Nuclear Maturation Competence and Pronuclear Formation in Ovine Oocytes Supplemented with *L-ergothioneine* in Maturation Medium

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ABSTRAK

Agustin RP, Setiadi MA, Supriatna I. 2023. Kompetensi pematangan inti dan pembentukan pronukleus oosit domba yang disuplementasi L-ergothioneine pada medium maturasi. JITV 28(1):11-21. DOI: http://dx.doi.org/10.14334/jitv.v28.i1.3120.

Tujuan dari penelitian ini yaitu untuk mengetahui efikasi penambahan L-ergothioneine (LE) pada medium maturasi terhadap kompetensi pematangan inti dan pembentukan pronukleus pada oosit domba *in vitro*. Penelitian tahap 1 dilakukan untuk mengevaluasi tingkat maturasi inti oosit. Oosit dibagi menjadi empat kelompok dan dimatangkan selama 24 jam dalam medium maturasi yang disuplementasi 0 mM (kontrol), 10 mM, 15 mM, dan 20 mM LE. Penelitian tahap 2 dilakukan untuk mengevaluasi tingkat fertilisasi oosit. Oosit dibagi menjadi dua kelompok dan dimatangkan selama 24 jam dalam medium maturasi yang disuplementasi 0 mM LE (kontrol) dan 10 mM LE (dosis optimal dari penelitian tahap 1) kemudian diko-inkubasi dengan sperma dalam medium fertilisasi selama 12 jam. Hasil penelitian tahap 1 menunjukkan bahwa tingkat maturasi inti oosit dengan suplementasi LE 10 mM (88,74±1,79%) dan 15 mM (87,52±2,30%) pada medium maturasi berbeda nyata (P<0,05) dibandingkan dengan kelompok kontrol (78,55±1,78%) dan LE 20 mM (77,37±1,21%). Hasil penelitian tahap 2 menunjukkan bahwa tingkat fertilisasi oosit dengan suplementasi LE 10 mM pada medium maturasi menunjukkan persentase pembentukan dua pronukleus secara signifikan lebih tinggi (75,55±3,47%) daripada kelompok kontrol (63,78±3,49%). Kesimpulannya, suplementasi LE 10 mM pada medium maturasi mampu meningkatkan kompetensi pematangan inti oosit dan pembentukan pronukleus.

Kata Kunci: Domba, Fertilisasi *In vitro*, Maturasi *In vitro*, *L-ergothineine*, Oosit

ABSTRACT

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This study aimed to determine the efficacy of supplementing maturation medium with L-ergothioneine on nuclear maturation competence and pronuclei formation of ovine oocytes *in vitro*. In the first study, oocytes were divided into four groups and matured for 24 hours in a maturation medium supplemented with 0 mM (control), 10 mM, 15 mM, and 20 mM LE. In the second study, oocytes were divided into two groups and matured for 24 hours in a maturation medium supplemented with 0 mM LE (control) and 10 mM LE (optimal dose from the first study), then co-incubated with sperm in a fertilization medium for 12 hours. The results of the first study showed that the maturation rate of oocytes enriched with LE 10 mM (88.74±1.79%) and 15 mM (87.52±2.30%) in maturation medium was significantly different (P<0.05) compared to the control (78.55±1.78%) and LE 20 mM (77.37±1.21%). Furthermore, the results of the second study revealed that the fertilization rate of oocytes with 10 mM LE (75.55±3.47%) supplementation in maturation medium had a significantly higher percentage of two pronuclei formation than control (63.78±3.49%). In conclusion, supplementing 10 mM LE in a maturation medium improved oocyte nuclear maturation competence and fertilization rates.

Key Words: *In vitro* Maturation, *In vitro* Fertilization, L-ergothioneine, Oocyte, Ovine

INTRODUCTION

Some research has been conducted to develop genetic improvements and accelerate population growth in small ruminants such as sheep through reproductive technology. Sheep are domesticated, and ruminant mammals commonly kept as livestock and have tremendous potential for genetic improvement in meat and milk production. The genetic material of these animals needs to be improved to increase fertility and productivity in small animal farms. An assisted reproductive technology known as *in vitro* embryo production (IVEP) consists of direct follicle retrieval of immature oocytes for *in vitro* maturation (IVM), *in vitro* fertilization (IVF), i.e., co-incubation of *in vitro*-matured oocytes with capacitated sperm, and *in vitro* culture

(IVC) of zygotes to the blastocyst stage (Mondal et al. 2019; Soto-Heras & Paramio 2020). It is a valuable technique for understanding early embryonic development, with applications ranging from treating reproductive disorders to preserving gametes from animals with high genetic value (Rizos et al. 2008; Zhu et al. 2018). IVEP enables the birth of offspring from infertile, prepubertal, pregnant, lactating, and even dead or slaughtered animals. Moreover, this technology can be used as an action plan to save some endangered species (Paramio & Izquierdo 2016).

