

# Supplementation with Goat Follicular Fluid in the In Vitro Maturation Medium Toward Cumulus Expansion and Nucleus Transformation

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## ABSTRACT

The purpose of the research was to the influence of goat follicular fluid (GFF) with different levels in maturation medium in vitro on cumulus expansion and transformation of goat oocyte nucleus. The results of this study showed that cumulus cell expansion was related to the higher level of different GFF in maturation medium TCM-199. Cumulus cell expansion, and increased nucleus transformation occurs at level of 10% and decreased medium without GFF. This suggests that cumulus expansion and nucleus transformation decreases, because the need of oocyte is not supported by hormones and nutrients in the maturation medium.

**Key words:** GFF, TCM-199, cumulus expansion and maturation of the nucleus

## 1. INTRODUCTION

In vitro fertilization is one of reproductive biotechnologies that can be used to produce embryos by using ovaries that come from living or slaughtered livestock. Ovaries from slaughtering houses are cheap oocyte sources available in huge amounts and that can be used optimally. Oocytes can be obtained in huge amount through in vitro oocytes maturation. but this biotecnolgy is influenced by the culture media used. Then the media used to this purpose should be similar with the condition in the mother body (In-Vivo), which gives the needed nutrients for oocytes metabolism and development.

One of the ways to create a suitable condition to oocyte maturation is by giving protein, hormones, and developmental factors for the needed metabolism in vitro (Nasich, et al., 2001). Some hormones have roles in the oocyte. Maturation that is Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and estradiol (McNatty et al, 2007). FSH stimulates the granulose cells to synthesise and secrete estrogen that make the follicle develops, while the estrogen stimulates the granulose cell to proliferate, LH also stimulates the initial stadium of follicle development (Anwar, 2005). Follicular Fluid also has important role in the oocyte development, ovulation, ovum transport to oviduct (Purwaningtyas, 2006). Follicular fluid contains protein and estrogen and mucopolysaccharide that are excreted by granulose cells (Johnson, 2008). Other sources stated that follicular fluid contains gonadotropin hormones if FSH and LH that serve as stimulus of cumulus oophorus cell development (Hafez, 2000). The cumulus cell will experience expansion if stimulated by the gonadotropin hormone activities and metabolism.

One of growth factors that has important role in the oocyte maturation process in general added with serum, is recognized as the standard supplement because contains many growth factors (Sataines and Price, 2003). Beside that, the serum also provides some proteins called as the binding protein that carry small elements such as albumins such as bring vitamin, fatty acid, cholesterol, and hormones, fat in serum that is the sources of essential fatty acid, lection and cholesterol in general that are needed by oocytes. So far, the hormones and serum supplementation often be used in the maturation media of in vitro come from pharmaceutical industry such as BSA, FSH, Estradiol 17 $\beta$ , and hCG, with expensive price and today for hormones FSH, LH and estrogen that are used in the in vitro maturation still imported from a broad then at certain area difficult to obtain. Recall to the matters, so finding the alternative materials to replace the materials with optimum results.

One of alternatives that replaced the materials: Follicular fluid (FF), because FF contains materials that able to stimulate the oocyte maturation (Dode and Graves, 2001; Nicolas et al, 2005). Goat Follicular Fluid is alternative that can be used as the supplementation in the maturation media because cheap and easy to get. The research aimed at knowing the influence of GFF at the different level at in vitro maturation media to the cumulus expansion and nucleus transformation of goat oocyte.

## **2. MATERIALS AND METHOD**

### **2.1. Research materials**

The used materials in the research was are immature, good quality goat oocyte, aspired from follicle of 2-6 mm in diameter.

### **2.2. Research method**

The used method is the laboratory research. The given treatments were 5 treatments, consist of 1) 0% GFF; 2) 2.5% GFF; 3) 5% GFF; 4) 7.5% GFF and 5) 10% GFF. Each treatment was replicated 5 times.

### **2.3. Media making for the goat ovaries collection**

Making physiological NaCl of 100 ml by dissolving 0.9 g NaCl in aquades 100 ml. Before be brought to RPH, the physiological NaCl solution in the glass bottle was entered into thermos. During the ovaries collection added with 0.01 g streptomycin sulfate and 0.006 g penicillin G into the solution.

