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IMPROVEMENT OF SHEEP OOCYTE COMPETENCE IN VITRO THROUGH GLUTATHIONE ADDITION

Peningkatan Kompetensi Oosit Domba Secara *In Vitro* Melalui Penambahan Glutation

Mitha Yusmala[1], Iman Supriatna[2], Mohamad Agus Setiadi[2*]
[1]Animal Biomedical Science Study Program, Graduate School, IPB University
[2]Division of Reproduction and Obstetrics, School of Veterinary Medicine and Biomedical Sciences, IPB University
*Email: asetiadi@apps.ipb.ac.id

ABSTRACT
Oocyte competence is a primary factor for the success of embryo production *in vitro*. This research aims to determine the effect of supplementation glutathione in sheep oocytes' maturation and fertilization rates *in vitro*. In the first research, maturation oocytes were conducted in a medium supplemented with GSH at graded doses for 24 hours. In the second research, 1.0 mM GSH (the best dose from research I) was supplemented in the maturation medium, fertilization medium, and their combination for 14 hours. The higher oocyte maturation rates were significant ($P < 0.05$) in the GSH supplementation of 1.0 mM (87.00%). Furthermore, supplementation of GSH on the maturation medium resulted in a higher normal fertilization rate (56.43%) than in other groups. It is concluded that GSH supplementation on the maturation medium is more effective in increasing the competence of sheep oocytes to be mature and results in improved fertilization rates.

Keywords: *Fertilization, Glutathione, In Vitro, Maturation, Sheep oocyte*

ABSTRAK
Kompetensi oosit merupakan faktor utama keberhasilan produksi embrio secara *in vitro.* Penelitian ini bertujuan untuk mengetahui pengaruh suplementasi glutation terhadap tingkat pematangan dan fertilisasi oosit domba secara *in vitro.* Pada penelitian pertama, pematangan oosit dilakukan dalam medium yang disuplementasi GSH dengan dosis bertingkat selama 24 jam. Pada penelitian kedua, GSH 1,0 mM (dosis terbaik dari penelitian I) disuplementasikan dalam medium maturasi, medium fertilisasi, dan kombinasinya selama 14 jam. Tingkat pematangan oosit secara signifikan lebih tinggi ($P<0,05$) pada kelompok yang disuplementasi dengan 1,0 mM GSH (87,00%). Lebih lanjut, suplementasi GSH pada medium pematangan menghasilkan tingkat fertilisasi normal yang lebih tinggi (56,43%) dibandingkan dengan kelompok lainnya. Disimpulkan bahwa suplementasi GSH pada medium pematangan lebih efektif dalam meningkatkan kompetensi oosit domba untuk menjadi matang dan menghasilkan tingkat fertilisasi yang lebih baik.

Kata Kunci: *Fertilisasi, Glutation, In Vitro, Maturasi, Oosit domba*

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INTRODUCTION

Attempts to save animal or livestock genetic material continue to avert extinction. Reproductive biotechnology is a field that continues to be developed to support rare animal rescue programs and increase livestock populations. Through reproductive biotechnology, population increase and preservation of animal germplasm can continue to regenerate offspring more quickly and efficiently, and this can be done on a mass scale.

In vitro embryo production (IVEP) is an assisted reproductive biotechnology that consists of the stages of maturation in vitro, fertilization in vitro, and culture in vitro to produce embryos ready to transfer (Menchaca et al. 2018; Yusuf 2024). This biotechnology has many advantages compared to the natural mating system or previous generation biotechnology, one of which is utilizing ovarian waste from slaughterhouses that is no longer used to produce new individuals. This technology can also be widely applied as a model for other species, including humans and endangered animals. However, the success of IVEP is still relatively low compared with that of natural mating systems or previous generations of biotechnology (Souza-Fabjan et al. 2023). Many factors can affect the effectiveness of this technology, and it continues to be the subject of research and development. IVEP outcome is determined by the source of gametes (sperm and egg) and production environment, e.g., media composition, CO_2 incubator, and metabolites produced from the metabolic process.

