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by H Hasbi

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Maturation of sheep oocytes with antioxidant α -tocopherol which are activated by parthenogenesis

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Abstract. This study was conducted to investigate the competence of sheep oocytes which are matured with antioxidant α -tocopherol by parthenogenesis activation. Oocytes from slaughterhouse were matured in tissue culture medium (TCM)-199 supplemented with 10% fetal bovine serum (FBS), 10 IU/ml pregnant mare serum gonadotrophin (PMSG), 10 IU/ml human chorionic gonadotrophin (hCG), 1 μ g/ml estradiol, 50 μ g/ml gentamycin, and with or without 150 μ M α -tocopherol supplementation, then were parthenogenetically activated by exposing to 5 μ M calcium ionophore for 5 min, and in medium containing 5 μ g/ml cytochalasin B for 4 h. Furthermore oocytes were cultured in TCM 199 + 10% FBS either with or without supplementation of 150 μ M α -tocopherol. Result of this experiment revealed that the percentages of activated oocytes with supplementation of 150 μ M α -tocopherol only in maturation medium (63.41%) significantly higher ($P < 0.01$) than the control group (50.00%) and the supplemented only in culture medium (51.02%). Furthermore, no significantly difference ($P > 0.05$) in the number of oocytes with 1 pronucleus (PN), 2 PN, and more than 2 PN. It is concluded that the supplementation of 150 μ M α -tocopherol on the maturation medium could also increase the percentage of activated oocytes optimally.

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1. Introduction

In vitro embryo production (IVEP) techniques have long been successfully developed in several mammal species. With the IVEP techniques, sick animals, slaughtered, died suddenly or have reproductive problems, but have high genetic material can still be saved as an effort to preserve germplasm.

Oocyte maturation is the first and most critical stage in determining the success of embryo production *in vitro*. Oocyte maturation is not only related to the occurrence of GVBD and MII but also related to the ability of oocytes to be fertilized and the early development of preimplantation embryos. The process of oocyte maturation consists of nuclear and cytoplasmic maturation. Nuclear maturation includes various chronological changes in the meiosis stage, whereas cytoplasmic maturation involves the acquisition of oocyte biological competence which includes various structural and biochemistry



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changes in the cell that for oocytes to express their developmental potential after fertilization and are able to support the formation and development of preimplantation embryos [1]. The quality of oocytes resulting from in vitro maturation can be evaluated by activation both naturally and artificially. Naturally, spermatozoa activate matured oocytes through the process of fertilization. In addition, matured oocytes can also be activated by parthenogenesis using physical and chemical stimuli [2-4].

In the maturation process, the success level is still strongly influenced by many factors. One such factor is free radicals that are produced from the metabolic processes of an organism aerobically. Reactive oxygen species can cause damage to cell structures such as mitochondria and microtubules, and also disrupt normal cell function when critical concentrations of ROS are abundant. Protection mechanisms against ROS in in vivo systems include enzymes such as superoxide dismutase (SOD), glutaredoxin, and glutathione peroxidase and catalase which break down hydrogen peroxide into water [5]. α -tocopherol or commonly called vitamin E is one of the main lipid-soluble antioxidants in animal cells [6]. The antioxidant action of vitamin E can prevent peroxidative tissue damage due to free radicals, and vitamin E is believed to be the main free radical scavenger in mammalian cell membranes [7].

Protection of oocyte quality during IVM also determines the ability of fertilization and subsequent embryo development. Therefore, it is necessary to add secondary antioxidants such as α -tocopherol to the maturation medium and culture medium to protect mitochondria and microtubules against oxidative damage so as to improve the quality and competence of oocytes in vitro.

2. Material and methods

2.1. Material

Oocytes used in this study came from sheep ovaries obtained from slaughterhouse, Citaringgul Village, Babakan Madang District, Bogor Regency. Oocyte selection is based on a homogeneous cytoplasmic and compact cumulus cells.

