THE INFLUENCE OF EWE SERUM ON *in vitro* OOCYTE MATURATION AND EARLY DEVELOPMENT OF OVINE EMBRYOS

PENGARUH SERUM INDUK TERHADAP PEMATANGAN OOSIT DAN PERKEMBANGAN EMBRIO DINI DOMBA SECARA *in vitro*

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ABSTRACT

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The experiments were carried out to study the influence of different sera on *in vitro* maturation and early development of ovine embryos. Sera used in this study were FLS (Fetal Lamb Serum), ewe serum collected on Day 0 (ES-D0) and Day 6 (ES-D6) of oestrus cycles. Ovine oocyte were ma-tured and cultured in TCM-199 supplemented with 10 % of different sera. Results of this experiment showed that supplementation of ES-D0 or ES-D6 could support maturation rate (Metaphase-II) at 68.7% and 67.6%, respectively better than FLS (32.9%). The fertilization rate was significantly higher ($p<0.01$) in medium supplemented with either ES-D0 or ES-D6 than FLS, (30.7%, 65.4%, and 65.8% for medium supplemented with FLS, ES-D0, and ES-D6, respectively). On the other hand the effect of ES-D0 supplementation followed by ES-D6 on IVM and IVC yielded in embryos cleavage (47.6%) higher than those supplemented with ES-D6 followed by ES-D6 (41.6%) and ES-D0 followed by ES-D0 (28.7%). In conclusion, supplementation of ES-D0 or ES-D6 into maturation and culture medium have given better results on both maturation rate and early embryonic development.

Key words: IVF, ovine embryos, ewe serum

ABSTRAK

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Telah dilakukan penelitian mengenai pengaruh serum induk terhadap pematangan oosit dan perkembangan embrio dini secara *in vitro*. Untuk proses pematangan dan biakan *in vitro* digunakan serum yang diperoleh dari fetal domba (FLS), induk domba oestrus (ES-D0) dan pascaoestrus (ES-D6). Oosit dimatangkan dan dibiakkan didalam TCM-199 yang diimbuhi 10% FLS, ES-D0 atau ES-D6. Hasil penelitian menunjukkan bahwa penambahan 10% ES-D0 atau ES-D6 dapat meningkatkan pematangan oosit mencapai tahap Metafase-II secara nyata masing-masing sebesar 68,7% dan 67,6% ($p<0,01$) dibandingkan FLS (32,9%). Tingkat pembuahan *in vitro* dalam medium yang diberi ES-D0 atau ES-D6 secara nyata lebih tinggi ($p<0,01$) masing-masing 65,4% dan 65,8% dibandingkan. Sedangkan pengaruh kombinasi ES-D0 dan ES-D6 masing-masing untuk pematangan dan kultur embrio menghasilkan tingkat pembelahan embrio (47,6%) lebih baik dibandingkan kombinasi ES-D6 dan ES-D6 (41,6%) ataupun ES-D0 dan ES-D0 (28,7%). Disimpulkan bahwa penambahan ES-D0 atau ES-D6 dalam media pematangan dan biakan memberikan hasil yang lebih baik untuk tingkat pematangan dan perkembangan embrio dini domba secara *in vitro*.

Kata-kata kunci: IVF, embrio domba, serum domba

INTRODUCTION

In vitro maturation or *in vitro* fertilization (IVM/IVF) is a critical step for early development of embryos. The effect of various factors including organic salt (Kim *et al.*, 1993; Pinyopummintr and Bavister, 1991), carbohydrates (Kim *et al.*, 1993; Lim *et al.*, 1994), amino acids (Takahashi and First, 1992); macromolecules and serum component (Bavister *et al.*, 1992, Pinyopummintr and Bavister, 1991), growth factor (Flood *et al.*, 1993) and vitamins (Pinyopummintr and Bavister, 1991) on the preimplantation development embryos have been investigated.

Several different media have been used for successfully maturing cow or sheep oocyte *in vitro*. For full development and subsequent fertilization the medium must contain a serum (First and Parrish, 1987). Serum as a protein supplement provided a superior environment for bovine oocyte maturation when compared with bovine serum albumin (BSA) or Fetal Calf Serum (FCS) (Lebfried *et al.*, 1986; Sanbuissho and Threlfall, 1985). Blastocyst development from bovine follicular oocyte was stimulated following IVM and IVF in medium supplemented with cow serum obtained at prooestrus (Younis *et al.*, 1989), at estrous (Schellander *et al.*, 1989) or from Day-0 and Day-7 superovulated cow (Boediono *et al.*, 1994). On the other hand, glucose inhibited the early development of embryos from a number of mammalian species (Rieger, 1992). The purpose of this study was to investigate the supplementation of ewe serum on *in vitro* ovine oocytes maturation and early embryo development.

