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Supplementation of Follicle Stimulating Hormon Into *In vitro* Maturation Medium to Increase Oocytes Maturation and 4 Cell Stadium Embryo Development of Bligon Goat

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ABSTRACT

The study was carried out to investigate the effect of follicle stimulating hormon (FSH) into *in vitro* maturation medium to increase oocytes maturation and 4 cell stadium embryo development of Bligon goat. Goat ovaries were obtained from a slaughterhouse and transported to the laboratory in a flask of NaCl at temperature of 31 – 34°C. Oocytes were aspirated from 2 – 6 mm of follicles into a 3 mL syringe (23G needle) that contained Dulbecco's Phosphate-Buffered Saline. Oocytes were divided into three groups, i.e tissue culture medium (TCM) with FSH supplementation 0, 50, and 100 IU/mL. Oocytes were put into those medium and incubated on 39°C, 5% CO_2, and 95% humidity for 24 hours. Matured oocytes were fertilized with capacitated frozen thawed-semen and incubated on 39°C, 5% CO_2, and 95% humidity for 5 hours. Fertilized oocytes were washed for 3 times in TCM and incubated in the same condition for embryo culture. The data of FSH supplementation and embryo development were analyzed using randomized completely one way classification. The results showed that the percentages of mature oocytes from FSH supplementation 0, 50, and 100 IU/mL were 70,48±23,22, 78,48±15,80, and 80,29±12,86%, respectively. Cleavage rate of the two cells stage were 36,00±14,22, 44,00±33,94, and 57,45±31,78%, respectively, and for the 4 cells stage were 27,33±22,04, 35,33±40,73, and 39,45±20,38%. It is concluded that supplementation of FSH in the maturation medium could not increase the percentages of *in vitro* maturation and embryo development.

Keywords: Bligon goat, Oocytes, Follicle stimulating hormon, *In vitro* embryo production

Introduction

Goat play an important role as a source of revenue and spur a national program to increase per capita consumption of animal protein. The rate of goat population growth in the last five years relatively low, less than 3.5 percent per year. Bligon goat is a crossbreed between Ettawa goat with Kacang goat (Nuraini *et al*., 2014). Ovary as the by-products of goats from the slaughter process can be used for reproduction technology. One of the recent technologies that can increase the population and productivity of Bligon goat is the application of *in vitro* fertilization reproduction technologies (Herdis, 2014).

In vitro fertilization process involves the oocytes recovery from ovary follicles, oocytes *in vitro* maturation, sperm capacitation, *in vitro* fertilization, and *in vitro* culture of the fertilized oocytes until it become an embryo (Boediono *et al*., 2000). One of the main factor to improve an *in vitro* embryo production is by the hormone supplementation on the maturation medium. Reproductive hormones play an important role in livestock reproduction activities. One of the reproductive hormones is Follicle Stimulating Hormone (FSH).

Follicle stimulating hormone is used as an essential component in culture medium, culture processes, and for maintaining viability and oocyte growth. Follicle stimulating hormone with growth factor can stimulate cumulus cells to produce and secrete a hyaluronic acid that will disperse the cell, this process is called expansion (Ciptadi *et al*., 2011). Follicle stimulating hormone in maturation medium will stimulate and regulate chromatin condensation for meiotic division, besides FSH will stimulate cAMP and cumulus cell expansion (Wattimena, 2011). Optimal expansion of the cumulus cells appears to be essential for the successful of *in vitro* maturation, influence the process of *in vitro* fertilization and *in vitro* embryo development (Zhou and Zhang, 2005).

The process of embryonal development in mammals begins with the penetration of spermatozoa into the oocytes to form a zygote. Zygote will undergo a process of division by mitosis of one cell into two cells called blastomeres. Each blastomere then be split into 4, 8, 16 cells and so on (Hafez and Hafez, 2000).

Based on the study above, so have to investigate the effect of Follicle Stimulating Hormon (FSH) into *in vitro* maturation medium to increase oocytes maturation and 4 cell stadium embryo development of Bligon goat.

