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Research Article

In vitro Maturation of Ovine Oocytes Using Follicle Stimulating Hormone (FSH), Estradiol-17β, and Co-Culture of Fallopian Tube Epithelial Cells (FTEC) in Tissue Culture Medium-199 (TCM-199)

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ABSTRACT

This study was conducted to analyze the effect of FSH, estradiol-17ß, and co-culture of FTEC on ovine oocyte *in vitro* maturation. The material used was 2.290 oocytes taken from preantral follicles of 254 ovine ovaries. Study was carried out by testing four treatments: (T1) base medium (TCM-199, 5% FBS, $NaHCO_3$), (T2) base medium + FSH, (T3) base medium + 17-β estradiol, and (T4) base medium + co-culture of FTEC. Result showed that percentage of mature oocyte (Metaphase II) in this study was 40.55%, 61.62%, 40.34% and 58.52% for T1, T2, T3, and T4, respectively. Statistical analysis showed that medium maturation treatments were highly significant different ($P<0.01$) on oocyte maturation (Metaphase II) percentage. The base medium containing FSH and base medium containing co-culture of FTEC are better medium than the base medium or medium supplemented with estradiol-17ß for ovine oocytes *in vitro* maturation.

Key words: Maturation, Medium, Oocyte, Preantral follicles, Ovine

INTRODUCTION

Among factors determining the success of conducting *in vitro* fertilization are the oocytes maturity that is grown in a maturation medium cells or tissues outside the body (*in vitro*). Tissue Culture Medium 199 (TCM-199) has been widely used as a basic medium to grow somatic cells, it also should be able to grow oocytes that categorized as gamete cells. Considering *in vivo* grown cells is supported by a suitable environment. *In vitro* grown oocytes should replicate as similar as possible to the real *in vivo*. Therefore, TCM-199 should be combined with other substances such hormone and substances that secreted by epithelial cells.

In its implementation to optimize the *in vitro* maturation (IVM) of the oocytes, TCM-199 is combined with other substances such as Follicle Stimulating Hormone (FSH) (Rahman *et al*., 2008) Estradiol-17ß, or co-culture cells (Campbell and Scaramuzzi, 1991). Hormone and co-culture of Fallopian tube epithelial cells (FTEC) that was added into TCM-199 are able to stimulate the process of maturation and development of zygotes becoming an embryos (Abe and Hoshi, 1997). Studies upon the three substances added to TCM-199 have done with various methods, purpose, and results. However, researches on the combination of TCM-199 combined with FSH, Estradiol, and co-culture of FTEC is inconclusive.

As FSH is a hormone that functions to stimulate the growth of follicle and maturation of ovum in the ovaries (Reeves, 1987), FSH is possible to be added into TCM-199. Wandji *et al*. (1996) stated that FSH, Epidermal Growth Factor (EGF) and Basic Fibroblast Growth Factor (Bfgf) significantly increased the percentage of preantral follicles growth to the next phase resulting 82%; 72% and 93% for TCM-199+FSH, TCM-199+EGF and TCM-199+FSH+Bfgf, respectively.

The growth is characterized by the appearance of proliferation of the granulose cells. According to Krogenaes *et al*. (1994), the oocytes of deer (*Rangifer tarandus*) cultured in a medium supplemented with FSH produce optimal maturity (71%). Meanwhile, Staigmiller (1988) and Raju *et al*. (2013) reported that ovine oocytes cultured in a medium supplemented with FSH and LH hormones can reach optimal maturation levels, because these hormones were capable to induce nucleus maturation.

Similarly, Estradiol-17ß hormone can also be added into TCM-199. The Estradiol-17ß intended to increase the percentage of embryo maturation and acquisition.