Usually, the oocytes for IVEP are obtained from the ovarian waste of slaughterhouses. Mammalian ovaries contain many immature oocytes that can be used for reproductive technology, such as IVF (El-raey & Nagai 2014). Nevertheless, *in vitro*-matured oocytes usually have less developmental capacity than *in vivo*-matured oocytes due to abnormalities that occur during cytoplasmic maturation (Quan et al. 2017). Meanwhile, oocyte quality after *in vitro* maturation is essential in supporting fertilization and embryo development (Gulo et al. 2020). It is generally known that *in vitro* oocyte maturation is the crucial process in the whole procedure of IVEP since immature oocytes have to undergo synchronized nuclear and cytoplasmic maturation to acquire fertilization and developmental competence (Paramio & Izquierdo 2014; Soto-Heras & Paramio 2020).

Furthermore, compared to the developability between in vivo and *in vitro* generated oocytes, improper *in vitro* maturation is the leading cause of polyspermy after IVF (Zhu et al. 2018). During IVM, significant changes occur in the cytoplasm and nucleus of the oocytes. Subsequently, oocytes expel the first polar body and enter metaphase II (Zhu et al. 2018). These changes are crucial for promoting successful fertilization and embryo development (Quan et al. 2017). Therefore, IVM is essential for producing high-quality oocytes for IVF and determines the potential developmental ability of the oocyte (Zhu et al. 2018).

Shortly after the animal is slaughtered, most organs undergo ischemia, which reduces oxygen and energy supply, forcing a switch to anaerobic metabolism to maintain ATP and increase ROS production (Soto-Heras & Paramio 2020). Oxygen tension in the oviduct is 3–4 times lower than in typical IVM culture (3–9% vs. 20% respectively), and such high oxygen tension is thought to cause intolerable creation of ROS, including hydrogen peroxide (H_2O_2), hydroxyl radicals, and peroxyl radicals (Rocha-Frigoni et al. 2016). When ROS production exceeds a cell's antioxidant defense mechanisms, it leads to oxidative stress and disrupts the redox system's processes (reduction/oxidation). Furthermore, these radicals can interact with some molecules to accept electrons and stabilize due to their high reactivity and instability. These interactions, in turn, can trigger a series of interrelated events that can ultimately lead to cellular damage, such as the oxidation of amino acids and nucleic acids and lipid peroxidation (especially of membrane phospholipids). In addition, excessive amounts of ROS cause DNA damage by rupturing mitochondrial membranes, releasing cytochrome C and activating caspase cascades. These processes lead to apoptosis and unsuccessful IVEP (Rocha-Frigoni et al. 2016). Several studies have been performed to control ROS levels during *in vitro* systems. One of them is to change the composition of the maturation medium by adding an antioxidant to increase intracellular antioxidant capacity and neutralize free radicals (Gulo et al. 2020).

The natural amino acid L-ergothioneine (LE) is a potent OH^- scavenger and an inhibitor of iron- or copper ion-dependent OH^- production from H_2O_2 (Zullo et al. 2016). LE is a histidine-derived amino acid, having a sulfur atom in the second position of the imidazole ring. Mammals cannot produce ergothioneine; only several organisms synthesize this compound, notably filamentous fungi and *actinobacteria* (Bazela et al. 2014; Mishra et al. 2018). For this reason, mammals obtain ergothioneine exclusively from their feed, which includes oats, meat, corn, and mushrooms. Ergothioneine has been shown to neutralize singlet oxygen, hydroxyl radicals, hypochlorous acid, and peroxyl radicals and prevent the nitration of proteins and DNA caused by peroxynitrite (Zullo et al. 2016; Mishra et al. 2018). Compared to other traditionally known antioxidants such as glutathione (GSH), uric acid, and Trolox, LE has recently been shown to have the highest antioxidant activity and to remove the most active free radicals (Zullo et al. 2016). Notably, the two functional groups in LE significantly impacting *in vitro* oocyte maturation are the quaternary ammonium group N-$(CH_3)_3$ and L-thiol (SH) group. The quaternary ammonium group increases the ATP level in cells by transporting fatty acids into the mitochondria to support the oocyte's nuclear maturation (Bazela et al. 2014).

Meanwhile, the L-thiol group increase glutathione level to prevent oxidative stress and support pronuclear formation after *in vitro* fertilization (Bazela et al. 2014; Mishra et al. 2018). In addition, recent research suggests that altering the maturation medium can significantly affect the developmental capability of oocytes *in vitro* (Quan et al. 2017). Therefore, to determine the effects of L-ergothioneine supplementation in the maturation medium, this study examined the enhancement of nuclear maturation competence and fertilization ability characterized by forming two pronuclei.

MATERIALS AND METHODS

This study was conducted at the *In vitro* Fertilization (IVF) Laboratory, Division of Reproduction and Obstetrics, Department of Veterinary Clinic,

Reproduction and Pathology, School of Veterinary Medicine and Biomedical Science, IPB University, Bogor. The bioethical committee of IPB University approved all experimental procedures in this study (No. 218 – 2021 IPB).