2.2. Methods

2.2.1. Collection and in vitro maturation of oocytes. Sheep ovaries are brought from the slaughterhouse to the laboratory in 0.9% NaCl medium plus 100 IU/ml penicillin and 100 μ g/ml streptomycin. Oocytes collection was done by slicing method in phosphate buffered saline (PBS) with 10% fetal bovine serum (FBS) (Sigma, USA). The collected oocytes were washed in the collection and maturation medium twice of each, then maturation was carried out in tissue culture medium (TCM) 199 (Sigma, USA) supplemented with 10% FBS, 10 IU/ml pregnant mare serum gonadotrophin (PMSG) (Intergonan, Intervet Deutschland GmbH), 10 IU/ml human chorionic gonadotrophin (hCG) (Chorulon, Intervet international BV Boxmeer-Holland), 1 μ g/ml estradiol (Intervet international BV Boxmeer-Holland), 50 μ g/ml gentamycin (Sigma, USA), with or without 150 μ M α -tocopherol which is the best concentration in previous study. Oocytes were matured in petri dishes with \varnothing 35 mm (Nunclon, Denmark) in 50 μ l drop for 10-15 oocytes and covered with mineral oil (Sigma, USA), in 5% CO₂ incubator 38.5 °C for 24 hours.

2.2.2. Parthenogenesis activation. After 24 hours of maturation, cumulus cells surrounding the oocyte were removed by 0.25% hyaluronidase enzyme and subsequently exposed to TCM 199 + 10% FBS medium containing 5 μ M calcium ionophore for 5 minutes [8] at room temperature, continued in a medium of 5 μ g/ml cytochalasin B for 4 hours as was done on oocyte mice [9]. After activation, oocytes were washed twice and cultured in TCM medium 199 + 10% FBS [3] with or without the addition of 20 μ M α -tocopherol. Culture was carried out on a petri dish in 50 μ l drop for 10-15 oocytes and covered with mineral oil, and placed in incubator of 5% CO₂ at 38.5 °C for 20-24 hours.

2.2.3. *Evaluation of activation rate.* Cultured oocytes were placed at a drop of 0.7% KCl on glass objects that have paraffin and Vaseline cushion, then fixed by cover glass. The preparation was entered to a fixation solution containing acetic acid and ethanol (1:3) for 3 days. One hour before staining, the preparation soaked first in an absolute ethanol solution, after that it was stained with 2% aceto-orcein for 5 minutes. The staining agent was rinsed with 25% acetic acid and further morphological observed under a contrast phase microscope (Olympus IX 70, Japan). The activation rate was observed by the number of oocytes that form the pronucleus after parthenogenesis, while the number of formed pronuclei (1 PN, 2 PN, >2 PN) was rated based on the ratio of formed PN amount to the overall activated oocytes.

3. Result and discussion

Cytoplasmic maturation can be known indirectly from the amount of blastocysts produced, the content of glutathione in oocytes, and also the percentage of pronucleus formation [10]. Furthermore, Hao et al. [11] and Ducibella et al. [12] reported that activated oocytes are characterized by a series of molecular and morphological changes such as the occurrence of cortical reactions, pronuclear formation, DNA synthesis, and cell division. In this study, observations of oocyte activation rate are based on pronucleus formation after parthenogenesis (figure 1).

