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In Vitro Ovine Embryo Culture: Effectiveness of TCM-199 in Combination with 17β-Estradiol Hormone and Co-Culture of FTEC

(KULTUR EMBRIO DOMBA SECARA IN VITRO: EFEKTIVITAS TCM-199 YANG DIKOMBINASIKAN DENGAN HORMON 17β-ESTRADIOL DAN KO-KULTUR FTEC)

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ABSTRACT

Objective of this research was to determine the effectiveness of various culture mediums on the cleavage to morula in *in vitro* embryo culture of ovine. A total of 1.064 zygotes (fertilized oocytes) were grown in three kinds of different embryo culture medium, which were Tissue Culture Medium 199 (TCM-199) (T1), TCM-199 + 17â-estradiol (T2) and TCM-199 + co-culture of Fallopian Tube Epithelial Cell (FTEC) (T3). Data were analyzed using a completely randomized design with ANOVA analysis and Honestly Significant Difference test. The variable observed were the percentage of embryo cleavage and morula. Results showed that T3 had the highest percentage (72.45%) of the cleavage occurred in zygotes cultured in the TCM-199 + FTEC co-culture medium, as well as embryo growth to the morula stage (75.75%). In contrast, zygotes cultured in T1 and T2 had lower cleavage and lower morula development than T3, as much as 27.38%; 47.82% and 58.15%; 66.66%, respectively. In conclusion, TCM-199 + FTEC co-culture medium is the best medium for embryo culture.

Keywords: culture medium; zygotes; cleavage; morula; ovine

ABSTRAK

Tujuan penelitian adalah untuk menentukan efektivitas dari berbagai media kultur terhadap cleavage sampai dengan terbentuknya morula yang dikultur secara *in vitro* pada embrio domba. Sebanyak 1.064 zigot ditumbuhkan dalam tiga media kultur embrio yang berbeda, yaitu TCM-199 (T1), TCM-199 + 17â-estradiol (T2) dan TCM-199 + co-culture FTEC (T3). Desain penelitian menggunakan Rancangan Acak Lengkap yang dianalisis dengan sidik ragam dan diuji lanjut menggunakan Uji Beda Nyata Jujur. Variabel yang diamati adalah persentase cleavage dan morula. Hasil menunjukkan bahwa persentase cleavage sampai perkembangan embrio ke tahap morula tertinggi terdapat pada T3 (72,45%; 75,75%), sedangkan persentase terendah cleavage sampai perkembangan embrio ke tahap morula secara berturut-turut terdapat pada T1 (27,38%; 47,82%) dan T2 (58,15%; 66,66%). Dapat disimpulkan bahwa media TCM-199 + co-culture FTEC adalah media terbaik untuk kultur embrio domba.

Keywords: media kultur; zigot; *cleavage*; morula; domba

INTRODUCTION

In the embryo transfer, there is one condition causing less successfully to achieve its goal. It is the provision of an adequate number of embryos, in term of quantity and quality. In *in vivo* techniques, the supply of embryos is slow due to limit of animal, tool and time. Meanwhile, in *in vitro* techniques, the acceleration of embryo supply can be achieved, but the obstacle encountered is the availability of effective medium for culturing the embryo, as the variety of embryo culture medium available is too diverse.

Verma and Singh (2014) defined that *in vitro* technique was the development ability of tissues or organs cultured in an artificial environment such in a culture medium outside the body of an animal. In animal husbandry, *in vitro* embryo production, both fresh and frozen using ovarian raw materials, has been carried out in the framework of an optimization program for increasing population and livestock quality to sustain community needs for future protein adequacy (Zhu *et al.*, 2018).

On its implementation, the successful of *In Vitro Embryo Production* (IVEP) has been identified to be influenced by several factors such as: the culture medium used for oocyte maturation, the fertilization medium, and the medium for embryonic development. Naturally, fertilized or ovulated ovum are positioned in the Fallopian tube lumen; thus, when they are to be cultured in *in vitro*, they should be supported by natural-like-environment in order to their viability. The new supported environment was formed by epithelial cell secretions of Fallopian tube to sustain ovulation process, zygotes development, and cleavage until morula/blastula stage. (Leese *et al.,* 2001)

The medium used for growing cells or tissues (oocytes, zygotes, and embryos) outside the body of animals *(in vitro)* that has been widely known and used is Tissue Culture Medium 199 (TCM-199). This medium contains combination of vitamins, amino acids, growing factor cells enriched with gonadotropin hormones or serum (fetal calve serum). However, the effectiveness of TCM-199 after being combined with other substances has not been conclusive for oocytes maturation and development. Under this circumstance, this experiment proposed two substances to be added to the TCM-199, hormone 17ß-estradiol and co-culture of Fallopian Tube Epithelial Cell (FTEC), in order to analyze their effectiveness.