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Estradiol-17ß functions is for maturing cells while they were in primordial phase, increasing the sensitivity of granulose cells to respond to Gonadotropin, and stimulating proliferation and differentiation of granulose cells when the oocytes were in the preantral follicle phase (Vanderhyden and Tonary, 1995; Beker *et al*., 2002). Previously, Staigmiller (1988) identified that embryo-culture medium enriched with 1µg/ml Etradiol-17ß resulted in 40% developed blastula of all embryos cultured in the medium.

Fallopian tube epithelial cells (FTEC) cultured in TCM-199 are capable to produce secretions in the form of materials that can form a suitable environment for oocyte maturation and embryo development. This stage takes place because in *in vivo* conditions, Fallopian tube plays a very important role in supporting reproductive processes such as final oocytes maturation process until they are fertilized by sperm and early development of the embryo (Tosti and Menezo, 2016).

Thus, co-culture of cells taken from their natural environment (granulose cells, FTEC, and uterine fibroblast cells) can be used for oocyte maturation medium and embryo growth. Bongso *et al*. (1990) reported that epithelial cells originating from Ampullary-Isthmic Junction (AIJ) which is grown and forms a cell layer can increase the percentage of human embryo growth to blastula stage (70%) compared to standard medium (33%). Meanwhile according to other researches, substances derived from co-culture secretion of FTEC contain various chemical elements such as proteins, ions, lipids, and many other chemical elements, so that their contribution to oocyte maturation and embryo growth is very essential (Gandolfi *et al*., 1989; Abe and Hoshi 1997).

Some researchers (Gandolfi *et al*., 1989; Hill *et al*., 1996) conducting studies on the use of co-culture of FTEC reported that secretions stimulated embryo development, as secretions contain non-serum glycoproteins, macro molecules, ions, and other triggering factors; besides, oviduct cell cultures have a pH and osmosis pressure that are suitable for embryo development. In addition, Chandra *et al*. (2012) proved that cow oviduct secretes a growth factor called Platelet Derived Growth Factor (PDGF) which plays a role in the early development of the embryo. Similarly, Li and Winuthayanon (2017) confirmed that oviduct cells secrete substances in the form of growth factors during embryo development process. Furthermore, they stated that the growth factors secreted by FTEC function as embryonic development and oocyte maturation process of this cell growing technique is known as co-culture medium. The use of co-culture of FTEC to support oocyte maturation and embryonic cells growth is significant in the *in vitro* fertilization program.

Given the explanation, the aim of this study was to analyze the effect of FSH, estradiol-17ß, and co-culture of FTEC on ovine oocyte *in vitro* maturation. This study is expected to contribute to the use of the right oocyte maturation medium in the context of implementing embryo transfer programs in terms of embryo procurement

MATERIALS AND METHODS

Sample Material, Chemical Material and Equipment

Material used in this study was 2,290 of grade A oocytes taken from preantral follicles of 254 ovine ovaries by aspiration technique. Collected oocyte then was placed into maturation medium (treatment). The chemicals used were TCM-199 (GIBCO), FSH (NISUI), Estradiol-17 ß (SIGMA), mineral oil (SIGMA), D-PBS (GIBCO), trypsin 0.25%, NaCl, KCl, $CaCl_2$, H_2O, $NaHCO_3$, $NaHPO_42H2O$, $MgCl_26H_20$, FBS (Foetal Bovine Serum), phenol-red, glucose, sodium pyruvate, kanamycin (SIGMA) NaCl 0.9%, deionized water, aquabidest, orcein 0.1%, hyaluronidase enzyme, acid acetate and alcohol.

The equipments used were dissecting microscope (Olympus SZ), inverted microscope (Olympus CK2) equipped with photographic equipment, laminar-flow hood (NUAIRE), magnetic stirrer, incubator (Imperial III), gaspack "OXOID", CO_2 tube, sterile room, HORIBA F-14 pH meter, autoclave, 0.22 µm (SIGMA) filter, filter paper, heater (FISHER), thermometer, analytical scales, timers, sterile petri dishes (CORNING) diameter 60 mm and 35 mm, 10 micropipette 100 µl (EPPENDORF), disposable Pasteur pipette (VWR SCIENTIFIC), pipette-tip, Erlenmeyer, 25, 50 and 100 ml vial bottles, test tube tubes, disposable syringes of various sizes 1, 5 and 10 ml, tweezers, scalpels, scissors, gloves and tissue.