As one of the components that form living things, oocytes play a crucial role in producing good-quality embryos. The quality of oocytes from they are released from the follicle to the maturation process is very susceptible to free radicals. Free radicals result from the high production of reactive oxygen species (ROS), which can't be controlled by antioxidant defense systems and can lead to pathological cell conditions (Takeshima et al. 2021). Normal cellular metabolic activities will produce ROS. ROS supports cellular signaling and affects various physiological functions, but uncontrolled production can cause failure, leading to cell death (Villalpando-Rodriguez and Gibson 2021). Furthermore, high ROS levels can cause oocytes to lose their competence so that they fail to mature, fail to be fertilized, and fail to develop into early embryos (Shi et al. 2025). Free radical damage can be prevented by increasing antioxidant levels.

Glutathione (GSH), a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), is the most abundant non-protein thiol in cells as an antioxidant to protect cells from oxidative stress (Forman et al. 2009; Lu 2013). Furthermore, GSH plays a role in detoxification, maintains intracellular redox status, and regulates cell proliferation, apoptosis, and immune function (Lu 2013; Adeoye et al. 2018). GSH is widespread not only in somatic cells but also in gamete cells. In gamete cells, GSH is important in binding or reducing ROS, thus improving the fertilization rate characterized by pronuclear formation to early embryonic development (Li 2018; Shirazi et al. 2018; Ren et al. 2021). GSH can prevent oxidative stress induced by excessive amounts of ROS during in vitro embryo production.

This study aimed to analyze the role of GSH in the maturation and fertilization system of sheep oocytes. The role of GSH will be observed based on the competence of the oocyte to mature and fertilize. The addition of GSH plays a role in neutralizing free radicals to improve oocyte maturation rate, thus producing oocytes that are competent for fertilization.

MATERIALS AND METHODS

Place and time of research

This research was conducted in the In Vitro Fertilization Laboratory, School of Veterinary Medicine and Biomedical Science, IPB University, from August 2024 to February 2025. This research was agreed upon by the Animal Ethics Commission of SKHB, IPB University (no.188/KEH/SKE/III/2024).

Oocytes collection

Ovaries obtained from the slaughterhouse were brought to the laboratory using a transport medium at 37℃ in less than 3 hours. The transportation medium consists of 0.9% Natrium chloride added with 100 IU/mL penicillin-G (Meiji, Indonesian) and 0.1 mg/mL streptomycin (Meiji, Indonesian).

Oocytes were collected from ovaries using a slicing technique with a blade in a petri dish containing collection medium. The collection medium was composed of PBS, adding 10% FBS (26140-087, Gibco-Stay Rd, USA), 100 IU/mL penicillin (P4687, Sigma-Aldrich, USA), and 0.1 mg/mL streptomycin (S9137, Sigma-Aldrich, USA) pre-equilibrated for at least 2 hours before use in a 5% incubator CO_2 at 38.5℃. Collected oocytes were selected using a stereomicroscope. Oocytes with homogeneous cytoplasm and compact cumulus cells were selected for maturation (Moulavi and Hosseini 2018).

In vitro maturation (IVM) of sheep oocytes

The maturation oocyte was used medium-199 (M4530, Sigma-Aldrich, USA) supplemented with 10% FBS (26140-087, Gibco-Stay Rd, USA), 0.02 AU/mL follicle-stimulating hormone (FSH) (Antrin R10 ・A, Kyoritsu Seiyaku, Japan), 10 IU/mL human chorionic gonadotrophin (hCG) (Intervet Boxmeer-Holland), and 50 µg/mL gentamycin (G1264, Sigma-Aldrich, USA). The medium was equilibrated in a 5% CO_2 incubator for at least 2 hours before use. Selected oocytes were divided into four groups, and each was matured in a droplet of maturation medium in a petri dish supplemented with 0 mM, 0.5 mM, 1.0 mM, and 1.5 mM of GSH (G4521, Sigma-Aldrich, USA) (Nugroho et al. 2017 with modification). The maturation medium was covered with mineral oil and then oocytes were incubated for 24 hours in a 5% CO_2 incubator.

Evaluation of nuclear maturation rates

Oocytes were denuded of cumulus cells using 0.25% hyaluronidase enzyme by repeated pipetting technique. The oocytes were washed three times in a collection medium and then placed in 0.5% KCl droplets on a slide that had been given vaseline pads and parafilm on the four corners. The slides were then covered with cover glass and fixed for 48-72 hours in acetic acid and ethanol (1:3) solution. Nuclear maturation rates were stained with 2% aceto-orcein staining. The slides were stained for 5-10 minutes and then rinsed using a 25% acetic acid solution. Then, all sides of the cover glass were treated with clear nail polish. The capability of an oocyte to reach metaphase II is an indication of oocyte nuclear maturation.