Adding hormone 17ß-estradiol or co-culture of FTEC to TCM-199 is presumably able to stimulate the process of maturation and development of zygotes into embryos. According Su *et al.* (2009) hormone 17ß-estradiol, played a role in maturing cells while they were still in the primordial phase, increased sensitivity of granulosa cells to respond to gonadotropins, and stimulated proliferation and differentiation of granulosa cells when oocytes were in the pre-antral phase. Meanwhile, FTEC cultured in TCM-199 produced secretions in the form of materials that can form an environment suitable for *in vitro* embryonic development. In the *in vivo* condition, the Fallopian tube plays a very important role for supporting the ongoing process of final oocyte maturation until it was fertilized by spermatozoa and the developed embryo start to grow. According Paik *et al.* (2012), epithelial cells of the Fallopian tubes and their secretion played a role in reducing polyspermia, which inhibited the process of embryos that were developed.

Given this explanation, objectives of this study was to determine the effectiveness of various supplementations in culture mediums on the cleavage to morula in *in vitro* embryo culture of ovine. This research is expected to contribute to the implementation of an embryo transfer program in terms of supplying embryos for small ruminants in particular or other ruminants in general.

RESEARCH METHODS

A total of 1.064 zygotes (fertilized oocytes) were used. Those zygotes then were grown in three kinds of different supplementation of embryo culture medium as treatment, which were TCM-199 (T1); TCM-199 + 17â-estradiol (1 µg/mL) (T2) and TCM-199 + FTEC co-culture (T3). The equipment used in this research consisted of dissecting microscope (Olympus SZ), inverted microscope (Olympus CK2) with photograph accessories, laminar-flow hood (NUAIRE), OXOID gasspack, CO_2 tube, HORIBA F-14 pH meter, autoclave, distillatory, filter 0.22 ìm (SIGMA), sterile petri dish (CORNING) of diameter 60 mm and 35 mm, 10-100 ìL micropipette (EPPENDORF), Pasteur pipette disposable (VWR SCIENTIFIC) and pipette-tip.

Preparation of FTEC Co-culture

Fallopian tube epithelial cell (FTEC) was preparing by flushing the ovine fallopian tube using *Trypsin* enzyme. Then, it was collected in petri dishes that contain TCM and was cultured in incubator at 37°C for 24 hours.

***In vitro* embryo culture method**

A total 10-20 zygote were placed into petri dish containing 100 ìL medium and mineral oil. Then it was incubate at 37°C for 144 hours using CO_2 incubator. Medium was replaced every 48 hours after being cultured. Observation upon the stages of embryo cleavage and development from the initial stage to the morula stage was determined based on the estimated time of cleavage and development of the embryo, as shown in Table 1. The division was observed for 5-6 days by observing the blastomeric cell division using dissecting microscopes from one cell to two cells, four cells, eight cells, 16 cells, 32 cells and more than 32 blastomeric cells (Park *et al.,* 2009).

Measured Variable

Variable measured were percentage of cleavage and morula. Percentage of cleavage is calculated from the number of zygotes cultured, percentage morula is calculated from the number of cells division cultured. Stages of blastomeric cell division of the fertilized oocytes (zygote development), and cell division starting from two cells, four cells, eight cells, 16 cells, 32 cells and more than 32 cells (morula).

Statistical Analysis

Percentage of embryo cleavage and morula were analyzed by using descriptive analysis. Effect of culture media on the embryo cleavage to morula were analyzed using Analysis of Variance with by using SPSS 16.0. Difference in the least square means of treatments was tested by Honestly Significant Difference Test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Results showed that differences of medium cultured embryo used in this study was statistically significant (P<0.05) on cleavage and morula growth. T3 had the highest percentage of the cleavage (72.45%) occurred in zygote cultured, as well as embryo growth to the morula stage (75.75%). In contrast, zygotes cultured in T1 and T2 obtained lower cleavage and morula development, as much as 27.38%; 47.82% and 58.15%; 66.66%, respectively (Table 2).