MATERIALS AND METHODS

Oocyte collection and maturation

Ovaries were collected from the local slaughterhouse and were kept in saline (NaCl, 0.9% w/v, supplemented with penicillin 100 IUmL^{-1}; streptomycin 100 μgmL^{-1}) at 30 to 35 °C. The cumulus-oocyte complexes were aspirated from 1 to 5 mm follicles with a 10-mL syringe attached to 22-G needle. The resultant oocytes suspensions were mixed with modified-Phosphate Buffer Saline (m-PBS, Gibco, USA). After washing three times in maturation medium, oocytes were transferred to microdroplets of maturation medium (10 to 20 oocytes/100 μL droplets), covered with mineral oil (E.R. Squibb & Son, Princeton, USA). Maturation medium consisted of Tissue Culture Medium-199 (TCM-199, Gibco, USA) supplemented with 0.01 mgmL^{-1} follicle stimulating hormone (FSH, Denka Pharmaceutical, Japan), penicillin 100 IUmL^{-1} and streptomycin 100 μgmL^{-1} and 10% FLS, 10% ES-D0 or 10%ES-D6. Oocyte were maturated for 24 h at 39 °C in 5% CO_2 in air.

Sperm preparation and oocytes fertilization

Semen was collected from rams using artificial vagina. Spermatozoa were washed twice by centrifugation (500*G*, 5 min) in 2-5 mM caffeine in Brackett and Oliphant's medium (Caff-BO; Brackett and Oliphant, 1975). The resultant sperm pellet was resuspended in Caff-BO supplemented with 1% BSA (Sigma, USA) and 20 □gmL^{-1} heparin (Shimizu Pharmaceutical, Japan). Following preincubation, sperm suspension with concentration of 5 x 10^6 spermatozoa mL^{-1} were added to each 50 μL fertilization droplet containing 10 to 20 matured oocytes (washed twice in fertilization medium). After 8 h insemination oocytes with adherent cumulus cells were washed by repeated pipetting in culture medium and transferred for further development into a microdroplet, culture medium consisted of medium TCM–199 supplemented with either 10% ES-D0 and 10% ES-D6, 5 μgmL^{-1} insulin (Wako Pure Chemical Industries, Osaka, Japan), penicillin 100 IUmL^{-1} and streptomycin 100 μgmL^{-1}.

Collection of ewe serum

ES-D0 was collected from ewes in the time of oestrus, whereas ES-D6 on Day 6 after the onset of oestrus. The serum obtained was then heat-inactivated (56 °C, 30 min) before use.

Experimental design

Oocytes were matured in medium supplemented with 10% FLS, 10% ES-D0 or 10% ES-D6. Each treatment was repeated three times. Maturation and culture media were supplemented as follows: (1) Day 0 for both IVM and IVC; (2) Day 0 for IVM and Day 6 for IVC; and (3) Day 6 for both IVM and IVC. After 24 h of maturation the oocytes were fixed in acetic acid-ethanol (1:30 and stained with 1% aceto-orcein to determine the percentage developing to germinal vesicle break down (GVBD), Metaphase-I (Mt-I) and Metaphase-II (Mt-II) stages. Sixteen hours after fertilization the oocytes were fixed and stained to determine fertilization rate as indicate by the presence of two pronuclei. The embryos were observed at day-2 of fertilization to determine the cleavage rate and developing to the 2–8 cell stages.

Statistical analyses

The Chi-square test was used to test the significance of individual comparisons for the rate of maturation, fertilization and cleavage.

RESULT AND DISCUSSION

Table 1 showed that the maturation rate (Mt-II) of oocytes in media supplemented with ES-D0 or ES-D6 was higher ($p<0.01$) than that in medium supplemented with FLS (32.9%, 68.7%, and 67.6% in medium supplemented with FLS, ES-D0, and ES-D6, respectively). The present results suggested that ES-D0 or ES-D6 contained substances that enhanced the capacity to maturation of ovine oocytes *in vitro*.

