused local goats to be marginalized, especially since the local goat recovery center has not yet been done. Strengthening technology *in vitro* namely *In vitro* Maturation, *In vitro* Fertilization and *In vitro* Culture which is a chain of producing large numbers of Kacang goat embryos, is needed to help these goats so they are not marginalized and extinct.

*In vitro* maturation process is the initial stage to produce oocytes as a source of gametes for the production of embryos *in vitro*. In the *in vitro* maturation process a stable culture system and quality of oocytes are needed as a good source of oocytes. There are several things that can increase the success of oocyte maturation, namely oocyte selection, hormonal stimulation in the medium, and medium supplementation [2].

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The process of maturation *in vitro* includes the process of maturation in the nucleus and cytoplasm [3]. In *in vitro* maturation, the role of cumulus cells is helpful during the process of maturation. During the process of maturation, nucleation and cytoplasmic maturation occur, so that the oocyte has sufficient competence to enter the next stage, namely the process of fertilization and embryo development. Oocytes will reach the stage of metaphase II which is characterized by nuclear maturation and molecular changes [4].

Cumulus cells have an important role during oocyte growth and maturation. Cumulus cells are mediators providing energy transport, micronutrients, and/or carrier molecules for oocyte development and mediate the effect of hormones on the cumulus-oocyte complex. As a transport mediator, cumulus cells play an important role in oocyte maturation *in vitro* and subsequent development through gap junctions [5].

Fetal Calf Serum is a protein that is often added to culture media to support the development and division of cells, oocytes or embryos. The addition of FCS to the maturation media is very important in the process of cumulus expansion induced by FSH. The use of FCS also increases the viability of cumulus cells and accelerates the process of division of meiosis I in cow and hamster oocytes [6].

Oocytes that have complex cumulus oocytes (COCs) and are bright and transparent, have a homogeneous cytoplasm will produce oocytes with a very good level of maturity. At the end, haploid oocytes are produced [7].

## Materials and methods

The research was carried out in the Biomedical Laboratory of the Faculty of Medicine, Universitas Airlangga. This research received ethical clearance number: 1.KE.061.04.2019 released by the Animal Care and Use Committee, Universitas Airlangga, Faculty of Veterinary Medicine. This study uses a Completely Randomized Design (CRD), assuming all treatments are given equally from sampling to workmanship and laboratory conditions. The sample of this research was the Kacang goat oocyte obtained from an abattoir. The sample of this study was divided into 2 treatment groups namely treatment group I (T1), namely cumulus oocyte complex of the Kacang goat surrounded by cumulus of more than 3 layers, saturated in EBSS medium and added 7% Fetal calf Serum. Treatment group II (T2) was a bare oocyte or cumulus complex of Kacang goats surrounded by 1 layer of cumulus cell, saturated in EBSS medium and supplemented with 7% Fetal calf Serum. Maturation was carried out in a 5% $CO_2$ incubator at 38.5$^{o}$C for 22 hours. The data obtained were then analyzed using a two-paired Independent Samples T-Test with $p < 0.05$. Independent-Samples T-Test is a parametric test. The stages of this research are as follows: medium preparation, goat oocyte collection, *In vitro* Maturation of Kacang goat oocytes, examination of the level of maturity of the goat oocyte.

### Medium Preparation

Media maturation was prepared by making EBSS media drops plus 7% Fetal calf Serum, HCG Hormones and PMSG on disposable petridish with micropipette. The medium droplets are then covered with mineral oil, and incubated in an incubator with 5% $CO_2$ at 38$^{o}$C for 22 hours before being used for *in vitro* maturation.