### **2.4. Preparation and characterization of goat follicular fluid**

The FF collection by aspiration from small follicle (<2 mm) and big (2-6 mm) by using needle with syringe size of 18 G. FF aspiration based on Robert and Echterkamp (2003). The GFF of the aspiration then be centrifuged of 600Xg for 10 minutes to eliminate debris and filtered with 0.22  $\mu$ m, in activation in 56 for 30 minutes (Malekshah dan Moghadam, 2005) and stored in the freezer. FF characterization done in the Biochemical Laboratory according to the procedure of Nicolas et al (2005) with minor modification.

### **2.5. Media making for oocyte maturation**

Dissolving 0.95 g TCM-199 into 100 ml aquades of free ion, then added with streptomycin sulfate (1 ml land GFF) suitable with the tried treatment. After mixed with the average medium then the pH was measured then be filtered by milipore at air flow laminar. After that, making 2 media in the big petri dish contain 2 ml media without paraffin oil, then making media drop at the small petri dish. The first media drop contain 2 drops media of 50  $\mu$ l while the second drop media contain 4 drop media 50  $\mu$ l that is closed with the paraffin oil. Then the maturation media is stored in the incubator CO<sub>2</sub> with temperature of 38°C for (26) hours before be used.

### **2.6. Oocyte collection**

The oocytes were collected by aspiration, using needle of 18G. Before aspiration, the laboratory media is sprayed by alcohol 70% to remove contaminants. By using gloves that have been sprayed by alcohol 70%, paper, tissue paper and sterile pincers, ovaries were taken by syringe that has contained 0.5ml washing medium (TCM-199), ovaries that contain follicle was stuck and aspired by FF based on Robert and Echterkamp 2003. The oocyte aspiration results about 5-10 ovaries were entered into reaction tube that has prepared in the water bath.

The oocyte washing for 3 times by using TCM-199 media. Then, it is precipitated for 10 minutes then the upper part was removed then the lower part be entered into petri dish. The oocyte selection, done by using inverted microscope by moving it, by using Pasteur pipette and microhematocryte. The oocytes were counted and classified under stereomicroscope (56x) according to Abd Allah (2009) in to three categories Based on their granulose-oocyte cell layers that surround the oocyte, the oocytes then be grouped into three categories Masudul Adhesion as follows: (C+) granulose enclosed oocytes (C+/-) partially granulose enclosed oocytes (whenever there were granulose cell-free regions on the oocyte surface) and (C-) granulose free-oocytes In the research, the used oocyte for maturation is oocyte that included in the C+ criteria. Evaluation of cumulus expansion. The matured oocyte then be observed the cumulus expansion by using inverted microscope by magnification of 400x, then be clarified in three level (1) no cumulus cell expansion (2) partly expansion (3) full expanded (Wahjuningsih, 2005).

### **2.7. Evaluation based on metaphase level II**

To know the metaphase level II done by staining with aceto-orcein 1% (Martino et al, 1994). After be cultured for 26 hours at the oocyte done cumulus cell denudation by mechanical ways (pipette), by using pipette of 110-120  $\mu$ m in diameter (that suitable with oocyte size). Then it is fixated for 3 days in the acetate – methanol solution (1:3) and stained with aceto-orcein (1%) orcein in 45% acetate acid. After one minute the stain is removed by aceto-glycerin and observed by inverted microscope with magnification of 400x.

### 3. RESULTS AND DISCUSSION

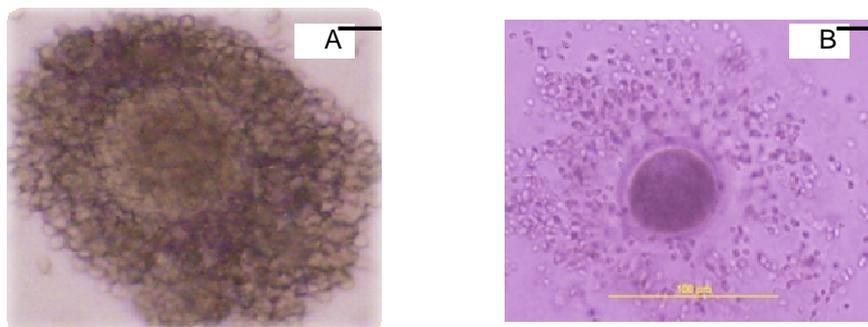
#### 3.1. Expansion of oophorus cumulus

After oocyte maturation process for 26 hours, then be evaluated for the oocyte maturation process based on expansion of the oophorus cumulus. The oocyte maturation expansion results in vitro that is supplemented by goat follicular fluid (GFF) with different level at maturation media was presented in table 1.