Research design

This research was divided into two experiments. In the first experiment, we evaluated the efficacy of L-ergothioneine (LE) (Sigma-Aldrich, E7521) supplementation in a maturation medium on the nuclear maturation competence of ovine oocytes. The selected oocytes were collected, divided into four groups, and then matured in a maturation medium supplemented with LE 0 mM (control), 10 mM, 15 mM, and 20 mM for 24 hours in a CO_2 5% incubator at 38.5°C. Afterward, the oocyte's nuclear status was observed under a phase contrast microscope (Olympus IX 70, Japan). The doses used in this research referred to the previous study by ÖZtürkler et al. (2010) and Mishra et al. (2018). The optimum dose obtained from experiment 1 was then used in experiment 2. Ten replications were carried out in the first experiment, and the experimental design used in experiment 1 was a completely randomized design (CRD).

In the second experiment, we assessed the potency of LE supplementation in a maturation medium on the pronucleus formation of ovine oocytes after *in vitro* fertilization (IVF). Oocytes were collected, selected, and then *in vitro*-matured in a maturation medium supplemented with LE 0 mM (control) and 10 mM (the best dose obtained from the first experiment). Oocytes were then fertilized *in vitro* for 12-13 hours in a CO_2 5% incubator at 38.5°C. Pronucleus formation was observed under a phase contrast microscope (Olympus IX 70, Japan). Eleven replications were carried out in the second experiment, and the experimental design used in experiment 2 was a completely randomized design (CRD).

Oocytes collection

In less than five hours, ovaries from sheep were collected from a slaughterhouse and transferred to the laboratory using a transport medium at 35-37 ˚C. Streptomycin sulfate (MEIJI, Indonesia) 0.1 mg/mL and penicillin-G (MEIJI, Indonesia) 100 IU/mL are added to 0.9% sodium chloride (WIDATRA, Indonesia), which serves as the transport medium. The method of oocytes collection (cumulus-oocyte complexes or COCs) refers to Bhakty et al. (2021), which is performed by slicing and flushing on a collection medium containing Phosphate-Buffered Saline (PBS) enriched with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, F7524), 100 IU/mL penicillin-G (Sigma-Aldrich, P4687) and 0.1 mg/mL

streptomycin sulfate (Sigma-Aldrich, S9137). Oocytes were collected in a Petri dish containing 2 mL of collection medium and selected under a stereo-microscope according to the evaluation criteria. Oocytes with at least three layers of compact cumulus cells and homogeneous cytoplasm should be used for maturation.

Oocyte maturation *in vitro* (IVM)

The maturation medium was equilibrated for two hours in a CO_2 incubator. Then, the selected COCs were washed three times with a maturation medium before the maturation process. The maturation medium contained Tissue Culture Medium 199 (TCM-199) (Sigma-Aldrich, M4530) supplemented with 10 % FBS, 0.1 IU/mL Follicle Stimulating Hormone (FSH) (Vetoquinol N-A inc, Canada), 10 IU/mL Human Chorionic Gonadotrophin (hCG) (ChorulonTM, MSD Animal Health), and 50 g/mL gentamycin (Sigma-Aldrich, G1264). COCs were then placed in 100 µL droplets (10-15 oocytes) of maturation medium in Petri dishes covered with mineral oil (Sigma-Aldrich, M5310) and incubated in a 5% CO2 incubator at 38.5˚C for 24 hours.

Evaluation of oocyte maturation

After 24 hours of incubation, COCs were repeatedly pipetted into a PBS medium containing 0.25% hyaluronidase (Sigma-Aldrich, H3506) to remove cumulus cells and obtain denuded oocytes. The denuded oocytes were washed three times in collection medium, then placed in a drop of KCl on a glass slide padded with paraffin and vaseline (1:9) on the edges and covered with a coverslip. A fixative solution with a 3:1 ratio of absolute ethanol and acetic acid was used to fix the slide for 48–72 hours. The slide was then stained with 2% aceto-orcein (Sigma-Aldrich, St. Louis, O7380, USA) for 2-3 minutes, rinsed with acetoglycerol decolorizer (consisting of acetic acid, glycerol, and distilled water), and examined with a phase-contrast microscope (Olympus IX 70, Japan). Depending on the stages of nuclear maturation, oocytes were classified as a germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase and telophase I (A/T), and metaphase II (MII). Oocytes that reached the M II stage were indicative of mature oocytes.

In vitro fertilization (IVF) of oocytes

Matured oocytes were fertilized using frozen semen from one ram obtained from an artificial insemination center in Lembang, Bandung, West Java, Indonesia. The frozen semen was thawed in water at 37˚C bath for 30 seconds and then centrifuged at 500 x g at 28˚C for five minutes in PBS media enriched with 0.2% Bovine Serum

Albumin (BSA) (Sigma-Aldrich, A7030). The supernatant was discarded; approximately 200 µL remained homogenized with the pellet. Sperm concentration was calculated to determine the volume of IVF medium and sperm to obtain a final sperm concentration of 5×10^6 sperm/mL (Bhakty et al. 2021). In addition, 0.1 IU/mL heparin (Sigma-Aldrich, H5515) and 10 µM hypotaurine (Sigma-Aldrich, H1384) was added to the sperm suspension and then kept in a CO_2 incubator until the matured oocytes were washed three times in IVF medium. Finally, droplets containing 100 µL of processed sperm were co-incubated with matured oocytes covered with mineral oil in a 5% CO_2 incubator at 38.5˚C for 12-13 hours.