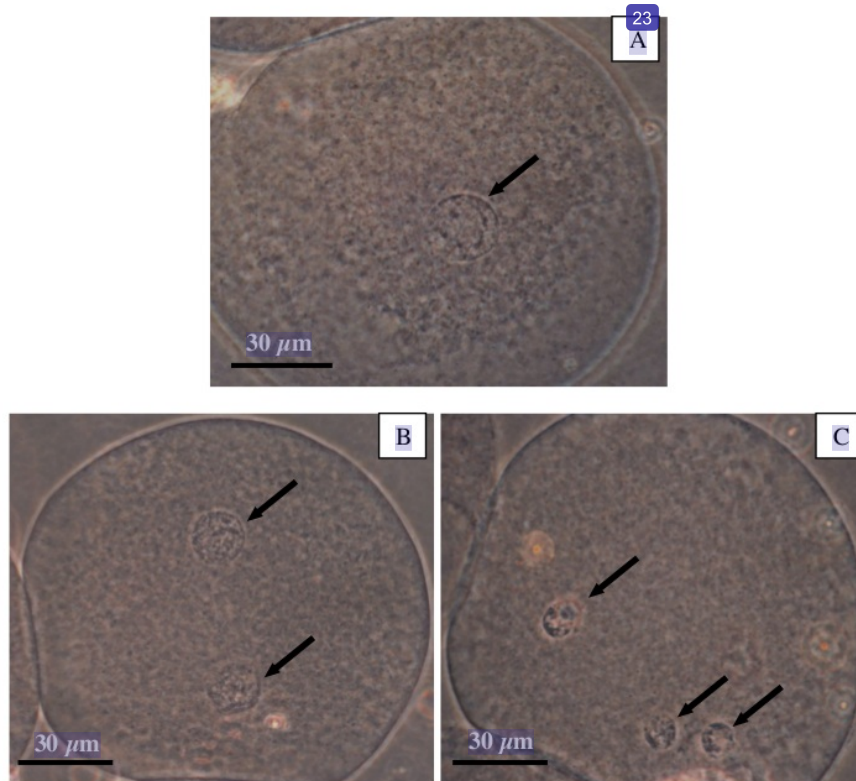


Figure 1. Pronucleus formation after parthenogenesis. A. 1 PN, B. 2 PN, and C. >2 PN, PN: pronucleus (arrow).

The level of oocyte activation in various treatment combination of 150 µM α-tocopherol supplementation in maturation and culture medium after parthenogenesis can be seen in table 1.

Table 1. Formation of pronucleus after activation in medium with the addition of α-tocopherol

Treatments	Number of Oocytes	Pronucleus Formation (%)				Activation Rate (%)
		0 PN	1 PN	2 PN	>2 PN	
A	50	25 (50.00)	12 (48.00)	7 (28.00)	6 (24.00)	25 (50.00) ^a
B	41	15 (36.59)	13 (50.00)	8 (30.77)	5 (19.23)	26 (63.41) ^b
C	49	24 (48.98)	9 (36.00)	9 (36.00)	7 (28.00)	25 (51.02) ^a
D	41	11 (26.83)	12 (40.00)	11 (36.67)	7 (23.33)	30 (73.17) ^b

PN: pronucleus, A: control (without addition of α-tocopherol both on maturation and culture medium), B: addition of α-tocopherol only on maturation medium, C: addition of α-tocopherol only on the culture medium, D: addition of α-tocopherol on maturation and culture medium. Values with different superscripts in the same column differ significantly (P<0.01).

The results of this study showed that the activation rate on the addition of α-tocopherol only to the maturation medium (treatment B) was high significant different (P<0.01) compared than control (treatment A). However, the addition of α-tocopherol only to the culture medium (treatment C) was not significantly different from that control (treatment A). While the addition of α-tocopherol to the medium of maturation and culture (treatment D) was also significantly higher compared to control (treatment A) and the addition of α-tocopherol only to the culture medium (treatment C), but it was not significantly different from the addition of α-tocopherol only to medium of maturation (treatment B). This shows that the addition of 150 µM α-tocopherol did not have a major effect when added to the culture medium, although there was a trend towards an increase in the number of activated oocytes. This proves that 150 µM α-tocopherol more optimally increases the number of activated oocytes when added to the maturation medium. The addition of α-tocopherol to the maturation medium is more optimal to increase the number of activated oocytes compared to the addition to the culture medium, this is thought to be caused because except time of oocytes exposure with α-tocopherol in culture medium relatively short, it is also caused because during maturation the oocytes undergo various changes in both the nucleus and cytoplasm, so protection is needed for the cell structure to maintain its ability. The process of changes includes RNA synthesis activity [13] which is characterized by a nuclear change from the diploten prophase meiosis I stage to metaphase II, where oocytes that reach the MII stage in the presence of polar body I are mature oocytes and are ready to be fertilized [14]. Whereas the cytoplasm experiences an increase in oocyte biological competence which includes various changes in structure and biochemical in the cell that allows the oocyte to express its developmental potential after fertilization and is able to support the formation and development of preimplantation embryos [1]. The process of cytoplasmic maturation is characterized by the accumulation of protein and mRNA, the development of calcium regulatory mechanisms, changes in the activity of maturation promoting factor (MPF) and mitogen activated protein kinase (MAPK) and redistribution of cellular organelles [15].

Roushandeh et al. [16] explained that free radicals (ROS) can cause damage to cell structure such as mitochondria and microtubules, and also disruption of normal cell function. Microtubules play a role in the reconstruction and proper placement of chromatin during the process of maturation of meiosis [1], and are also responsible for the movement and placement of pronuclei in bovine oocytes resulting from parthenogenesis [17]. Furthermore Kitagawa et al. [18] reported that α-tocopherol is able to protect cell membranes from damage caused by ROS, thus increasing the ability of embryonic development.