### Oocyte collection

Kacang goat ovaries are obtained from abattoirs. The ovaries are brought to the laboratory in a flask containing 0.95% physiological NaCl with a warm temperature of 37$^{o}$C. After arriving in the laboratory, the ovaries are cleaned from the hanging device and washed with physiological NaCl 0.95% and 100 µl gentamycin until clean. Oocyte collection is done by aspiration using a syringe with an 18G needle containing 1 ml medium EBSS. And then, the collected oocytes are inserted into a sterile petri dish and followed by observation under a complex of 2 or more microscopes to observe oocyte grade oocytes. Only oocytes surrounded by cumulus complexes of more than 3 layers are used for *in vitro* maturation. Oocytes collected half to be frozen by vitrification method and half to oocyte maturation.

### Maturation *in vitro*

Oocytes are surrounded by more than 3 layers of cumulus, washed 3 times with EBSS media before maturation. Oocytes are transferred to the previously prepared maturation media, and incubated in a 5% $CO_2$ incubator at 38$^{o}$C for 22 hours until expansion of cumulus cells occurs. Then oocyte maturity level is examined.

**Examination of the level of oocyte maturation of Kacang goat**

After 22 hours of culture, observations were made to see the level of oocyte maturity using Nikon Diaphot 300 microscope with magnification of 100 x and 400 x. To observe Polar body I, a denudation was performed to remove cumulus cells.Chemical denudation usually uses the enzyme hyaluronidase (HYASE-10x ™, Vitrolife®), the oocyte is immersed in a medium containing the hyaluronidase enzyme and left for 30 seconds then transferred to the culture medium [7].

## Results

**Oocyte maturity level**

The level of oocyte maturity obtained from maturation between treatment group I and treatment group 2 is different. The number of mature oocytes marked by the formation of Polar Body I in the treatment group I was higher than in the treatment group 2. In the treatment group 2 the number of ooites that did not mature after *in vitro* maturation was higher than in mature oocytes. For more details can be seen in table 1 below.

| Group | Number of Oocytes | Mean±Standart Deviation Mature Oocytes | Immature Oocytes | Probability (p≤0,05) |
|---|---|---|---|---|
| T1 | 162 | $96^{a}$± 2.00 | $66^{a}$± 2.65 | .021 |
| T2 | 158 | $78^{b}$± 2.00 | $80^{b}$± 2.51 | .045 |

**Table 1**. Mean and Standart Deviation Maturation Level of Kacang Goats Before and After Vitrification.

Different superscripts in the same column show significant differences (p <0.05)

T1: Cumulus oocyte complex. Kacang goat surrounded by cumulus of more than 3 layers, is saturated in EBSS medium and added 7% Fetal calf Serum.

T2: Bald oocytes or cumulus goat complex Beans are surrounded by only 1 layer of cumulus, saturated in EBSS medium and supplemented with 7% Fetal calf Serum.

The number of mature oocytes in the treatment group I ($96^{a}$±2.00) was higher than the treatment group II ($78^{b}$±2.00), While the number of immature oocytes in group I ($66^{a}$±2.65) was lower than in treatment group II ($80^{b}$±2.51). The mature oocytes are marked by the formation of Polar body I and cumulus expansion, whereas in oocytes which did not mature after *in vitro* maturation was not found in the formation of Polar Body I as shown in Figure 1 and Figure 2.

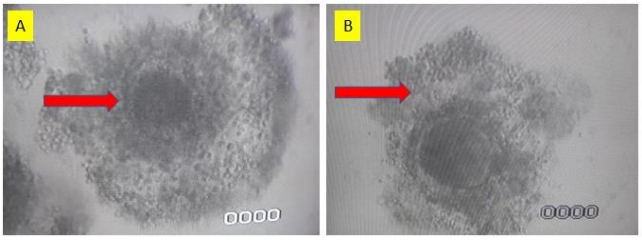


**Figure 1.** Kacang goat oocytes after *in vitro* maturation for 20 hours. The figure shows that cumulus cells spread equally to surround oocytes. A: Oocyte B: Cumulus cells.