Treatment of oocytes maturation

Four treatments and 35 replications were applied for oocyte maturation that was shown in Table 1. Experimental design used in this study was completely randomized design. The entire treatment medium has a molarity of 320 mOsm and pH 7.3.

In Vitro oocytes maturation

In this study, 2.290 oocytes were divided into four groups and then cultured in the oocyte maturation medium according to the treatment. Before being cultured, oocytes (preantral follicles) collected were rinsed using treatment medium as much as three times, transferred to treatment drops medium in petri-dish, which each drop of medium consisted of 100 µl of the treatment medium. Then it was covered with mineral oil, and cultured as much as 10 to 20 oocytes for each drop of medium. The petri-dish containing oocytes was cultured in CO_2-incubator containing 5% CO_2 at 38^0C for 24 hours. After 24 hours, staining was carried out using 1% aceto-orcein staining agent.

Variables observed

After 24 hours of *in vitro* maturation process, oocyte was observed using a microscope and classified into four stages, which were Germinal Vesicle (GV), Metaphase I (MI), Anaphase I (AI) and Metaphase II (MII) as shown in Table 2 (Chye, 1986). GV stage as the initial stage of oocyte development is characterized by the visible nucleus in the cytoplasm. MI stage is characterized by the presence of homologous chromosomes in pairs in the equatorial. AI stage is characterized by the presence of homologous chromosomes, each at opposite poles. MII stage, as the last observation stage, is when the chromosome position in the equatorial. Percentage of oocyte of each stage then was measured.

Statistical analysis

Percentage of maturation oocyte at GV, AI and MI were analyzed using descriptive analysis. The effect of the treatment on oocyte maturation at MII were analyzed using Analysis of Variance (ANOVA) with by using SPSS 16.0

procedure. The study consisted of four treatments and 35 replications. Difference in the least square means of treatments was tested by Honest Real Difference test (Steel and Torrie, 1980).

RESULTS

Result showed that the number and the percentage of the oocytes were varied among treatments, as shown in Table 3. Percentage of mature oocyte (MII) in this study was 40.55%, 61.62%, 40.34% and 58.52% for T1, T2, T3, and T4, respectively. Statistical analysis showed that medium maturation treatments were highly significant different ($P<0.01$) on oocyte maturation (MII) percentage. T2 and T4 had significantly ($P<0.01$) higher mature oocyte (MII) (61.62%; 58.53%) compare to T1 and T3 (40.55%; 40.34%). While T2 was not different ($P>0.05$) with T4, as well as T1 compare to T3.

The percentage of oocytes at GV is 3.21% (T1), 1.95% (T2), 4.54% (T3) and 2.08% (T4). The percentage of oocytes at GV in T1 and T3 is higher than T2 and T4; yet, the result was not the best in term of oocytes maturation at MII. This finding suggested that different treatments did not fully affect the number and the percentage of oocytes maturation from GV to AI.

DISCUSSION

The percentage of oocytes maturation from GV to AI in four treatments was inconsistent. It indicate that unknown initial stage of oocytes and its duration needed to be mature in this study influence the inconsistency of the oocytes maturation. The initial stage of oocytes when started to be cultured possibly scattered from GV to AI (mostly) and from GV to MII (small part). As a result, when oocytes were cultured in four different treatments of oocytes maturation medium, the initial stage of the maturation was not consistent.

Furthermore, dismissal and evaluation of oocytes maturation after being cultured for 24 hours resulted in cessation of oocyte maturation process in certain phases. Nevertheless, indication showed that dominant response due to treatments occurred in maturation stages between AI and MII, as proven by a sharp increase in percentage from AI to MII in four treatments.