**Table 1. Goat oocyte maturation results by using GFF with different levels**

(GFF)	Σ Tested Oocyte	Cumulus expansion quality %		
		A	B	C
0	37	6/37 (16.39) <sup>a</sup>	8/37(21.94) <sup>a</sup>	23/37 (61.67) <sup>b</sup>
2,5	38	6/38 (15.63) <sup>a</sup>	18/38 (46.71) <sup>ab</sup>	14/38 (37.66) <sup>a</sup>
5	27	12/27 (46.67) <sup>a</sup>	9/27 (34.89) <sup>a</sup>	5/27 (18.44) <sup>a</sup>
7,5	24	11/24(47.33) <sup>b</sup>	7/24 (30.00) <sup>a</sup>	5/24 (22.67) <sup>a</sup>
10	23	14/23(60.33) <sup>bc</sup>	5/23 (22.33) <sup>a</sup>	4/23 (17.33) <sup>a</sup>

Explanation: Different superscript at the same column for each treatment showed significant differences ( $P < 0.01$ ). Quality A: Oophorus cumulus expand wholly, Quality B: Oophorus cumulus expand partly, Quality C: Oophorus cumulus not expand



**Fig 1. Goat oocytes (A). Before in vitro maturation, the cumulus cell do not expand, (B). After in vitro maturation, cumulus cell expand wholly and seem clear and transparent (400X).**

From table 1. It is seem that the maturation medium that is supplemented GFF 10% of the big average results, to the wholly cumulus expansion of A quality, compared with the treatment P0 without GFF supplementation. The higher GFF level that produce oocyte percentage increase with wholly cumulus expansion (quality A). It was occurred because the component that contained in the follicular liquid supported the oocyte growth such as FSH, LH, estradiol factor and various other factors so the cooperation of gonadotropin hormones with the growth factors during maturation process able to trigger the oocyte development become mature. It is along with the research of Roimil Latifa (2007) showed that GFF contain hormone FSG 1.7 ng, LH 0.22 ng, progesteron 27 ng and estrogen 11.500 ng and growth factor that serve as stimulus for the cumulus cell development at the oocyte maturation. Because of that, the high maturation level at the cultivation media with GFF level of 10% showed that the GFF contain component that able to support the oocyte maturation process. One of factors that heighten the oocyte maturation level is the hormone contents that contained in the GFF. One of indications that be used to know the success level of in vitro maturation is the occurrence of cumulus cell (fig 1).

#### 3.2. Nuclei maturation in oocyte

The oocyte maturation results are important stage to determine the success of in vitro fertilization (IVF). In the oocyte fertilization period usually be valued based on 2 criteria, at nuclear maturation and cytoplasmic maturation, at the nuclear maturation, the oocyte experiences various process, from the germinal vesicle break down (GVBD), chromosome condensation, spindle formation process at metaphase I, homolog chromosom separation process with the body polar estruction, up to metaphase II (Kubelka, et al, 1995). However at cytoplasmic maturation, based on the development abilities level of oocyte from the beginning of the process, is the critical point from the oocyte survivability after inseminated (Younes et al, 1989). After maturation in the suitable medium, oocyte nuclei will experience the development up to metaphase II and ready for the next process.

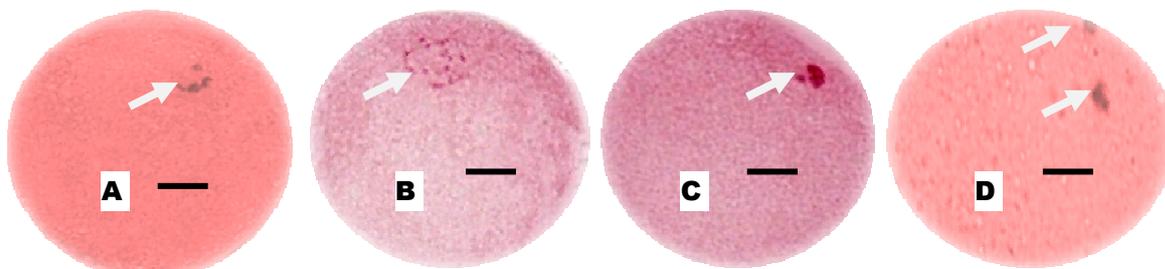
**Table 2. The nuclear maturation level of goat oocyte at the medium that is supplemented GFF with different levels**