Evaluation of oocyte fertilization

The formation of the pronucleus was used to evaluate the fertilization rate. Normal fertilization was distinguished by forming two pronuclei (2PN) after staining with 2% aceto-orcein, while polyspermy refers to oocytes containing more than two pronuclei (Catalá et al. 2012).

Statistical Analysis

Nuclear maturation rate data were expressed as percentages and statistically analyzed using Analysis of Variance (ANOVA) with a significance level of 95%. Duncan's Multiple Range Test (DMRT) is used when there is a difference between the treatments. In addition, data on fertilization rates were also presented as percentages and statistically analyzed using an independent t-test. Data were processed using SPSS version 27 software.

RESULTS AND DISCUSSION

Nuclear maturation competence of ovine oocytes supplemented with LE in the maturation medium

In this experiment, comparisons were made between oocytes matured in four doses of LE. Regarding the assessment of nuclear maturation (germinal vesicle, germinal vesicle breakdown, metaphase I, anaphase/telophase I, and metaphase II), after 24 hours of incubation, the percentage of oocyte maturation reaching metaphase II (MII) between experimental groups are shown in Table 1. In addition, the microscopic representation of the stages of nuclear maturation of the ovine oocyte in this study is shown in Figure 1.

The data in Table 1 show that the percentage of oocytes that could reach the MII stage ranged from 77.37 to 88.74%. The highest percentage of oocytes that

reached the MII stage was shown in group LE 10 mM group (88.74±1.79), while the lowest percentage was shown in LE 20 mM group (77.37±1.21). In this study, the addition of LE 10 mM in IVM medium was significantly different (P<0.05) from control and LE 20 mM but showed no significant difference (P>0.05) if compared with LE 15 mM (87.52±2.30). At the same time, the percentage of oocytes reaching MII in the LE 20 mM group showed a downward trend (77.37±1.21%) compared to the other groups. Therefore, supplementation of 10 mM to 15 mM LE in the maturation medium most likely promoted the nuclear maturation of sheep oocytes *in vitro*. In comparison, supplementation of 20 mM LE demonstrated a decrease. Therefore, instead of 15 mM LE, supplementation with 10 mM LE in IVM medium was chosen for further study (*in vitro* fertilization) since the smallest dose already gave the best result.

The chromatin configuration allows for evaluating and classifying the oocyte nuclear maturation status into various stages. A large nucleus with an intact envelope and filamentous chromatin characterizes oocytes at the germinal vesicle (GV) stage. In contrast, oocytes with slightly condensed or clumped chromatin without a visible nuclear membrane are classified as germinal vesicle breakdown (GVBD). After GVBD, oocytes enter the metaphase I (MI) stage, characterized when chromosomes become strongly condensed and positioned in the ooplasm on the peripheral of the metaphase plate with no polar body (PB). Furthermore, the complete sets of chromosomes are pulled to each opposite pole or toward the centrioles. A new nuclear membrane is formed around the clumps of chromosomes (anaphase-telophase I). This phase occurs briefly, and it is rarely found in this study. Later, the existence of a metaphase plate with two chromatin masses positioned in the ooplasm's periphery followed by the ejection of the first polar body (represented by a dense chromosome set), categorized as a matured metaphase II (MII) oocyte. Those with altered or absent nuclear structures were considered degenerated (Shirazi et al. 2010; Landim-Alvarenga and Maziero 2014). Immature oocytes (GV-A/TI) are incapable of fertilization and embryo development. Hence immature oocytes must undergo proper oocyte nuclear (MII) and cytoplasmic maturation to obtain their developability (Zhu et al. 2018).

The present study aimed to attenuate OS during *in vitro* maturation in an ovine model by LE supplementation (10 mM, 15 mM, and 20 mM) to improve oocyte quality and successful *in vitro* fertilization. In this study, different concentrations of LE during IVM showed that supplementation of LE at doses of 10 and 15 mM significantly (P<0.05) improved the nuclear maturation of sheep oocytes compared with other concentrations (0 mM and 20 mM). This finding agrees with ÖZtürkler et al. (2010), who found that the percentage of oocyte maturation was significantly higher

Table 1. Nuclear maturation rate of ovine oocytes supplemented with LE in maturation medium*

Treatment	Number of oocytes	Status of nuclear maturation (% mean±SEM)					D
		GV	GVBD	MI	A/T	MII	
LE 0 mM	175	0.00±0.00[a]	4.65±1.15[ab]	15.60±2.03[b]	1.20±0.84[a]	78.55±1.78[a]	0.00±0.00[a]
LE 10 mM	177	0.00±0.00[a]	0.50±0.50[a]	10.76±1.74[ab]	0.00±0.00[a]	88.74±1.79[b]	0.00±0.00[a]
LE 15 mM	172	0.67±0.67[a]	2.10±1.15[a]	8.55±1.68[a]	1.17±0.79[a]	87.52±2.30[b]	0.00±0.00[a]
LE 20 mM	179	0.71±0.71[a]	8.14±2.32[b]	12.96±2.31[ab]	0.82±0.54[a]	77.37±1.21[a]	0.90±0.60[a]