Statistically, T3 proved to be no different ($P>0.05$) with T1 at MII. This finding suggested that adding steroid hormones (estradiol 17-ß) to the oocyte maturation medium did not affect oocytes maturation in MII, as estradiol-17ß is known to have a role in the early stage of oocyte maturation (Salehnia and Zavareh, 2013). Moreover, the ovaries collected in this study had passed their primordial phase, while their initial stage were unidentified; therefore, the effect of estradiol 17-ß in T3 was not significant ($P>0.05$) with T1 at MII. This finding was in line with Cui *et al*. (2013) that estradiol 17-ß is a primary estrogen that one of its functions is to mature cells when they are still in the primordial phase.

Although estradiol-17ß is unable to stimulate oocyte to grow to MII, its existence is needed to increase granulose cells sensitivity to respond Gonadotropin hormone (increase sensitivity of FSH and LH receptors) (Homer *et al*., 2017) and stimulate the proliferation and differentiation

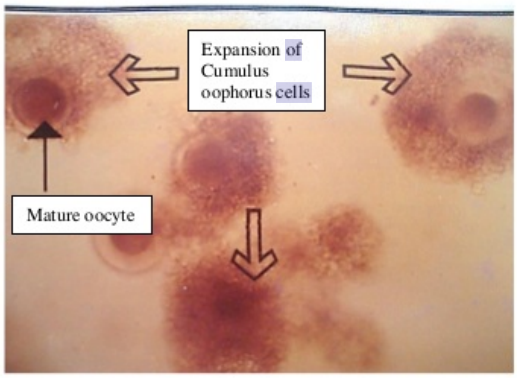

Fig. 1: A number of mature oocytes are being cultured in maturation medium containing FSH (100x magnification).

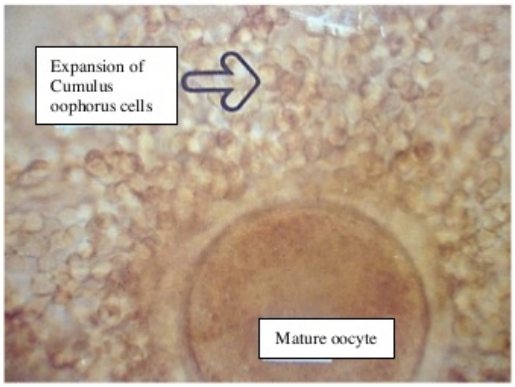

Fig. 2: Mature oocyte characterized by perfect expansion of cumulus oophorus cells (400x magnification).

of granulose cells when the oocytes are in preantral phase (Orisaka *et al*., 2009). Therefore, the role of adding steroid hormones (estradiol-17ß, progesterone) into the base medium was to prepare the oocytes in the fertilization process, as steroids have an essential function in the process of capacitation and the acrosome reaction on spermatozoa (Ickowicz *et al*., 2012).

The percentage of the phases of oocyte maturation from GV to AI in T2 and T4 was low; GV (1.95%; 2.08%); MI (18.50%; 17.11%); and AI (17.61%; 23.21%), but at MII the percentage of oocyte maturation (61.62%; 58.53%) increased, as there was a highly significant difference ($P<0.01$) among the four treatments (Table 3). T2 and T4 had significantly ($P<0.01$) higher mature oocyte (MII) (61.62%; 58.53%) compare to T1 and T3 (40.55%; 40.34%). Meanwhile, T2 was not different ($P>0.05$) with T4, as well as T1 compare to T3. This proved that T2 and T4 responded positively to the maturation of oocytes compared to T1 and T3.

Although, statistically, there was no difference between T2 and T4, the percentage of oocytes in T2 tended to be higher than T4 (61.62% vs 58.53%) at MII. This suggested that medium added with FSH increased the percentage of ovine oocytes maturation to MII.

