THE MATURATION RATE OF DIFFERENT OOCYTES QUALITY IN INDONESIAN THIN TAILED SHEEP CULTURED *IN VITRO*: AN INITIAL STEP FOR FURTHER USE IN EMBRYO PRODUCTION

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ABSTRACT

To preserve and further use of oocytes of Indonesian Thin Tailed Sheep (ITTS), its quality which determined by the presence of cumulus complex cell (CC) layers need to be considered for *in vitro* maturation (IVM). This study aims to know whether oocyte quality has effect on IVM rate of ITTS. Further result of the IVM, would be use in the production of *in vitro* embryos to keep the Indonesian indigenous animal genetic. Oocytes were grouped into grade A - (oocytes with >2 CC layers) and grade B (<2 CC layers). The IVM outcome were evaluated into three categories namely mature (M-II), undeveloped (UD) and degenerated (DG) oocytes after 24 h of culture. In total, 228 oocytes were collected; 143 and 85 oocytes were grouped into A and B grades, respectively. The IVM rate in grade A vs B showed oocytes which developed into M-II, UD and DG were 57.34% *vs* 43.53 % (P< 0.05); 22.38% *vs* 37.65% (P<0.01) and 20.28% *vs* 18.82% (P<0.01), respectively. The results show that a greater number of grade A oocytes was recovered, and that the percentage of mature oocytes after IVM in these oocytes was higher that that achieved by grade B oocytes.

***Key words*:** Oocyte quality, *in vitro* maturation, Indonesian thin tailed sheep

Introduction

In mammals, although ovaries produce large number of oogonia, female produce limited number of mature gametes during estrus cycle. In every estrus cycle, though dozens of antral follicles were recruited, selected, then developed into dominant follicles, however, most often, only one dominant follicle will be fully matured, ovulated, and fertilized. The large majority of primordial follicles were atretic (Britt, 2008), even those have same biologic and genetic potential to develop into mature oocytes. Attention to utilize and retrieve immature oocytes would exploit female genetic potential for further research in offspring production, gamete preservation, and embryo study. To address that potential benefits, immature oocytes must be matured by IVM before proceeded to the next purposes. The outcome of IVM is mature oocytes in meiotic phase II (M-II) which is indicated by the presence of first polar body (PB). During IVM, immature oocytes continuing its development from GV state to M-II, and will remaining in this stage until activated by natural or artificial activation (Alberio *et al.,* 2001). Thus, IVM being a crucial step for in vitro embryo production (Widyastuti *et al.,* 2017) that affect to the further embryo survival as well as fetal development (Krisher, 2004).

Previous studies showed that IVM outcomes were influenced by many factors such as culture condition, including culture media component and atmosphere, and the presence of cumulus cells representing oocytes quality (Appeltant *et al.,* 2015; Mahmoudi *et al.,* 2005; Warriach and Chohan, 2004). Considering the first step of IVM is oocytes collection, oocytes quality needs to be paid in attention. Cumulus cells are necessary for meiotic and developmental competence of oocyte growth and maturation, in this regard number of CC layer, cumuli quality, and cumulus expansion are determined the success of IVM and required for viability of mature and fertilized oocyte (Nevoral *et al.,* 2014).

Indonesian Thin Tailed Sheep is an indigenous breed, belongs to small ruminant and mostly reared in West Java Indonesia. Due to its size (small), easy to raise, and prolific, this sheep plays an essential role in small holder farmer such as saving, investment, manure production, religion aim, meat source and urgent cash (Udo and Budisatria, 2011). Additionally, ITTS is genetically reported resistant to fascioliasis (Pleasance *et al.,* 2011) which gives point to promote local breed cultivation. It is reported that *Fasciola hepatica* and *Fasciola gigantica* were classified by World Health Organization as tropical disease which has high risk to infect millions of people, therefore this parasites infection is categorized as one of health problem due to the increasing demand of animal-derived food product (Cwiklinski *et al.,* 2016).

The increasing demand of sheep meat tend to increase the slaughtering number in both male and female. At this point, females left hundreds of immature oocytes that still have possibility to be utilized or even preserved. Considering the aforementioned facts, this study aims to investigate the effect of ITTS oocyte quality, indicated by the presence of CC layers, to the IVM outcomes of ITTS.