Growth of Zygote to Cleavage

Statistical analysis showed that the percentage of cleavage taken place on the T1, T2 and T3 was different (P <0.01). The results of the influence of T3 and T2 on the percentage of cleavage showed different (P<0.01). In T1, the percentage of cleavage was lower (27.38%) than T2 = 58.15% and T3 = 72.45 %. The resulting division of the embryo into two cells, four cells, and eight cells as shown in Figures 1, 2 and 3.

Growth of Cleavage to Morula

The T3 was found to be highly significant difference from basic medium, while T1 was difference (P<0.01) from T2, and T3 was difference (P<0.01) from T2. This finding suggested that the effect of medium tested on the percentage of the embryo cleavage to be morula stage was different. The cells division process and their development into morula stage of T1 was not as good as that of T2 and T3 (Table 2). This outcome was presumably caused by the basic medium (TCM-199) in T1 without being supplemented by any substances consisted only organic salt, amino acids, and vitamins, while the medium of T2 and T3 contained additional substances of estradiol hormone and Fallopian tube co-culture. Even though T1 and T2 might also support cell division process until morula stage (Figure 4), but their effectiveness was not as good as T3.

The medium of T1, which was TCM-199 containing organic salt, amino acids, and vitamins, and of T2, containing of TCM-199 added with estradiol hormone. As estradiol hormone functions to prepare reproduction organ of oviduct for gestation period/process, its effect on supporting embryonic division until reaching

Table 1. Time of observation of division and development of ovine embryo based on Sugie *et al.* (1980)

Observation (dayth)	Embryonic stages
0-2	2 cells
1-2	4 cells
2-3	8 cells
3-4	Early morula
4-5	Compact morula
5-6	Late morula