Materials and Methods

The study was conducted from June to December 2014 at Balai Embrio Ternak, Cipelang, Bogor, and Animal Physiology and Reproduction Laboratory, Faculty of Animal Sciece, Universitas Gadjah Mada, Yogyakarta.

Materials

The material used in this study is ovarian of Bligon goat which amounted to 30 ovaries from 15 of Bligon goats. Goat ovaries were obtained from a slaughterhouse (which is located on Babadan Baru, Jl. Kaliurang KM. 7, Sleman, Yogyakarta).

Methods

***In vitro* maturation.** *In vitro* maturation medium used was tissue culture medium (TCM) with supplementation of FSH 0, 50, and 100 IU / mL, then prepared three tissue culture dish (TCD, Falcon, United States) size 35 mm x 10 mm for *in vitro* maturation process of oocyte, each marked V / 0, V / 50, and V / 100. In each TCD dropped 10 µl x 3 drop TCM (Gibco, USA) as maturation medium. Mineral oil is poured as much as 100 µl in TCD, so that the entire surface of the TCM drop is submerged.

Oocytes that have been washed with DPBS (DPBS, Gibco, USA) were transferred in a TCM drop on all TCDs. Oocytes were supplemented with FSH (Folltropin-Vetrepharm®, Canada) obtained from porcine pneumonia selection). The placement of the oocytes to the treatment, ie TCD marked V / 0 filled with treated oocytes without supplementation of FSH (0 IU / mL); V / 50 filled with oocytes with supplementation of 50 IU / mL FSH; and V / 100 filled oocytes with supplementation of 100 IU / mL FSH. Oocyte maturation was done by placing oocytes on TCM medium then oocyte incubated in CO2 (Cole Parmer, USA) incubator at 39°C, 95% humidity and 5% CO2 for 24 hours (Widayati, 1999).

Measurement of cumulus cell expansion. Oocytes performed observations and measurements of cumulus cells under a stereo and optilab microscope (Cole Parmer, USA). The measurements of cumulus cell expansion are the difference in the diameter of oocyte cumulus cells before mature and the diameters of oocyte cumulus cells after expansion (Widayati *et al*., 2014).

Preparation of sperm. Frozen semen is melted in waterbath, then a semen was inserted on a centrifuge tube. The semen supplemented with semen washing medium (sperm rinse, Vitrolife, Sweden) 0.5 mL and centrifuged at 1800 rpm for 10 min. The upper liquid (supernatant) is taken with a pasteur pipette carefully. Washing is done twice in the same way. Double washed sediment plus 0.5 mL of fertilization medium (G - FertTM, Vitrolife, Sweden). Suspension of spermatozoa made 50 µl spermatozoa drops in disposible TCD size 35 x 10 mm, then covered with mineral oil. Spermatozoa 10 µl drops with a concentration of 2.8 x 106 / mL incubator incorporated CO2 temperature 39°C, 5% CO2, 95% humidity for 3 hours. In order for simultaneous sperm and maturation capacitation of the oocyte, the sperm preparation was treated 3.5 hours before the oocyte maturation time ended (Widayati, 1999).

Fertilization and embryo culture. Oocytes that have been maturified were washed with TCM 3 times, then the oocytes were put in 50 µl drops of spermatozoa suspension. The fertilization process was treated in CO2 incubator at 39°C, 5% CO2, 95% humidity for 5 hours (Gordon, 1994).

In vitro fertilized oocytes were washed with TCM 3 times. The oocyte was put in to 50 µl drops of G - 1TM at a disposible TCD of 35 x 10 mm, then kept in a CO2 incubator at 39°C, 5% CO2, 95% humidity. Embryonic development is observed every 24 hours. Medium is replaced every 48 hours. Media G - 2TM v3 (Vitrolife, Sweden) was used the second day after culture (Gordon, 1994).