FF	Σ Tested Oocyte	GV	GVBD	M-1	M-2
0	18	5/14 (35.35)	4/14 (29.90)	5/14 (34.75)	0/18 (0.00) <sup>a</sup>
2,5	17	6/17 (35.00)	5/17 (30.00)	3/17 (18.33)	3/17 (16.67) <sup>a</sup>
5	30	0/30 (0.00)	10/30 (33.33)	10/30 (33.33)	10/30(33.33) <sup>a</sup>
7,5	17	0/17 (0.00)	2/17 (12.67)	5/17 (29.33)	10/17(58.00) <sup>b</sup>
10	28	0/28 (0.00)	5/28 (18.57)	6/28 (20.63)	17/28(60.79) <sup>b</sup>

Explanation: Different subscript at the same column for each treatment, showed significant differences and (GV) Germinal, (GVBD) Germinal Vesicle Break Down, (M-1) Metaphase I, (M-II), Metaphase II.

Based on the variance analysis results in the research showed that the maturation level percentage of the goat oocyte that reached metaphase II at the basic media of TCM -199 supplemented with GFF with different level is different significantly ( $P < 0.01$ ) to the M-II, treatment P 4 (10%) GFF reached highest average of 60.79%. While at the other treatment no significant differences ( $P > 0.05$ ), can be proven that the increase and decrease of metaphase II percentage depend on the GFF level that is given in the TCM-199 media. The results showed that the level of GFF 0%, 2.5%, 5%, 7.5%, and 10% showed the presence of average value differences to the metaphase II. The higher GFF level the higher percentage at metaphase II, so in the research GFF level 0% (without GFF giving) the lowest percentage value of the nuclear transformation that obtained at metaphase II (0%). It is caused by medium without GFF supplementation unable to reach metaphase II because the oocyte needs is not support by hormones and nutrients in the maturation media. The results suitable with that reported by Choi et al (2001) that the hormone dosage addition able to increase the cumulus cell expansion and the nuclear transformation.

The research after maturation 26 hours in the media TCM-199 that is supplemented by GFF with different level. The maturation level is valued by calculating the oocyte amount at each meiosis division from GV, GVBD, M-1, and M-II. GV stage where it is marked with the nuclear membrane and nucleus seem clearly at the edge, GVBD is marked with the nuclear membrane torn and the can not be seen clearly, M-1 is marked with the homolog chromosome that is paired and paralel with the equatorial plane and M-II (mature oocyte) is marked with the pole I and the similar chromosome arrangement with M-1 level (Zeidan et al, 2011). The nuclear development stage can be seen in the figure 2.



**Fig 2: Oocyte nuclear status after in vitro maturation**

The arrow showed the nuclear status at the stage (A). (GV) Germinal Vesicle (B). (GVBD) Germinal Vesicle Break Down (C). (M-1) Metaphase I (D). (M-II) Metaphase II = scale line 50 um

In general, the mammalian oocyte development up to ovulation experience 2 rest phases, that is the first meiosis prophase stage and metaphase stage II (Whitaker, 1996). It is indicated that the nuclear oocyte at the rest condition or GV at the first meiosis division. Then the meiosis process will be continued, initiated with the torn of nuclear membrane known as GVBD, at the stage, there will be chromosome condensation then oocyte enter the rest stage at the metaphase II (Kidson, 2005).

During ovulation, oocyte at the metaphase stage II up to activation at the oocyte to continue the development at the initial meiosis, oocyte is controlled by maturation M-phase Promating factor (MPF) whose activities increase during GVBD maximum at metaphase I and decrease at metaphase II (Crozet, Dahirel and Gall, 2000). The oocyte difficulties to increase at metaphase II is expected because of the inability of oocyte to form maturation promation factor (MPF) at GVBD level because MPF has role in the phoporilation that important in the oocyte maturation (Gall et al, 1996). When the maturation process of oocyte in vitro does not give appropriate situation for oocyte although the nuclear cell has reached metaphase II, but the next results will

decrease the IV quality value (Kidson, 2005). Because of that the in vitro fertilization quality can be improved by culture at optimum condition.

#### 4. CONCLUSIONS

Based on the research results, it can be concluded that the GFF level 10% in TCM-199 able to improve the goat oocyte percentage reach cumulus expansion and metaphase II. Oocyte from the in vitro maturation uses IVM media with the GFF supplementation 10% is suggested for IVF.

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