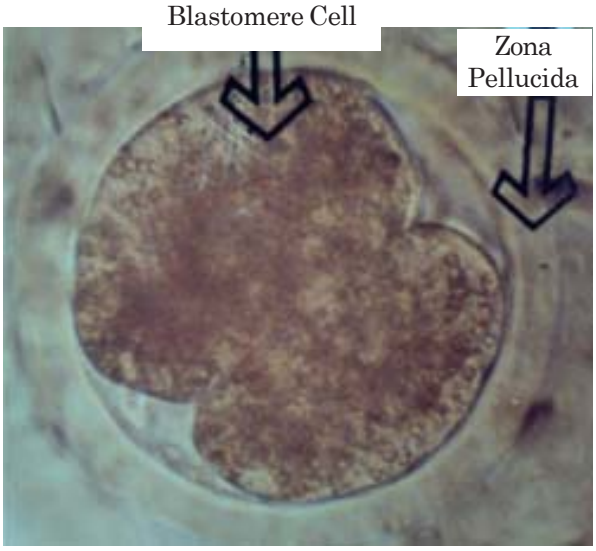

Figure 1. Two cell ovine embryo division

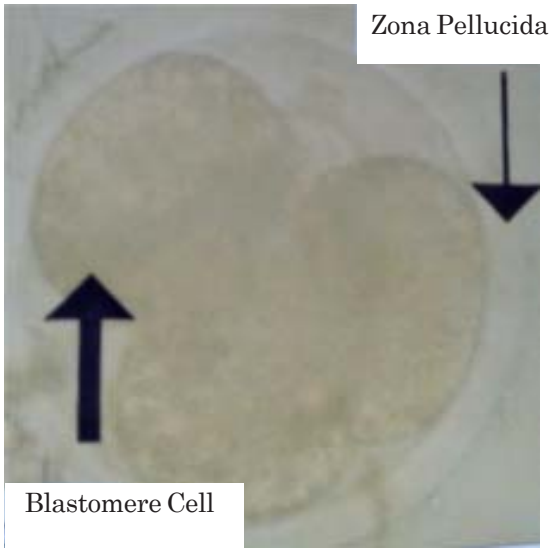

Figure 2. Four cell ovine embryo division

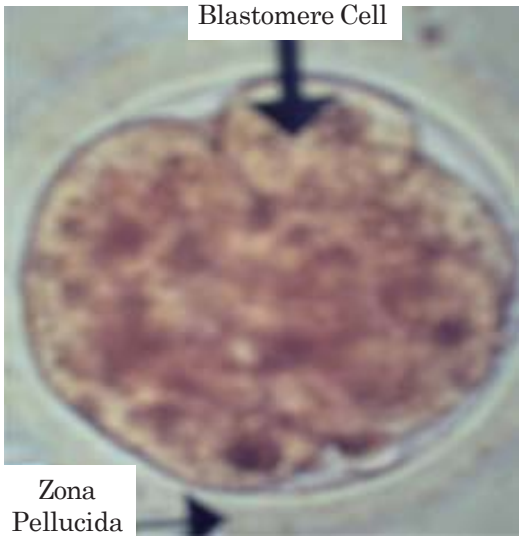

Figure 3. Eight cell ovine embryo division

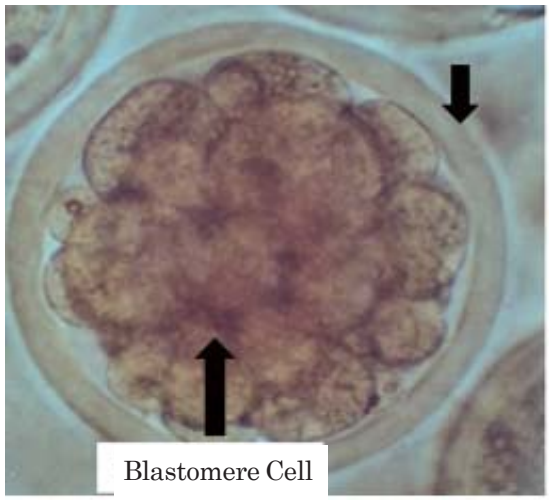

Figure 4. Compact morula stage of ovine embryo

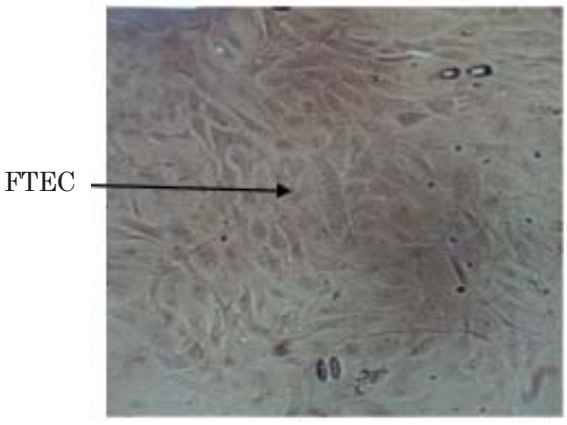

Figure 5. The co-culture medium of FTEC as a result of culturing FTEC for 24 hours.

the stage of morula was not effective (Li and Winuthayanon, 2017). In this study, the dosage of estradiol-17ß used in the culture medium of ovine embryo was 1 µg/mL, which was relatively high. In fact, using excessive dosage of 17ß-estradiol negatively affects embryonic cell division which is developing into morula to blastula. According Tkachenko *et al.* (2015), as much as 1 µg/mL of 17ß-estradiol is often used in routine *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). According Ma *et al.* (2003), minimal dosage of estradiol that is required to induce uterine sensitivity for embryo development was ranged 1.5-3.0 ng. Furthermore, the result of this study showed

Table 2. Number and percentage of zygotes division and development into morula embryos in culture medium.

Treatment	n	Cleavage		Morula	
		Σ	(%)	Σ	(%)
T1 (TCM-199)	336	92	27.38[a]	44	47.82[a]
T2 (TCM-199 + 17ß-Estradiol)	227	132	58.15[b]	88	66.66[b]
T3 (TCM-199 + co-culture of FTEC)	501	363	72.45[c]	275	75.75[c]

Note: Different superscript in the same column indicate significant differences (P<0.01)

that T3 had the highest percentage of morula compared to other two treatments. The co-culture medium secrets growth-factors, glycoprotein, macro molecule and ions triggering early embryonal development (Ferraz *et al.*, 2017). In addition, FTEC has suitable pH and osmoses pressure for the development of the embryo (Rottmayer *et al.*, 2006). Division cells cultured in *in vitro* form a cell layer spreading in the bottom of the petri dish (Figure 5). According Li and Winuthayanon (2017) in *in vivo*, reproduction organ of Fallopian tube functions to support reproduction process; such as, spermatozoa capacitation, fertilization, and early-stage development of embryo, furthermore it was reported that FTEC of ovine secret specific protein that support embryo development even though functions of the protein has not been clearly identified. In *in vitro*, function of FTEC created micro environment by forming ions, amino acids, and glycoprotein suitable for embryo to grow (Aguilar and Reyley, 2005). Similarly, Gopalapillai *et al.* (2013) reported that a number of essential cells such Na^+, K-, Ca^{2+}, Mg^{2+}, PO_4^{3-}, Cl^-, and HCO^{3-} function as growth supporting embryo. Lawrenson *et al.* (2013) argued that secretion of FTEC in the form of ions that were essential for embryo development was known synthesizing new proteins in *in vitro* technique. Those ions, Na^+/K^- (16.6 meq/L), Ca^{2+}/Mg^{2+} (3.8 meq/L), Cl^- (119 meq/L) and HCO^{3-} (21.8 meq/L), function to maintain the stability of medium, permeability, and interaction among cells when morula process is taking place.