Evaluation of embryos. Evaluation of embryonic development treated using desiccated microscope refers to Gordon (1994) method. Embryo quality is determined based on several parameters such as flat surface, color, cell compactness, number of degenerated or "extruded" cells, the size of the number of "vesicles" (Widayati, 1999).

Data analysis. The research data obtained from 30 ovaries of Bligon goat in the form of maturation data and embryonic development data then analyzed with completely randomized design of direct pattern. Data on the quality of fertilization were analyzed descriptively. The mathematical model formula for the complete randomized design of the unidirectional pattern is:

$Yij = \mu + Ti + \varepsilon ij$

$i = 1,2, ..., t; j = 1,2, ..., n$

Yij = value of observation on the treatment of “i”, repeat “j” ; observation of mature oocyte percentage, fertilized oocyte, and *in vitro* embryo development

µ = common middle value

Ti = effect of the I, treatment; supplementation of FSH 0, 50, and 100 IU / mL

εij = random effect (experimental error) on the i-treatment and j- repeat

t = number of treatments
n = number of replications.

Result and Discussion

In vitro maturation of oocytes

The result of present study of oocytes *in vitro* maturation from female Bligon goats with FSH supplementation 0, 50, and 100 IU / mL on maturation medium showed no significant difference (Table 1), because the quality of oocytes used in the research is almost uniform, that is classes 1 and 2 derived from follicles with a diameter of between 2.0 and 6.0 mm (Camargo *et al*., 2006). Follicles with diameter 2 mm it's contain fully grown oocytes (have reached the first stage of the first meiotic prophase), so that it could cleaveage spontaneously. Bligon goat oocytes from follicles diameter less than 1.6 mm, it have not completed their growth phase, so they have not been able to carry out the first meiotic division (Rutledge *et al*., 1987 *cit.* Kusindarta, 2009), while the oocytes in the 6 mm diameter follicles have not atresia (Gordon, 1994).

The evaluation of *in vitro* maturation process was done by observing the expansion of cumulus cells by measuring cumulus cells before and after maturation which could be shown in Table 2. The average of cumulus cells expansion in mature oocytes from FSH supplementation 0 IU / mL, 50 IU / mL, and 100 IU / mL were 2.84, 2.69, and 2.98 µm, respectively.

Measures deviation could be shown by cumulus cells expansion. The cumulus cells expansion was one of maturation oocytes indication, otherwise an indication could be shown by corona radiata has bright color, pellucida zone visible, ooplasm looks clean, granulosa cell membrane expands well (Widayati, 2008).

In all treatments of FSH indicates that the oophorous cumulus expansion. This indicates that oocytes had undergone maturation. The most easily observed feature of mature oocytes were expansion of cumulus cells surrounding an oocyte and zona pellucida. Gordon (1994) states that evaluation of the maturation process is the rate of nucleus maturation, cumulus cell expansion, and morphological assessment methods. Oocytes that have been matured *in vitro*, so the cells surrounding the oocyte spread and there is a change in the perivitelin space that is the presence of polar body I (PB I) and the formation of metaphase II (M II) blastomers on the vitelin surface. In accordance with Linn *et al*. (2003) *cit.* Widayati (2008), good maturation could be shown by corona radiata has bright color, pellucida zone visible, and an existence of cumulus expansion.

The cumulus cells expansion is the identification of mature oocytes so that a wider expansion it indication the maturation rate. In this research, oocytes selected which surrounded by cumulus cells, because according to Kusindarta (2009), the cumulus cells surrounding the oocyte not only regulate the maturation level of the nucleus and preserve the oocyte's life but also important to stimulate cytoplasmic maturation. During *in vitro* maturation oocytes, cumulus cell changes occur and interactions between follicular cells and oocytes. Furthermore, cumulus cells and serum at a short time (2 hours) is required for the release of Polar Body I (PB I) at *in vitro* maturation oocytes and also helps increase penetration of oocyte by spermatozoa. The cumulus cells play an important role to improving the normal cytoplasmic maturation of oocytes for pronucleous formation and to continue development ability.