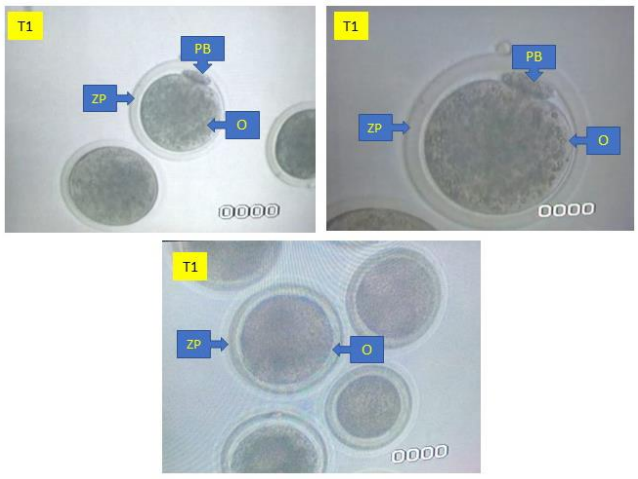


**Figure 2**. Group T1, Oocytes in the Metaphase II stage marked by Polar Body I (arrows), Group T2 oocytes not yet mature did not find Polar Body I (Observation with magnification of 100x and 400x; Nikon Diaphot 300).

## Discussion

The results showed that the level of oocyte maturity of the treatment group I, namely oocytes that have cumulus more than 3 layers, when matured, give a higher level of mortality than the bare oocytes that are saturated oocyte. Oocytes quality is classified based on the number of cumulus cell layers surrounding the oocyte. The number of cumulus layers surrounding the oocyte decreases during storage, thereby affecting the number of oocytes that reach the metaphase II phase. The number of cumulus cell layers surrounding the oocytes (3-5 layers) significantly affected the number of oocytes reaching the MII stage. Long storage time can reduce the quality of oocytes. Mature oocytes are the product of the first meiotic division, the secondary oocyte and the first polar body (PB I), which are located between the plasma membrane and the zona pellucida in the perivitelline space [8].

Decreasing in oocyte quality can be a type of follicular degeneration, besides showing pyknosis, oocytes also show irregular granulosa cells and decreased cellular level density [9].

The data in the table above shows that oocytes surrounded by cumulus cells of more than 3 layers give a higher rate of oocyte maturation than those of bald oocytes that are matured *in vitro*. In treatment group II there was a decrease in the ability of developing oocytes to reach MII, this was indicated by the number of oocytes that did not mature more. The thickness of the cumulus cells surrounding the oocytes is needed as a transport route for nutrition from the oocyte's external environment. The success of oocytes to reach the stage of meiosis division is strongly influenced by microtubules and microfilaments, as the process progresses, the oxygen demand of the oocytes will increase. Incubation temperature and media storage are the main factors influencing complete oocyte maturation [10].

Cumulus cells have an important role during oocyte growth and maturation. Cumulus cells are mediators providing energy transport, micronutrients, and / or carrier molecules for oocyte development and mediate the effect of hormones on the cumulus-oocyte complex. As a transport mediator, cumulus cells play an important role in oocyte maturation *in vitro* and subsequent development through gap junctions. Cells communicate with a gap junction built by the connexin43 (Cx43) protein [5].

Cx43 remains in the complex cumulus oocyte until after the expansion of cumulus cells. This implies a constant level of communication between cumulus cells and oocytes after maturation. The presence of gap junctions after maturation of complex cumulus oocytes as evidenced by Cx43 expression may indicate that communication between oocytes and cumulus cells and / or between cumulus cells through gap junctions [5].

One of the micro factors of the oocyte environment that supports oocyte maturation is cumulus cells. As a functional unit of the ovarian follicle, cumulus cells play an important role during oocyte growth and maturation as indicated by the significance between the rate of cumulus cell expansion and the quality of oocyte maturation. The development of cumulus cells such as expansion and production of extra cellular matrices can be stimulated through the activation of the Epidermal Growth Factor (EGF) family [5].

The regulation of nucleus maturation involves cytokines affecting between several metabolic pathways in granulosa and oocyte cells. Metabolic pathways in granulosa cells are regulated by cell surface receptor binding. Molecules such as adenosine, uridine, hypoxanthine, and their metabolites diffuse between granulosa cells and oocytes [11].