According to Giacomo (2016) and Pan and Li (2019), FSH stimulates gametogenesis process, as in the initial stage, it increases the levels of cyclic adenosine

Table 1: The number of oocytes cultured in the treatment maturation medium.

Treatment	n	Maturation Medium
T1	528	Basic Medium (TCM-199, 5% FBS, $NaHCO_3$)
T2	562	Basic Medium + FSH
T3	528	Basic Medium + Estradiol 17-ß
T4	672	Basic Medium + co-culture of FTEC

Table 2: Determining oocytes maturity based on the signs seen after being cultured for 24 hours (Chye, 1986).

Phase	Cumulus Oophorus	Corona Radiata	Oocyte
1	Tight	Tight	Not clear
2	Loose expanded	tight	Not clear
3	Loose expanded	little expanded	Vaguely
4	Loose and slightly slimy	little expanded clearly	Polar body is not visible
5	Loose slimy	Loose clearly	Polar body I appears (MII)
6	Slightly slimy cumulus	Loose, aggregate	Polar body I is clear (MII)
7	Little, without cumulus	Varies	Degeneration, pycnotic

Description: Phase 1-3 = Immature oocyte; Phase 4-5 = mature oocytes; Phase 6 = Over matured oocytes; Phase 7 = Degeneration.

Table 3: The Number and the Percentage of the Oocytes Maturation According to Treatment Medium and Oocytes Maturation Stages after being Cultured for 24 hours.

Treatment	Stages								Total
	GV		MI		AI		MII		
	n	%	n	%	n	%	n	%	
T1	17	3.21	94	17.80	203	38.44	214	40.55[a]	528
T2	11	1.96	104	18.50	99	17.62	348	61.92[b]	562
T3	24	4.55	132	25.00	159	30.11	213	40.34[a]	528
T4	14	2.08	115	17.19	156	23.21	387	57.60[b]	672

Different superscripts in the same column show differences; (GV = Germinal vesicle, MI = Metaphase I, AI = Anaphase I, MII = Metaphase II).

monophosphate (c-AMP) in the cumulus cells surrounding the oocytes. Then, when the c-AMP level decreases, Germinal Vesicle Breakdown (GVBD) takes place. Similarly, Yan *et al*. (2016) stated that c-AMP is a nucleotide derived from ATP through the work of the Adenylate Cyclase enzyme that has an essential function in a number of hormones to be processed. The level of intracellular c-AMP can increase or decrease by the influence of various hormones and its effect is varies among tissues.

Yan *et al* (2016) explained that the interaction of hormones with their receptors causes either activation or inactivation of Adenylate Cyclase resulting in the level of c-AMP in the cytoplasm to increase or decrease. The activation or inactivation of Adenylate Cyclase depends on the group of hormones that interact; hormones that stimulate (FSH, ACTH, LH) or hormones that inhibit Adenylate Cyclase (acetylcholine, somatostatin, angiotensin II). Meanwhile, according to Lee *et al*. (2007), medium containing FSH increases the effectiveness of the oocyte maturation process, as FSH in the medium causes the expansion of cumulus cells surrounding the oocytes, which in turn spurs the process of capacitation of spermatozoa, fertilization, and embryo development.

In ovine, FSH can induce granulose cells from small growing follicles to produce estradiol; however, large amounts of insulin and FSH are needed for large follicles (Campbell and Scaramuzzi, 1991). Likewise, according to Moor and Trounson (1977), in *in vitro* conditions, the presence of FSH in oocyte maturation medium stimulates the occurrence of meiotic division of the oocyte nucleus. The meiotic division in oocyte nucleus is driven by the presence of maternal nutrients and proteins as physiological effects of cumulus/granulose cells stimulated by FSH through the process of protein phosphorylation/ dephosphorylating. Further, the nutrients and ingredients needed by oocytes are distributed through microvilli in tissues that penetrate the zona pellucida.