The cumullus cells play an important role for maturation oocytes by affecting the meiosis continuation and affecting the cytoplasmic maturation. The function has a correlation with gap junction and metabolic ability. Physical contact between oocyte and cumulus cells for nutrient transfer and important factors for oocytes development. The increase of maturation rate in the co-cultured oocytes, due to the production of paracrine factors by the addition of cumulus cells, which transfer of the denud oocyte by gap junction. This as evidence of cumulus cells not only increases IVM but also saves oocytes from degeneration (Hassan and Kazim, 2004).

Table 1. Percentage of mature oocytes with FSH supplementation 0, 50, and 100 IU/mL after *in vitro* maturation

FSH hormone (IU/mL))	N (Replication)	Total number of immature oocytes, 0 hour (cell)*	Total number of mature oocytes, 24 hours (cell)*	Percentage of mature oocytes, 24 hours (%)
0	5	25	15	70,48±23,22
50	5	28	21	78,48±15,80
100	5	32	25	80,29±12,86

* : Total number of immature oocytes (0 hour) and mature (24 hours) used for basic calculation of 2-cells and 4-cells cleavage percentage).

Table 2. Measure of cumulus cells expansion

FSH hormone (IU/mL)	Diameter of immature cumulus cells, 0 hour (µm)	Diameter of mature cumulus cells, 24 hours (µm)	Cumulus cells expansion (µm)*
0	2,14±1,67	2,84±1,71	0,71±0,60
50	1,71±0,49	2,69±0,69	0,98±0,68
100	1,55±0,66	2,98±1,05	1,43±0,75

* : Cumulus cells expansion= deviation between the diameter of immature cumulus cells with the diameter of mature cumulus cells (figure 1, 2, 3).

***In vitro* fertilization and *in vitro* embryo development**

The number of fertilized oocytes is shown in Figure 4. Fertilization in mammals consists of three stages: (1) spermatozoa migration between cumulus cells, (2) migration of spermatozoa through pellucida zone, and (3) fusion of ovum plasma membrane and spermatozoa. Spermatozoa can penetrate cumulus cells because of the spermatozoa movement itself and assisted by hyaluronidase enzymes present in the acrosomes (Widayati *et al*., 2007).

The success rate of fertilization is marked by the process of penetrating spermatozoa into the ovum, in this phase a fusion of male pronucleus with female pronucleus. The result of this fertilization phase is the formation of a zygote. The next phase, the zygote develops into a new individual through the cleavage process (Campbell, 2004). The results of cleavage *in vitro* 2 cells and 4 cells in Bligon goat oocytes with follicle stimulating hormone (FSH) doses of 0, 50, and 100 IU / mL in maturation medium showed no significant difference (Table 3).

The results of embryo development *in vitro* stage 2 cells and 4 cells in Bligon goat oocytes with FSH supplementation of 0 IU / ml, 50 IU / ml, and 100 IU / ml were (36.00±14.22%,

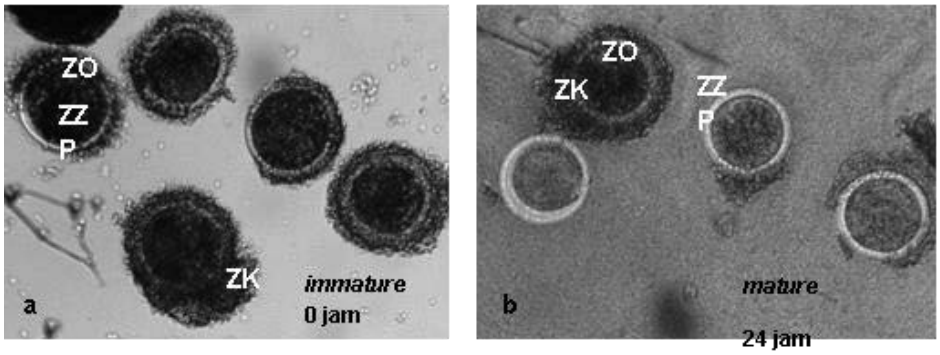

Figure 1. Immature oocytes of Bligon goat without FSH supplementation (FSH 0 IU/mL) with intact cumulus cells (a) and mature oocytes of Bligon goat with the expansion of cumulus cells. Magnificient 10 x 16, O =oocytes, K=cumulus cells, ZP=zona pellucida.