On the other hand, the addition of 150 µM α-tocopherol in the medium of maturation, culture and both did not show a significant effect (P>0.05) on the number of formed pronuclei. The number of formed pronuclei in oocytes resulting from parthenogenesis varies from 1 PN, 2 PN, and >2 PN. The

diploid embryo (2 PN) produced by parthenogenesis resembles that of a normal fertilized embryo and has a similar developmental ability. Liu et al [19] reported that diploid embryos from parthenogenesis had development capabilities that were almost the same as embryos from in vitro fertilization to the blastocyst stage (87±3% vs 88±4%). Diploid embryos resulting from parthenogenesis usually result from the inhibition of polar body II release by cytochalasin B [20]. Cytochalasin prevents actin polymerization and causes dramatic changes in the cytoskeleton [9], disrupting the microfilament, thereby effectively inhibiting the removal of the second polar body without any influence on the formation and movement of the pronucleus [21].

Whereas haploid embryos (1 PN) have a lower development ability compared to diploid embryos [15, 21] in rat, pig, cattle, and human [19, 11, 23]. Haploid embryos from parthenogenesis are only able to develop to the morula stage (78±5%) and very few are able to reach the blastocyst stage [19, 11] or even none at all [24]. The low development ability of haploid embryos resulting from parthenogenesis is due to the high incidence of apoptosis as indicated by the occurrence of condensation or fragmentation of the nucleus and/or DNA [19] and chromosomal abnormalities [25]. In cows, haploid embryos have few microphiles and lipid droplets so that the availability of nutrients is limited [22]. Likewise, polyploid embryos (>2 PN) have a lower development ability than diploid embryos. Polyploid embryos will usually also experience chromosomal abnormalities [25], develop abnormally and occur irregularities on chromosomes associated with tripolar spindles formation and are not recommended for transfer because there is a great possibility of chromosomal abnormalities in their development. Usually triploid embryos can develop to the stage of blastosis and implantation if transferred but generally experience an abortion or in some cases can cause trophoblastic diseases such as choriocarcinoma [18] and molar pregnancies [26].

Parthenogenesis is a biological phenomenon in which embryonic development is initiated without passing through the fertilization process by sperm. Embryo parthenogenesis only has the ability to grow until early or mid-pregnancy and in mammals there has never been a natural birth. Embryo parthenogenesis develops abnormally and development stops after organogenesis occurs in the 10th day of mice [27] and the 25th day in sheep [28]. Pregnancy failure is related to genomic imprinting, where all genetic material comes from females and there is no male contribution. This results in the development of extraembryonic tissue including the placenta [29]. Although the ability of development is low, embryo parthenogenesis can be used for stem cells, therapeutic cloning [29, 30], and is also important for nuclear transfer applications [31].

In this study, the matured oocytes were activated with 5 µM calcium ionophore for 5 minutes combined with 5 µg/ml cytochalasin B for 4 hours. According to Heytens et al. [32] that activation can only be done on mature oocytes or those in the MII stage which are characterized by high levels of the cyclin B/cdc1 complex and also the level of maturation promoting factor (MPF). During MII arrest, cytochalasin B prevents MPF destruction by maintaining anaphase-promoting complex (APC) in an inactive state.

Oocyte activation with calcium ionophore for 5 minutes can stimulate release of intracellular Ca²⁺ from storage in endoplasmic reticulum and also helps influx of extracellular Ca²⁺ ions [33, 24]. Increased intracellular Ca²⁺ is caused by the activation of diacylglycerol (DG) and inositol 3-phosphate (IP3) induced by G-protein through phospholipase C (PLC). Increased intracellular Ca²⁺ then stimulates increased protein kinase C (PKC) activity in mouse oocytes which in turn induces exocytosis of cortex grains [34]. In addition, increasing of Ca²⁺ ions causes activation of calmodulin-dependent protein kinase II which functions to stimulate damage to cyclin-B, and inactivation of p35cdc2 kinase. As a result, MPF activity decreases, and then meiotic division can restart [35, 3]. This study used a combination of cytochalasin B because in a preliminary study the level of oocytes activation with single calcium ionophore was very low or did not exist at all (unpublished data).

4. Conclusion

Addition of 150 µM α-tocopherol to the maturation medium can increase the number of activated oocytes.

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