Materials and Methods

Ovaries and oocytes collection

Sheep ovaries were collected from local abattoir, brought to the laboratory within 2 hr in warm (38ºC) saline (0.9 % NaCl) supplemented with 100 IU/ml penicillin (MERCK, Germany. Cat. No. 5161-25MU) and 100 μg/ml streptomycin (Sigma-Aldrich, USA. Cat. No. 5711-100GM). Once arrived, three times washing in fresh and warm saline were performed to the ovaries. Oocytes were collected using slicing method in collection medium containing Phosphate Buffer Saline (PBS)

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(Sigma-Aldrich, USA. Cat. No. P3813) supplemented with 5 % Bovine Calf Serum (BCS) (Sigma-Aldrich, USA. Cat. No. 12133C) under stereo microscope (Nikon SMZ-10). Oocytes were then evaluated on its cytoplasm homogeneity, only oocytes with homogenic cytoplasm were proceed to the grade classification based on the presence of intact cumulus cells (CC) layer. Grade A was indicated by more than two CC layers while grade B when CC is less than two layers (Bakri *et al.,* 2016). Following the classification, oocytes were washed three times using maturation medium then cultured.

***In vitro* maturation and development stage evaluation**

In this study, oocytes were cultured in groups of 10 in maturation medium containing modified TCM-199 (Thermo Fisher Scientific, USA. Cat. No. 31100035), 10 % BCS (Sigma-Aldrich, USA. Cat. No. 12133C), 10 mIU/ml Follicle Stimulating Hormone (FSH) (Sigma-Aldrich, USA. Cat. No. F2293), and incubated in 38.5°C, 5% CO_2 for 24 hr. The maturation was performed in 35 mm petri dish (Nunclon® MERCK, USA. Cat. No. D7804-500EA) and maturation medium drops (each 100 µl) were covered with sterile mineral oil (Sigma-Aldrich, USA. Cat. No. M8410). On the next day, CC were removed by incubating the oocytes in 1 % Hyaluronidase (Fischer Scientific, USA. Cat. No. ICN10074080) at 37°C for 5 min followed by rigorous pipetting. After CC removal, oocytes were observed and then categorized into oocytes with 1st polar body extrusion means developed or mature (M-II), undeveloped (UD), and degraded (DG) oocytes indicated by vacuolated and shrunk cytoplasm.

Data analysis

In this study, the rate of IVM were calculated in percentage by dividing the number of oocytes with its development statues post IVM, with the total number of immature oocytes in each grade. Five batches for IVM were performed during the study and arranged as replication. Moreover, statistical comparison between oocytes grade in each post IVM outcome was done by *Chi Square* and $P< 0.05$ was considered as significant different.

Results and Discussion

A total of 228 oocytes were recovered from collected ovaries, 143 oocytes were belonging to grade A and 85 oocytes were grade B. Figure 1 shows the different oocyte grades based on the number of CC layers, with grade A oocytes (Fig. 1A) having more intact CC layer compared to B grade ones (Fig. 1B). The IVM outcome (Table 1) shows that grade A oocytes resulted more ($P<0.05$) matured oocytes (M-II) as indicated by the presence of 1st polar body (Fig. 2A) when compared to grade B. Moreover differences ($P<0.01$) also observed in both UD and DG which shows higher in grade B oocytes. In this study UD was indicated by no visible polar body (Fig. 2B) and DG was observed by visible shrunk cytoplasm (Fig. 2C).

In vitro maturation (IVM) is one of the most important assisted reproductive technologies that archived huge improvement in the field of animal breeding and conservation of genetic recourses. Although several investigators studied IVM process intensively to be applied commercially in many farm animals, it is still not a conservative option in both breeding industry and genetic recourses conservation of Indonesian indigenous breed. To increase the application of IVM oocytes to produce more i*n vitro* embryo production, for both breeding propose and genetic conservation, quality of the obtained oocytes is imperative. Different morphological markers have been proposed to evaluate the oocyte quality (Wang and Sun, 2007) as noninvasive method. For example, continuous multilayer cumulus, bright and homogeneous ooplasm are considered to be as a good sign for immature oocytes quality. On the other hand, incomplete and granulated or too less CC layers, dark oocyte cytoplasm is a bad sign and pointed to lower quality immature oocytes. Hence, the presence of CC is essential for the success of IVM in several animals, mirroring oocytes quality and the IVM outcomes.