The mechanism for keeping oocytes in a resting state is still being studied, but there are two theories that are often used namely, adenosine stimulates oocyte adenylate cyclase through surface receptors on oolemma, and hypoxanthine prevents cAMP hydrolysis, high cAMP keeps meiosis resting. Adenosine can participate directly in keeping meiosis at rest by converting it to ATP, the substrate for adenyl cyclase in the oocyte. Purines can also participate in cell signaling via G-protein in oolemma and plasma membranes of cumulus cells [12].

*In vivo*, nucleus maturation starts from the primary oocyte in the antral follicle to a secondary oocyte, a process of meiosis, with transformation. This process follows the surge in FSH and LH, just before ovulation. The germinal vesicle membrane is fractured, and the nucleus develops from the first dictate prophase meiosis state and then rests again in metaphase II. LH surges will increase cAMP in oocytes. This process involves intracellular messengers such as $Ca^{2+}$ and $H^{+}$, and a series of complexes that have accumulated in oocytes, including maturation promoting factor (MPF) and cytostatic factor (CSF). LH surges and progesterone secretion, causing oocytes to enter the Germinal Vesicle Breakdown (GVBD) stage. The form of chromatin in the nucleoli is enlarged and meiosis goes back [4,13,14].

LH surges during folliculogenesis cause morphological changes of cumulus cells and secretion of hyaluronic acid in the intracellular space. Oocytes also produce cytokines that initiate the production of hyaluronic acid and matrices that convert solid granulosa cells into diffused and dispersed. The syncytial connection between the oocyte and cumulus cells is lost, intercellular communication through the gap junction is stopped, metabolites and molecules can no longer enter the oocyte. This event triggers meiosis division [12].

During oocyte growth, the size of the golgi apparatus increases and develops around the cortex of the oocyte. The golgi apparatus secretes glycoproteins into the zona pellucida and forms cortical granules that can be found on the oocyte surface. Cortical granules contain enzymes that will be released when fertilization occurs to change the zona pellucida. The endoplasmic reticulum extends into the cytoplasm to form a dense membrane toward the cortex. Endoplasmic reticulum will be involved in calcium excretion for cortical granular exocytosis. An important feature during cytoplasmic maturation is the translation of mRNA during stable and inactive oogenesis [15,16].

Oocyte cell membranes are composed of a mixture of proteins and membrane lipids. Lipid levels in the membrane occupy 20 to 80%, depending on the cell type and cell function. Lipids play a role in membrane flexibility, whereas membrane proteins regulate the chemical content in cells and regulate the chemical transport of membranes. Phospholipids are the main component in cell membranes. The lipid bilayer (lipid bilayer) is semi permeable. Only a few molecules can diffuse through the membrane. Cholesterol is the largest lipid component of cells and is evenly distributed so as to prevent the membrane from becoming hard caused by phospholipids that are tightly bound [17].

The structural proteins in oocytes composed of proteins play an important role in maintaining the shape and size of cells. Receptors on protein cell membranes play an important role in cell communication to the external environment by the use of hormones, neurotransmitters and other molecular signaling. Membranes can repair themselves post ICSI by the way lipid molecules contained in membranes regulate their respective positions to reduce exposure from their hydrophobic regions to water[17].

The biochemical and structural changes that occur during the process of oocyte maturation affect the ability of oocytes to increase the concentration of calcium in the cytoplasm. The structure of the Endoplasmic Reticulum will change during the process of maturation and will affect the ability of the Endoplasmic Reticulum to release calcium. Increased calcium concentration is important in maintaining oocyte quality[18].

## Conclusions

The study was that the level of oocyte maturation with thick cumulus was better than bald oocytes. Cumulus has a very important role in the maturation process of Kacang goat oocytes. The level of oocyte maturity can support embryonic development after IVF.

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## Declaration of Interest

The authors declare no conflict of interest.

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