LE= L-ergothioneine; GV= germinal vesicle; GVBD= germinal vesicle breakdown; MI= metaphase I; A/T= anaphase-telophase I; MII= metaphase II; D= degeneration. [a, b,] different superscripts in the same column showed significant differences (P<0.05). *This study was carried out with ten replications
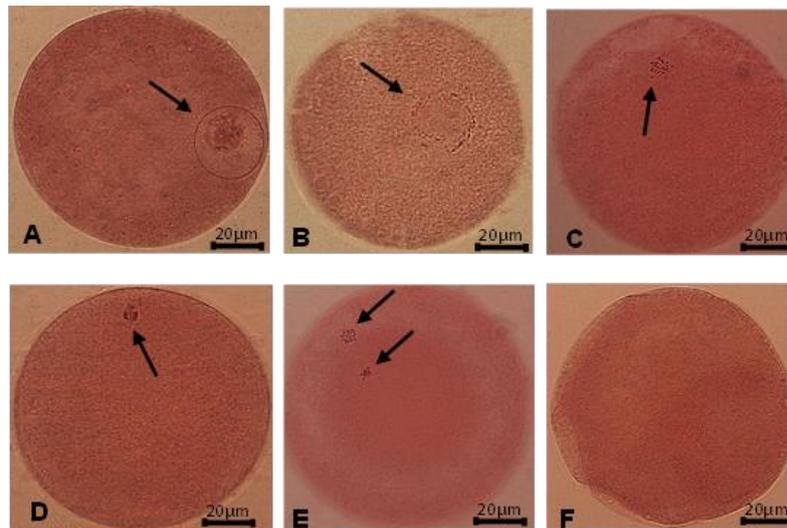

Figure 1. Nuclear maturation status of oocyte using 2% aceto-orcein staining. A= germinal vesicle; B= germinal vesicle breakdown; C= metaphase I; D= anaphase-telophase I; E. metaphase II; F= degeneration. Images were taken using a phase-contrast microscope. Arrows indicate the oocyte's nuclear maturation status.

in the LE 10 mM group compared with other groups (control and L-ascorbic acid 10 mM). The possible explanation is the role of LE as a direct ROS scavenger (Franzoni et al. 2006) and its ability to prevent DNA damage and apoptosis (Paul & Snyder 2010). Secondly, proteins involved in antioxidant cellular pathways, such as superoxide dismutase (SOD), GSH reductase, and catalase, are upregulated by LE (Li et al. 2014; Kerley et al. 2018). In contrast, Mishra et al. (2018) showed that the maturation rate was not affected by LE supplementation (5 mM and 10 mM) in the maturation medium (82.96-84.11%). However, supplementation of LE (10 mM) during the maturation period showed a significantly higher blastocyst rate than the other groups with lower concentrations (0 mM and 5 mM).

As we know, LE is a naturally occurring, water-soluble amino acid found mainly in fungi and derived from histidine, which mammals ingest exclusively through their diet (Bazela et al. 2014; Mishra et al. 2018). LE has two functional groups: the quaternary ammonium group N–$(CH_3)_3$ and the L-thiol (SH) group. The quaternary group of LE transports fatty acids to mitochondria and increases cellular ATP levels. The thiol group suggests an antioxidant function (Bazela et al. 2014). Amino acids play an essential role in cellular processes serving as the basis for producing proteins, energy, organic osmolytes, and intracellular buffer (Sutton et al. 2003). Amino acids added to the maturation medium serve as a vital energy source for both cumulus cells and oocytes to promote nuclear maturation (Songsasen 2012; Bahrami & Cottee 2022). In addition, amino acids act as glutathione (GSH) precursors and increase GSH levels to prevent oxidative stress in oocytes (Bahrami & Cottee 2022), and apparently, ATP is necessary for GSH production (Xie et al. 2016).

Oocyte maturation is highly dependent on energy metabolism because all the dynamic processes that occur during the development of the nucleus and cytoplasm require a lot of energy from various substrates such as carbohydrates, amino acids, and lipids (Songsasen 2012; Xie et al. 2016). It is well known that numerous organelles, such as the mitochondria, endoplasmic reticulum, and Golgi complexes, increase in number during oocyte growth, demonstrating the importance of protein synthesis and energy production in this process