The presence of multi-CC layers during IVM in this study, resulted better number of matured oocytes (Table 1). The result agrees with the similar study in other ruminants as cattle (Auclair *et al.,* 2013), pig (Alvarez *et al.,* 2009), and buffalo (Warriach and Chohan, 2004). In Indonesian animal, the present study also in agreement with the previous review by DaBroi *et al*. (2018) that CC is one of critical factors which determine the oocyte quality. It is explained that CC have contribution to support oocytes metabolism through intracellular communication *via* gap junction. In detail, CC are connected to the oocyte by the presence of connexin which enable the transfer of small molecules needed for oocytes development.

In the antral follicle, oocytes are surrounded by multilayer of CC, in a structure called “cumulus-oocyte complex (COC)”. Harmonized communications take place in the COC through *connexin* based gap junction channels between the oocyte and surrounding CC. The channel is an interaction between CC and oocyte which occurs *via* cytoplasmic extensions of CC that go through zona pellucida and building up a strong contact with the oocyte membrane. This contact then allowing the formation of intracellular communications, leading to launch metabolic coupling between the female gamete and the surrounding CC (Salustri, 2000). Through gap junction channels, bidirectional exchange of some molecules can be come to pass including ATP, ions (such as calcium, sodium, and chloride), cAMP, and some nutrients, such as pyruvate and lipids. Thus, the presence of CC participates in oocyte growth, development, and maturation at both levels of nuclear and cytoplasmic, as well as the further oocyte and resulted embryo competence (Conti, 2010; Johnson *et al.,* 2007; Salustri, 2000).

During maturation, CC contributed to oocyte maturation by induction meiotic resumption and support cytoplasmic maturation. Meiotic resumption occurs by disappearing meiosis inhibition substance and this due to the role of Luteinizing Hormone Receptor, while cytoplasmic maturation indicated by glucose metabolism into pyruvate, and cystine into cysteine lead to the deposition of glutathione (GSH) into the oocyte (Tanghe *et al.,* 2002). High level of GSH in oocyte cytoplasm is known to be associated with high development capacity of oocyte to from pronuclei after fertilization.

Numbers of metabolites secreted by CCs during IVM were

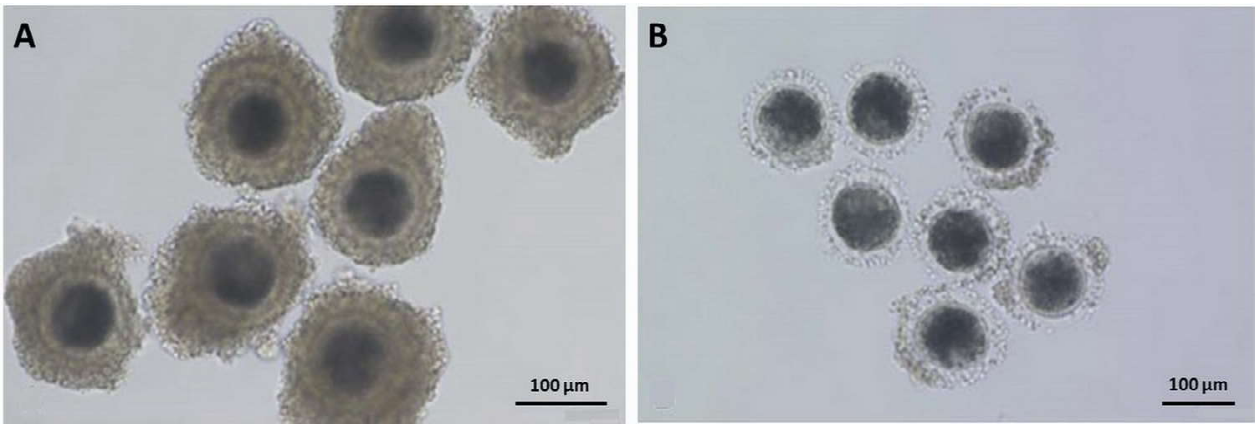

Fig. 1: The Indonesian Thin-tail sheep oocyte grade prior to IVM. A. Oocytes with grade A, B. Oocytes with grade B oocytes. (Scale bar = 100 µm).

Fig. 2: The IVM outcome of Indonesian Thin-tail sheep. A. Mature or developed oocyte, B.Undeveloped and C. Degenerated oocyte. (Arrow = 1st polar body; scale bar = 20 µm).

Table 1: *In vitro* maturation outcome of oocytes in the different grade.