Furthermore, Hafez (1993) described that the granulose cells found around the pellucida zone forming a junction in the form of microvilli extension that penetrates the zona pellucida towards the cytoplasm in the oocyte which functions to deliver nutrients and maternal protein. This finding was similar to the previous results of Moor and Trounson (1977), Crosby *et al*. (1981), Pugh *et al*. (1991), and Powel and Rexoad (1992), who succeeded in increasing the percentage maturation of ovine oocytes by adding FSH to the maturation medium. Sophon *et al*. (1993) who added FSH to the medium proved to increase the percentage of pig oocytes maturation to MII; meanwhile, Lu (1987) succeeded in increasing the percentage maturation of cattle oocytes in a medium supplemented with FSH.

The oocyte maturation process begins with the presence of a perfect GV. This stage is characterized by a core membrane and nucleoplasm intact and chromatin that spreads evenly, then followed by membrane decay and oocyte nuclei (Slavik and Fulka, 1992). In the next stage, oocytes develops into MI, AI, and MII. Under microscopic observations, the oocyte maturation in this study was characterized by the perfect expansion of the cumulus cells (Figure 1 and 2).

The perfect expansion of cumulus cells indicated that processes in the oocytes or in the cumulus cells were taking place. According to Da Broi *et al*. (2018), cumulus cells performing in the maturation process of oocytes have function as somatic cells and these cells respond to stimulus from endocrine, paracrine, and autocrine. These activities occur to support oocyte activities such as metabolism, growth, meiotic division, and maturation process.

The perfect expansion of the cumulus cells occurred after the oocytes were cultured for 24 hours in the maturation medium. This finding was supported by the one of Barrett and Albertini (2010) in mice oocytes by administering 0.5 µg/ml FSH in th maturation medium resulting in the best response to the expansion of cumulus cells. Sharma *et al*. (1996) also showed that the optimal maturity level of goat oocytes is characterized by the perfect expansion of cumulus cells after being cultured for 24 hours. The same condition occurred at maturation oocytes of bovine (Arredondo and Seidel, 1994). The expansion of cumulus cells was likely useful for providing and paving the way for spermatozoa to penetrate directly to receptors in the zona pellucida to facilitate the fertilization process.

In this study, T4 resulted in a high percentage of oocytes maturation until MII compared to T1. The *in vivo* oocytes maturation occurs since the oocytes are inside the ovary, then continued until they are ovulated and place in the Fallopian tube. According to Hafez (1993), in an *in vivo* condition, the ovulated oocytes are already in MII and the oocytes at this stage will be received by Fallopian tube for the subsequent meiotic process until fertilization.

The Fallopian tube with various components produced by its epithelial cells secures an appropriate and suitable environment for the development stages of oocyte maturation until the embryo is formed (Songsasen and Wildt, 2007). In the Fallopian tube, the oocytes undergo optimal maturation before being fertilized by the spermatozoa (Álvarez *et al*., 2013). The support of FTEC in stimulating oocyte maturation is to provide an appropriate environment by securing the substances needed by the oocytes (Sirard, 2011).

In this study, T4 highly significant (P<0.01) increased oocyte maturation, co-culture of FTEC can secrete substances needed in oocyte maturation in the form o ns, growth factors, and several amino acids (Gandolfi *et al*., 1989). The results of this study were in line the finding of Hill *et al*. (1996) showing that the co-culture of FTEC originating from the ampoule of estrus ovine oviduct contains Estrus-associated Glycoprotein (EGP) which is able to stimulate the maturation process of ovine oocytes.

Conclusions

The maturation oocyte medium containing FSH has the highest percentage of oocytes maturation to MII and is followed co-culture of FTEC medium. Both are better medium for ovine oocytes *in vitro* maturation compared to the base medium or medium supplemented with estradiol-17ß. The supplementation of estradiol-17ß into the medium is only beneficial at the beginning of the oocyte development stage and is unable to increase oocyte maturation until MII.

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