(Songsasen 2012; Warzych & Lipinska 2020). Most of the cellular energy (about 90%) is generated by mitochondria through oxidative phosphorylation, which can cause a significant amount of ROS (Bazela et al. 2014). Meanwhile, increased oocyte ROS levels can disrupt the maturation-promoting factor and trigger programmed cell death (Rakha et al. 2022). The main cause of triggering apoptosis is often excessive ROS production and mitochondrial damage. ROS can cause the oxidation of mitochondrial pores due to the disruption of mitochondrial membrane potential, triggering the intrinsic apoptotic process (Simon 2000; García-Martínez et al. 2020). In addition, mitochondria possess DNA (mtDNA) that is distinct from nuclear DNA, and mtDNA is more prone to breakage because it lacks histones for its protection. In addition, mitochondria lack the DNA repair mechanisms of the nucleus (Paul & Snyder 2010). Although mitochondria have antioxidant defense mechanisms that can detoxify the amount of ROS produced, some ROS manage to evade these processes and damage proteins, lipids, and mtDNA (Bazela et al. 2014). The primary ergothioneine transporter is OCTN1 (organic cation transporter novel-type 1), encoded by the gene slc22a4, which has a beneficial role in LE activity (Cheah & Halliwell 2021). OCTN1 is abundantly expressed in mitochondria (Lamhonwah & Tein 2006; Paul & Snyder 2010). Therefore, supplementing the maturation medium with LE could protect mtDNA from OS and improve mitochondrial quality, which could support cytoplasmic maturation of the oocyte, as mitochondrial distribution is a dynamic process and one of the essential factors in oocyte quality (García-Martínez et al. 2020). Moreover, increasing the number and quality of mitochondria positively affects both the maturation rate and the formation of two pronuclei (Opiela & Kitska-Ksiizkiewicz 2013).

The L-thiol (SH) group of LE implies an antioxidant role and cytoprotective potentials against various cell-stressing factors (Bazela et al. 2014). Thus, LE acts as an antioxidant and effective free radical scavenger. Mishra et al. (2018) reported that LE supplementation during IVM can increase the activity of the enzyme GSH peroxidase, leading to a significant upsurge of GSH in oocytes and, consequently decrease in intercellular ROS, which prevents apoptosis in cells. Furthermore, the free radical scavenging properties of LE neutralize the production of ROS by oocytes throughout the maturation period and protect cell organelles such as mitochondria, which are the primary source of ROS, enabling oocytes to reach the next developmental stage (Bazela et al. 2014). Accordingly, LE acts as an antioxidant and an anti-apoptotic substance. From this study, adding 10-15 mM LE during IVM can promote the successful nuclear maturation of the ovine oocyte, making the oocyte suitable for fertilization by sperm and allowing the formation of two pronuclei achievable. This study

suggested that under *in vitro* conditions, the components of the maturation medium are essential in supporting oocyte maturation to the M-II stage, with antioxidants playing an important role because oocytes are highly susceptible to oxidative stress (Rakha et al., 2022). Therefore, enrichment of the maturation medium with antioxidants, co-enzymes, amino acids, or vitamins can enhance the success of oocyte maturation (Rao et al. 2002; ÖZtürkler et al. 2010). Moreover, the selection of antioxidants and their concentrations are essential. In addition, better information about antioxidants and their mechanisms is needed to support the production of high-quality oocytes and successful maturation and fertilization *in vitro*.

In this study, supplementation of 20 mM LE in the maturation medium shows a decrease in the percentage of oocyte maturation compared to lower doses (LE 10 and 15 mM). Most likely, it is because LE is primarily stored in the body with minimal metabolism (Gründemann 2012). Consequently, a change in the balance between antioxidants and prooxidants may lead to toxicity reported at greater doses. In addition, it is well known that too many antioxidants in the media can cause adverse effects by altering the redox state and weakening the disulfide bonds in proteins, leading to denaturation or inactivation (Guérin et al. 2001; Zullo et al. 2016).

Fertilization capability of ovine oocytes supplemented with LE in the maturation medium

The evaluation of pronucleus formation after fertilization of *in vitro*-matured oocytes by frozen sperm with a concentration of 5×10^6 sperm/mL and incubated for 12-13 h showed in Table 2. Fertilized oocytes are characterized by the formation of pronucleus (PN). Regular fertilization is characterized by two pronuclei (2PN) consisting of one female and one male pronucleus. Oocytes with more than two pronuclei (>2PN) were categorized as polyspermy (Catalá et al. 2012). A microscopic image of the formation of the ovine oocyte in this study is shown in Figure 2.

Table 2 shows that there was a significant difference (P<0.05) in total fertilization, formation of two pronuclei (2PN), and one pronucleus (1PN) between the control group and LE 10 mM (optimum dose obtained from experiment 1). In contrast, there was no significant difference (P>0.05) in the formation of more than two pronuclei (polyspermy) between the two groups. The supplementation of LE 10 mM during maturation resulted in a significantly (P<0.05) higher percentage of total fertilization (83.91±2.04%) and the formation of 2PN (75.55±3.47%) compared with the control group (74.07±1.06% and 63.78±3.49%, respectively). At the same time, the percentage of 1PN was higher in the control group (21.62±2.04%) than in the LE 10 mM group (11.35±1.54%).