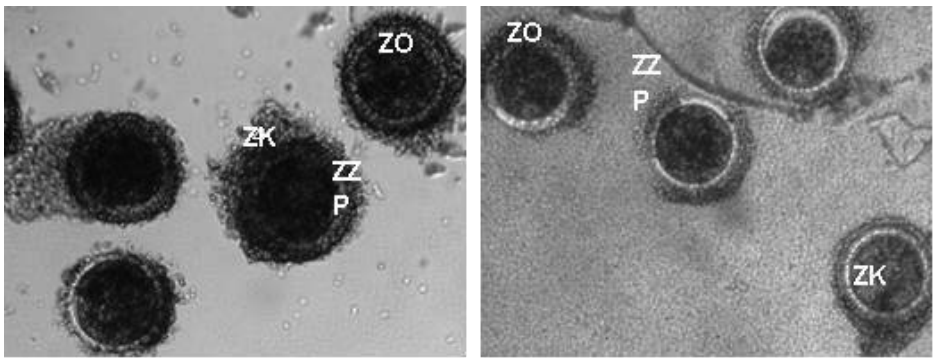

Figure 2. Immature oocytes of Bligon goat with FSH 50 IU/mL supplementation with intact cumulus cells (a) and mature oocytes of Bligon goat with the expansion of cumulus cells. Magnificient 10 x 16, O =oocytes, K=cumulus cells, ZP=zona pellucida.

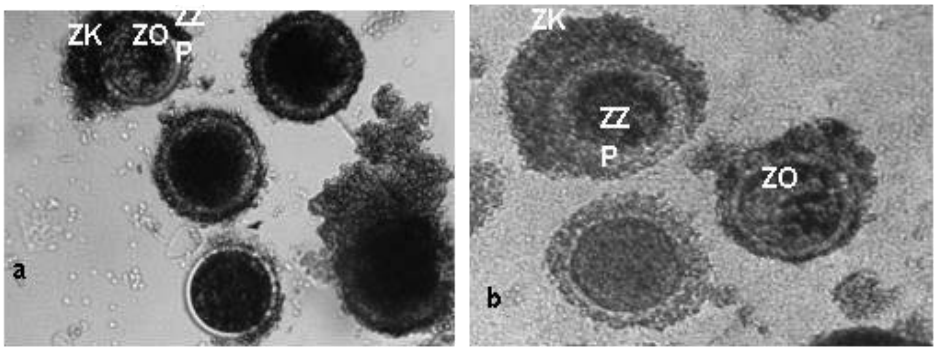

Figure 3. Immature oocytes of Bligon goat with FSH 100 IU/mL supplementation with intact cumulus cells (a) and mature oocytes of Bligon goat with the expansion of cumulus cells. Magnificient 10 x 16, O =oocytes, K=cumulus cells, ZP=zona pellucida.

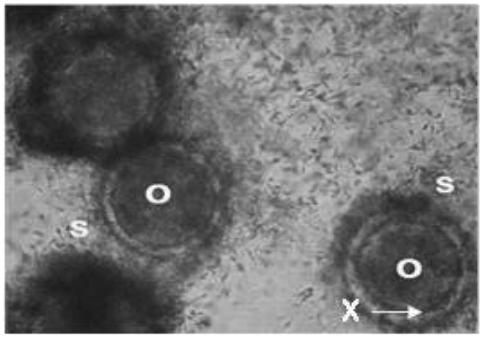

Figure 4. *In vitro* fertilization on the medium (magnificent 10 x 16) O = oocytes, S = sperm, X = spermatozoa migration) get near to cumulus oocyte complex.