S	Grade A	Grade B	Chi-Square	P value
	---------- n (%) ----------			
M-II	82 (57.34)	37 (43.53)	10.0093	0.04
Undeveloped	32 (22.38)	32 (37.65)	30.7089	<0.01
Degenerated	29 (20.28)	16 (18.82)	28.1408	<0.01
Total	143	85		

related to amino acid, carbohydrate, energy, and lipid metabolism pathway (Uhde *et al.,* 2018). These essential nutrients for oocyte development were secreted by CC, and CCs that interact each other and with oocyte through gap junction communication (Zhou *et al.,* 2016), therefore the loss of cumulus during maturation is directly related to poor oocyte quality as well as its further development competence (Dhali *et al.,* 2017). At this point, we can conclude that sufficient nutrition for oocyte metabolism resulted better oocyte development competence as confirmed in Fig. 1 and 2, where better maturation rate was achieved in the oocytes with more CC layer (Grade A) compared to the less one (Grade B). While oocyte maturation is the last step of female gamete development, the mature oocyte is the first step to expect good result, embryos, of *in vitro* fertilization (IVF). Intact CC layer act as a marker of good oocytes quality (Uyar *et al.,* 2013) leading to better IVF result as demonstrated by Dhali *et al.* (2017).

In the molecular perspective, the cumulus cells gene expression could be a molecular marker for oocytes quality (Racowsky and Needleman, 2018). For instance, the expression of GSTA1, CLU, PGR, FSHR, and SMAD2 genes in CC during IVM were related to oocyte nuclear maturation, development competence, cumulus apoptosis rate, and progesterone synthesis (Salhab *et al.,* 2011). In addition, previous study performed by Dhali *et al.* (2017) showed the downregulation of FSHR, LHR, and BCL2 gene expression in poor quality oocytes compared to good quality one during IVM. As we know, FSH and LH play an important role for oocytes to achieve M-II phase as mentioned by Farin *et al.* (2004).

An anti-apoptotic, BCL2, gene expression was found to be significantly higher in mature oocyte with more CC. It is indicating the role of CC in oocyte and embryo development competence (De Bem *et al.,* 2014). The expression BCL2 is related to the presence of more antioxidant and low oxidative stress in the oocyte with more CC layer during IVM. As studied previously by Amin *et al.* (2014) and Linares-Otoya *et al.* (2018), set of antioxidant gene such as NRF2, CAT, PRDX1, SOD1 and TXN were found down regulated in cell under oxidative stress. Under oxidative stress, cells also found to be suppressed mitochondrial activity as the indicator of high production of reactive oxygen species (ROS) (Prastowo *et al.,* 2016). In general, more ROS lead to the higher expression of BAX, the BCL2 downstream gene, results in more apoptotic cell (Li *et al.,* 2004). Therefore, IVM of oocytes with the addition of antioxidant resulted better oocyte maturation rate (Barakat *et al.,* 2018; Veshkini *et al.,* 2018), because the use of antioxidant during IVM reduce oxidative stress either by

decreasing ROS or increase antioxidant level in oocytes (Veshkini *et al.,* 2018). Moreover, the protection role of CC to counter ROS effect during IVM was found to be associated with the accumulation of antioxidant such as GSH in the oocytes (Tatemoto *et al.,* 2000). These previous results might explain why more CC layer resulted better in number of oocytes reach to M-II stage when compared to the oocytes with less CC as our finding in this study. Indeed, these molecular mechanisms need to be explored to confirm that in the ITTS IVM occur the same mechanism, but at least we already have the molecular marker candidate for the further study.

To the best of our knowledge, specific information of IVM of oocytes in ITTS as Indonesian indigenous animal with a special trait resistant to fascioliasis is still very limited and has not well documented. Hence, the present study tries to capture the potential use of matured oocytes *in vitro* for further use in embryo production. In the future such information would be important for design breeding strategic in conservation as well as the utilization of local animal genetic resources keeping sustain. Moreover, this study then could be a steppingstone strategy to recover female genetic material for animal production, gamete or genetic preservation and embryology study in Indonesian indigenous animal.

Conclusions

According to the study, the presence of CC surrounding oocytes during IVM achieved better result according to the number of matured oocytes. More CC layers in the oocytes of ITTS breed gives better percentage of mature oocytes after IVM indicated by more matured (M-II) oocytes. At least, the ITTS matured oocytes from our work, visually, could meet with the need of IVF input for embryo production.

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