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Table 2. Fertilization rate of ovine oocytes supplemented with LE in maturation medium*

Treatments	Number of oocytes	Total Fertilization (%mean±SEM)	Pronucleus formation (%mean±SEM)			D
			2PN (Normal)	>2PN (Polyspermy)	1PN	
LE 0 mM	152	74.07±1.06[a]	63.78±3.49[a]	10.29±3.91[a]	21.62±2.04[a]	4.32±1.56[a]
LE 10 mM	150	83.91±2.04[b]	75.55±3.47[b]	8.36±2.58[a]	11.35±1.54[b]	4.73±1.46[a]

LE= L-ergothioneine; 1PN= one pronucleus; 2PN=two pronuclei; >2PN= more than two pronuclei; D= degeneration. [a,b] different superscripts in the same column showed significant differences ($P<0.05$). *This study was carried out with eleven replications
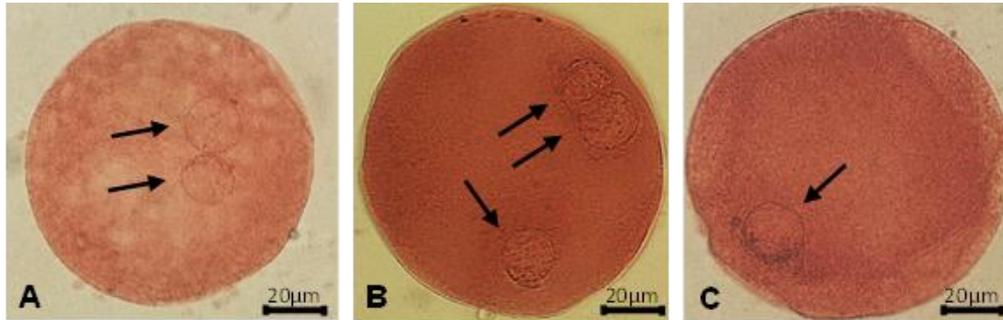

Figure 2. The pronucleus formation of ovine oocytes after *in vitro* fertilization. A. two pronuclei; B. more than two pronuclei (polyspermy); C. one pronucleus. Arrows indicate pronucleus.

In this study, the supplementation of 10 mM LE to the maturation medium significantly increased the formation of two pronuclei after IVF compared to the control group (0 mM LE). The plausible explanation is that LE increases the GSH content in oocytes during IVM, improving oocyte quality and supporting their subsequent development (Hidaka et al. 2018; Anchordoquy et al. 2019). An essential component of the successful formation of two pronuclei is proper cytoplasmic maturation supported by the synthesis of intracellular GSH during IVM (García-Martínez et al. 2020) as incomplete or abnormal oocyte cytoplasmic competence causes inhibition of sperm head decondensation (Catalá et al. 2012). Moreover, the formation of the male pronucleus after fertilization depends on the adequate amount of GSH reservoirs acquired during the maturation period since GSH plays an essential role in the development, upkeep, and defense of the meiotic spindle towards oxidative stress (García-Martínez et al. 2020). In addition, a study by Mishra et al. (2018) stated that LE supplementation in a maturation medium could support cytoplasmic oocyte maturation by increasing intracellular GSH. Increased intracellular GSH in matured oocytes can significantly increase the percentage of male pronucleus (MPN) formation after IVF (Urdaneta et al. 2004; Anchordoquy et al. 2019). In addition to oocyte activation, GSH is involved in sperm decondensation and conversion of the sperm head, which fertilizes the oocyte into the male pronucleus (De Matos et al. 2002; Rodríguez-Gonzáles et al. 2003). GSH content has been shown to increase in vivo as the oocyte approaches the time of ovulation, protecting it at later stages of fertilization (Anchordoquy et al. 2019). In addition, GSH in the intracytoplasmic protects cells from oxidative damage, and high intracytoplasmic GSH in oocytes after IVM remains constant after fertilization and disappears at the 6 to 8-cell embryonic stage (Urdaneta et al. 2004).

The incidence of polyspermy in our study ranged between 8.36–10.29%. In comparison, the polyspermy rate in the survey by Gulo et al. (2020) showed a range between 8.5–14.3%, while the range in the study by Anzalone et al. (2021) was 6.5–17.8%. This finding indicated that the polyspermy occurrence in this study is still in the normal range. The prevalence of polyspermic penetration in ovine oocytes has been known as a significant problem in IVF that also has been observed in certain other species. It results in early embryonic lethality or embryo developmental arrest, which reduces the fertilization rate of oocytes (Bragança et al. 2021).