Furthermore, Nerkar and Gadegone (2013) stated that some mammalian species have specific glycoprotein secreted by Fallopian tube. The increased glycoprotein by 50-100 ng/mL is able to increase the percentage of embryo division to be in blastula stage. In addition, Li and Winuthayanon (2017) proved that ovine oviduct secrets estrus-associated glycoprotein (EGP) functioning to trigger mitosis division at the early development of embryos. According to Li *et al.* (2007), glycoprotein and amino acids generated from Fallopian tube was needed in the early stage development embryo of mice. Kim *et al.* (2011) explained that amino acids secreted by FTEC of ovine were alanine, arginine, asparagine, aspartate acid, threonine, glutamine, glutamate acid, glycine, histidine, isoleucine, leucine, lysine, methionine, sistein, phenylalanine, proline, serine, taurine, tryptophan, tyrosine and valine. Meanwhile, the glycoprotein is a protein oligosaccharide chain (glycan) covalently bonded by its polypeptide. Glutamine that derived from TCM and also secreted by FTEC co-culture function as the source of amine cluster which was used to support transmission process to synthesize other amino acids in the growing embryonic cells. According Ishiwata *et al.* (2000) and Newsholme *et al.* (2003) the importance of glutamine to cell survival and proliferation *in vitro*. Meanwhile, Xu *et al.* (2018) stated that oligosaccharide chain has an important role in affecting embryonic growth and differentiation in addition to its responsibility in other biologic activities. Almost all plasma protein, except albumin, are in the form of glycoprotein including certain hormones such gonadotropin chorionic.

In *in vitro*, the environment was manipulated by the TCM-199 for culturing cells taken from FTE as the cells grew and formed a layer of cell (Lawrenson *et al.*, 2013). The cells growth in a layer secrete growing factor substances at the process of cells developing, this finding was similar to Tyson and Novak (2014). Insulin and insulin-like growth factor signaling increases proliferation and hyperplasia of the ovarian surface epithelium and decreases follicular integrity through upregulation of the

PI3-kinase pathway. King *et al.* (2013) stated that the growing factor secreted by FTEC is called insulin-like growth factor (IGF). The IGF triggers embryonic development before it implanted uterine of mice.

Moreover Ando *et al.* (2000) stated that insulin hormone improved capability of glucose transport and metabolic process in the embryonic division until morula-blastula stage. In addition, FTEC provide environment suitable for *in vitro* embryonic development. This might happen as cells forms a cell layer (monolayer) being able to secrets embryonic growing factors called epidermal growth factor (Zeng and Harris, 2015). In this study, dosage of 17ß-estradiol used as medium is good enough. Excessive dosage of estradiol will affect the division of embryonic cells and affect its development to the morula-blastula stage. Ma *et al.* (2003) 17â-estradiol plays a role in preparing the channels of female reproductive organs in order to prepare for pregnancy (Banciu *et al*., 2018).

The effect of FTEC culture on cleavage obtains a higher percentage. It can be understood because naturally the initial process of division occurs in the Fallopian lumen after the oocyte is fertilized, then slowly goes towards the cornua of the uterus to obtain implantation sites. The results of this study are similar to the opinion of Lawrenson *et al.* (2013) which stated that the usefulness of FTEC culture can produce secretions that can stimulate early embryonic development, because they contain non-serum glycoproteins, macro molecules, ions, other stimulating factors and also had suitable pH and osmoses pressure. In this study, the FTEC culture medium obtained a higher percentage of cleavage (72.45%). Data on the percentage of embryo development to the morula stage showed that the treatment of FTEC culture medium obtained a higher percentage (75.75%), compared to the medium containing estradiol-17ß (66.66%).

CONCLUSION

Based on the results of this study, it can be concluded that TCM 199 co-cultured with FTEC is the best medium for ovine embryo culture.

SUGGESTION

Further research is needed to be done by adding growth factor into FTEC co-culture medium for the best possible result of *in vitro* ovine embryo culture.

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