Table 1. Pronuclear status of oocytes 24 h after incubation in maturation medium supplemented with different sera

Sera	No. of oocyte	Pronuclear status (%)*		
		GV/GVBD	MT-I	MT-II
FCS	76	20 (26.3)	31 (40.8)	25 (32.9)^a
ES-D0	80	9 (11.3)	16 (20.0)	55 (68.7)^b
ES-D6	74	9 (12.2)	15 (20.3)	50 (67.6)^b

*GV/GVBD = Germinal Vesicle /Germinal Vesicle Break Down; MT = Metaphase; $^{a\text{-}b}$Values within column with different supercripts are differ significantly ($p<0.01$)

Oocytes matured in medium containing ES-D0 or ES-D6 also qualitatively differed to those matured in FLS, since the cumulus of oocytes matured in FLS was only expanded in the most outer layers, as reported in our previous study (Djuwita *et al.*, 1998).

As shown in Table 2, fertilization rate in medium supplemented with ES-D0 or ES-D6 was 65.4% and 65.8% respectively, and was significantly higher ($p<0.01$) than that in medium supplemented with FLS (30.7%). Addition of serum during maturation of primary oocyte prevented zona hardening and enhanced the potential of mouse oocyte for fertilization and development. Sera might account for the variability of the bovine IVF results which may be affecting the zona penetrability (Younis *et al.*, 1989). The low fertilization rate in medium containing FLS might due to the low quality of the matured oocytes as shown in Table 1.

Table 2. Fertilization rate of oocytes after maturation in media supplemented with diffterent sera

Sera	No. of oocytes	Fertilization rate (%)
FLS	75	23 (30.7)[a]
ES-D0	78	51 (65.4)[b]
ES-D6	76	56 (65.8)[b]

[a-b]Values within column with different supercripts are differ significantly ($p<0.01$).

The proportion of embryos cleaved in media supplemented with various combinations of ES-D0 and ES-D6 which were used for IVM and IVC was shown in Table 3. Combination of ES-D0 and ES-D6 yielded higher number of embryos cleaved.

Table 3. Number of embryos cleaved in media supplemented with different sera

Sera used in		No. of	No. of embryos
IVM	IVC	ocytes	cleavad (%)
ES-D0	ES-D0	80	23 (28.7)[a]
ES-D0	ES-D6	84	40 (47.6)[b]
ES-D6	ES-D6	60	25 (41.6)[b]

[a-b]Values within colums with supercripts are differ significantly ($p<0.05$)

Results above supported the early study of Moor and Trounson (1977) who revealed that hormonal and follicular factors were found to affect *in vitro* maturation of sheep oocyte. Cleavage rate after IVF of oocytes matured in medium supplemented with FCS without hormonal addition was very low, while addition of LH and E2 increased the cleavage rate. Similar results also has been reported that *in vitro* fertilization and cleavage rate of bovine oocyte matured in medium supplemented with estrous cow serum increased significantly than those with FCS (Schellander *et al.*, 1990; Boediono *et al.*, 1994). The above results suggested that estrous or post-estrous ewes serum might contain high level of LH which was required for the oocytes *in vitro* maturation .

It has been also reported that heat-treatment of serum did not only inactivated the complement system but also altered the beneficial components of serum for embryos development (Lim *et al.*, 1994). The variability of protein sources explained the contradictory results obtained for the culture of sheep embryos (Betterbed and Wright, 1985). The composition of different lots of serum from the same species could vary widely, so did the ability of different lots of serum to support the embryos development *in vitro* (Batt *et al.*, 1993). Because of the inability to predict an appropriate protein source for the optimum embryo development *in vitro*, protein source supplement would have to be determined empirically by carefully screening. Serum factors were still required for maximal blastocyst development (Benjamin *et al.*, 1993).

As reported by Thompson *et al.*, (1992) the presence of glucose at the concentration greater than 1.5 mM inhibited the development of one- and two-cell sheep embryos culture, indicated that premature utilization of glucose was detrimental to embryo development in culture. Glucose concentration analyses done in this study were 0.678 mM and 0.609 mM in ES-D0 and ES-D6, respectively. This value indicated that medium with supplemented ES-D0 and ES-D6 contained low concentration of glucose should be beneficial to oocyte maturation and culture of ovine embryos *in vitro*.

CONCLUSION

Supplementation of ES-D0 and ES-D6 into maturation and culture media has given better results on *in vitro* maturation and early embryonic development of ovine oocytes.

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