Furthermore, Mondéjar et al. (2013) stated that the leading cause of polyspermy is mainly due to deficient or belated zona pellucida hardening, and it appears to be connected to deficiencies in oocyte cytoplasmic maturation, which leads to difficulties in cortical granules (CGs) exocytosis (Batista et al. 2016; Evans 2020; Rojas et al. 2021). Conversely, Wang et al. (2003) mentioned that immature oocytes displayed higher percentages of polyspermic penetration after IVF in most mammals than mature oocytes. Immature oocytes cannot prevent polyspermy due to the CGs' failure to release after sperm entry, resulting from incomplete

oocyte activation (Wang et al. 2003). Oocyte activation is a coordinated and orchestrated process with predetermined outcomes consisting of meiosis II (MII) resumption, the extrusion of second polar body (2ndPB), cortical granule exocytosis which alters zona pellucida to prevent polyspermy and cytoskeletal reorganizations (Swain and Pool 2008; Kashir et al. 2022). Additionally, Wang et al. (2003) explained that immature oocytes have less calcium oscillation in response to sperm penetration, calcium injection, and injection of inositol 1,4,5-trisphosphate (IP$_3$) since immature oocytes contain fewer IP$_3$ receptors in their cortex, which reduces their capacity to respond to activators. Furthermore, the amount of cortical endoplasmic reticulum responsible for calcium reserves is less in immature oocytes (Wang et al. 2003).

Cytoplasmic maturation is the fundamental process that has an impact on oocyte quality, involving the development of organelles, mainly cortical granules, Golgi complexes, endoplasmic reticulum (ER), and mitochondria, as well as the accumulation of mRNA, proteins, substrates, and nutrients (Sun et al. 2022). This circumstance demonstrated that reorganizing cytoplasmic organelles such as CGs is essential to produce more competent oocytes, which are required to induce cortical and zona reactions (Slavík et al. 2005). A proper CGs migration to the ooplasm is compulsory for upcoming exocytosis after sperm penetration, and this migration occurs during the final oocyte maturation (Wang et al. 2003). Contrarily, insufficient cytoplasmic maturation leads to improper fertilization due to abnormal signaling pathways and insufficient protein synthesis, which results in cortical response failure and polyspermic penetration (Swain & Pool 2008). In addition, Xia (2013) stated that there are three possible degrees of oocyte cytoplasmic maturation: immature, mature, and overmature. Oocytes with immature or over-mature cytoplasm (aging) tend to have polyspermy after IVF. Aging oocytes reveal biological abnormalities, including impairment in cortical granules reaction and zona pellucida hardening, mitochondrial malfunction, meiotic aberrations, and depleted ATP production and maturation-promoting factor (MPF) expression (Wang et al. 2017; Luo et al. 2020).

According to Bahrami et al. (2019) that the slicing technique to collect oocytes from the ovarium could be one of the reasons for polyspermy because this method might cause the release of oocytes from primordial follicles and only depend on the presence of thick layers of cumulus cells and homogeneous cytoplasm, allo wing these oocytes to be classified as grade A. Consequently, 24 hours of IVM is likely insufficient to fully and concurrently mature the nuclear and cytoplasmic of these primary oocytes. Furthermore, oocytes that have reached nuclear maturity may not have reached cytoplasmic maturity (Lee et al. 2003; Abbara et al. 2018) since some aspects of cytoplasmic maturation are independent of the nuclear component even though cytoplasmic components can be influenced by nuclear maturation (Swain & Pool 2008; Xia 2013). Consequently, depending on the oocyte's cytoplasmic maturation level, meiotically matured oocytes with first polar body appearance may not have all the essential maternal constituents needed for subsequent fertilization, PN formation, and embryo development (Swain & Pool 2008; Sun et al. 2022).

This study found one pronucleus (1PN) formation in control and LE 10 mM groups. The main reasons for unsuccessful IVF include failure in sperm chromatin decondensation, a lack of male pronuclear (MPN) formation, and ineffective oocyte activation (Shirazi et al. 2018). In theory, the failure of oocyte activation resulted from Phospholipase C zeta (PLCζ) as a Sperm-borne Oocyte-activating Factor (SOAF) unable to permeate the sperm plasma membrane (Ramadan et al. 2012). Furthermore, the oocyte contents, particularly glutathione (GSH), appear to be necessary for weakening the disulfide bond in the sperm nucleus and facilitating the formation of male pronuclear throughout fertilization (Shirazi et al. 2018). Some possibilities have been reported that one pronucleus (1PN) zygotes might develop from parthenogenetic activation, aberrant formation of nuclear envelopes such as asynchrony of the pronuclear emersion, belated pronuclear formation, and fusion between male and female pronuclei (Van Der Heijden et al. 2009; Hirata et al. 2020). Parthenogenetically activated oocytes would result in a haploid embryo, and only maternal chromatin is decondensed due to the failure of sperm nucleus decondensation (Hirata et al. 2020). Contrarily, Van Der Heijden et al. (2009) revealed that several 1PN zygotes are of paternal origin and typically formed when parental chromatin fuses following sperm penetration. Furthermore, asynchronous pronuclear development and male and female pronuclear fusion indicate that fertilization has succeeded, and the following embryos could be diploid (Hirata et al. 2020). A report by Itoi et al. (2015) proposed that IVF-derived 1PN embryos are presumably generated as the male and female chromosomes are surrounded by the same nuclear membrane due to sperm entering the oolemma from various locations, including sites near the spindle apparatus in the ooplasm.

CONCLUSION

In conclusion, the supplementation of 10 to 15 mM LE in the maturation medium increases oocyte nuclear maturation of oocytes. In addition, supplementing 10 mM LE in the maturation medium improves the oocyte fertilization rate.

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