44.00±33.94%, 57.45±31.78%) and (27.33±22.04%, 35.33±40.73%, 39.45±20.38%) respectively, statistically shown there was no significant differences.

In vitro embryo development at 2 cells and 4 cells stage statistical analysis showed no significant difference, this is due to the quality of embryos in which the result of embryo quality is not good, it causing many degenerate embryos. The embryos produced in this research for control treatment (dosage 0 IU / ml) morphologically showed uniform blastomer size, a tight and compact blastomer bond so that the blastomeres appeared out of the bond, the blastomers were not intact, and the blastomer color was less bright. While for treatment with FSH supplementation (doses of 50 IU / ml and 100 IU / ml) morphologically showed uniform blastomer size, tight and compact blastomer bonds, intact blastomers, blastomers rather bright colors and spherical round embryos (Figures 5, 6, and 7).

The embryo development stage achieved in this research up to 4 cell divisions then degenerated, because the nutritional needs, especially energy, rise to the stage of 8-16 cells because of the decrease in the ratio of adenosine triphosphate (ATP) or adenosine diphosphate (ADP), so if the energy requirement is not sufficient for the embryo during incubation, so the embryo could not develop properly (Widayati, 1999). Supportive factors for *in vitro* maturation, such as *in vitro* maturation medium (Hammam *et al*., 2010), contamination risk and culture conditions (Sagirkaya *et al*., 2007). Supplementation of FSH on maturation medium resulted in a decrease of cleavage rate because the medium used for culture was G–1^{TM} (Vitrolife, Kungsbacka, Sweden). G–1^{TM} is a basal medium designed to aid the development and division of embryonic cells up to the 8 cell stage. G–1^{TM} contains carbohydrates, amino acids, and chelators at the onset of the embryo (Adifa *et al*., 2010). According to Gordon (1994), the cell culture medium required a combination of six major components: growth factor, adhesive factor, mineral, hormone, protein and vitamin. These

Table 3. Cleavage percentage of 2-cells and 4-cells of Bligon goat oocytes with FSH 0, 50, and 100 IU/mL supplementation into *in vitro* maturation medium

FSH hormone (IU/mL)	Jumlah oosit *Total number of oocytes in 2-cells cleavage, 48 hours (cell)**	*Percentage of oocytes in 2-cells cleavage (%)**	*Total number of oocytes in 4-cells cleavage, 72 hours (cell)**	*Percentage of oocytes in 4-cells cleavage, 72 hours (%)**
0	8	36,00±14,22	6	27,33±22,04
50	9	44,00±33,94	6	35,33±40,73
100	14	57,45±31,78	10	39,45±20,38

* : *Calculation of total oocytes and percentage of oocytes in 2-cells and 4-cells cleavage based on the result of mature oocytes (table 1).*

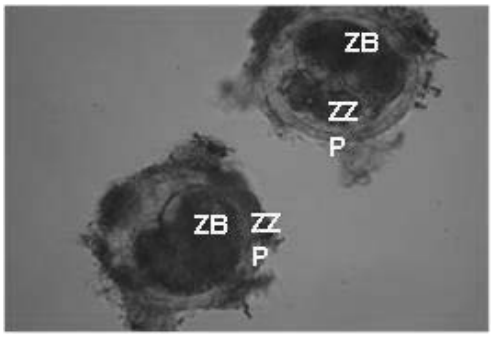

Figure 5. The embryo of Bligon goat on 2-cells stage after *in vitro* fertilization with FSH 0 IU/mL supplementation, ZP = zona pellucida, B = blastomer.

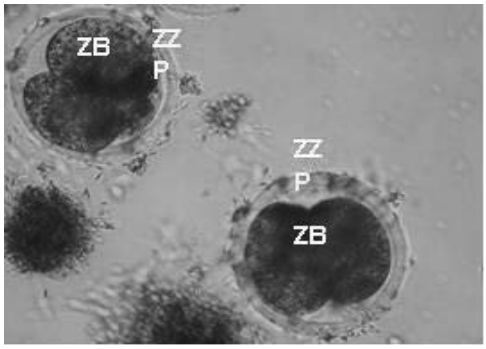

Figure 6. The embryo of Bligon goat on 2-cells stage after *in vitro* fertilization with FSH 50 IU/mL supplementation, ZP = zona pellucida, B = blastomer.

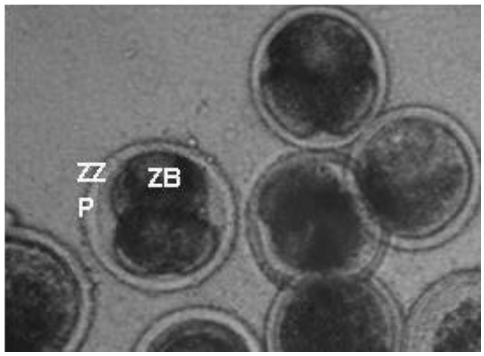

Figure 7. The embryo of Bligon goat on 2-cells stage after *in vitro* fertilization with FSH 100 IU/mL supplementation, ZP = zona pellucida, B = blastomer.

components are present in some commercial products used for cell culture, as well as no microbial contamination.

Based on the morphology, *in vitro* embryos on stage 2 cells and 4 cells included in quality A and B, and decreases the quality after the 8 cell cleavage phase. *In vitro* embryo development usually stops at the 8-16 cell phase. This gives a reference about the transition of control from mother to embryo. The development of embryo on stage 8-16 cells is a very sensitive period with the environment (Adifa *et al*., 2010).

According to D'Alessandro and Martemucci (2003), the classification of embryo quality based on morphology is: embryo quality of A (excelent), stage of embryo according to desired (morula, early blastocyst or blastocyst), no defect, spherical spherical shape, tight and compact, symmetrical shape, and slightly brighter colors; the quality of the embryo B (good), the stage of development of 16 - 32 cells, looks slightly defective as the release of one of the blastomers of the bond, and the asymmetric shape; the quality of embryo C (fair), the stage of development is somewhat late one to two days of the desired stage (8-16 cells), defects, some out blastomers, and the size of the blastomers are not as large or asymmetrical; the quality of embryo D (poor) and E (very poor).

Embryo evaluation includes: 1) Density of cells, normal embryo is denser than loss of cell mass; 2) Regularity of shape, shape like ball better than oval shape; 3) Variation in cell size, same blastomer size; 4) The color and texture of the cytoplasm, should be luminous and not too dark; 5) The presence of bubbles, in the cytoplasm should not contain even small bubbles; 6) The presence of depressed cells, should not contain depressed cells; 7) Normal embryo size; 8) Regularity of pellucida zone; 9) The existence of fragmentation of the cell, there should be no slight cell fragmentation from the periphery of the blastomer; and 10) the development of cells corresponding to the age of the embryo (Bearden and Fuquay, 1997).

According to Boediono *et al*. (2000) the results of research on embryo blastocyst production in goats about 11%. The development of *in vitro* embryos is strongly influenced by *in vitro* maturation results. Oocytes that do not reach mature stage or metaphase II can be stalled at previous stages such as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I, anaphase and telophase I (Setiadi and Karja, 2013).

These components are necessary for early stage embryonic development such as proteins and growth factors. The protein will be released when the embryo passes through the oviduct, then binds to the zona pellucida and enters the embryo cytoplasm. The protein and messenger ribonucleic acid (mRNA) found in the embryo is essential for the transcription process that occurs in the early stages of embryonic development. In case of failure of transcription process, the embryo division will be stopped and cause a blockade (Gordon, 1994).

Conclusion

The supplementation of Follicle Stimulating Hormon (FSH) into *in vitro* maturation medium could not increase the percentage of oocytes maturation and 4 cell stadium embryo development of Bligon goat.

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