In Vitro Fertilization (IVF)

Oocyte collection methods, selection, and maturation procedures were carried out based on the first study. Frozen semen was used Garut sheep semen (191316) from Lembang Artificial Insemination Center, West Java. Sperm preparation started by thawing frozen semen in a water bath at 37℃ for 30 seconds. The semen was placed into a 15 mL centrifuge tube containing 4 mL PBS supplemented with 0.2 % Bovine Serum Albumin (BSA) (A7030, Sigma-Aldrich, USA) and centrifuged at 1600 rpm for five minutes at 28℃. The supernatant was discarded, and approximately 200 µL of the pellet was diluted using a fertilization medium to get the $5x10^6$ spermatozoa/mL concentration. The fertilization medium refers to Suzuki et al. (2000) modified by supplementation of 0.1 IU/ml heparin (H5515, Sigma-Aldrich, USA) and 10 µM hypotaurine (H1384, Sigma-Aldrich, USA) based on Rahmatullah et al. (2022). The sperm were placed as drops in a petri dish and covered with mineral oil. After maturation, the oocytes were washed twice in the fertilization medium and transferred into the droplet. The oocytes were then incubated with sperm in a 5% CO_2 Incubator for 14 h.

Evaluation of fertilization rates

Oocyte denudation, fixation, and staining procedures were carried out as in the first study. Fertilization rate was observed by pronucleus formation. The formation of two pronuclei (2PN) is categorized as normal fertilization (Kemper et al. 2023).

Statistical analysis

The maturation rates data were evaluated by Analysis of Variance (ANOVA) with a significance level of 95%, with Duncan's Multiple Range Test (DMRT) as a further test if there were differences between treatments. The fertilization rates were analyzed using the Kruskal-Wallis test and a signifi-

cance level of 95%. If there were any significant differences between treatments, the Mann-Whitney test was used.

RESULT AND DISCUSSION

Maturation competence of sheep oocytes nuclear after GSH addition

The nuclear maturation of sheep oocytes was determined based on the capacity of the oocyte to reach MII. The addition of GSH with graded doses increased the potential of oocytes that reached MII from 73.41% to 87.00%, with the optimum dose being the addition of 1.0 mM GSH. This study's results align with Ren et al. (2021) that exogenous GSH supplementation increases the competence of ovine oocyte development to reach MII. In this research, the supplementation of GSH 1.0 mM (87.00%) in maturation significantly increased ($P < 0.05$) the percentages MII between 0.5 mM GSH group (73.41%), 1.5 mM GSH (78.23%), and control (70.50%).

Oocyte maturation is an important early stage because it determines the success of the following process capacity of in vitro fertilization and the early developmental stage of the embryo. However, not all selected oocytes can to be fertilized and develop into embryos (Jiang et al. 2023). Oocyte maturation is highly susceptible to radical free. Radicals free are generated by high ROS production and low antioxidant cellular defense systems (Wei et al. 2025).

ROS are byproducts of cellular metabolism that produce adenosine triphosphate (ATP). However, excessive or uncontrolled ROS production has an unfavorable effect on oocytes because it interferes with maturation and reduces oocyte competence. High ROS production can be dangerous for cells, damaging intracellular proteins, membrane lipids, and nucleic acid (DNA) (Jomova et al. 2024; Yang and Lian 2020). Excessive ROS levels also trigger increased spindle formation errors, mitochondrial dysfunction, and decreased oocyte maturation (Rakha et al. 2022). ROS accumulation can be regulated under physiological conditions by the enzymatic and non-enzymatic antioxidant defense systems (Ye et al. 2015). To prevent the negative effects of ROS and increase the competence of oocytes, it's very important to supplement the maturation medium with antioxidant or their precursors, such as N-Acetyl-L-cysteine (NAC) (Wang et al. 2025), melatonin (Qu et al. 2023), cysteamine (Gulo et al. 2020), and glutathione (GHS) (Ren et al. 2021).

Glutathione is a primary antioxidant that is synthesized in cells. GSH regulates the redox balance and prevents the formation of free radicals in cells. During IVEP, light and oxygen pressure cause oocytes to be susceptible to exposure free radicals. In the in vitro environment, oxygen pressure is approximately 20% higher than the uterus and oviduct, around 2% to 8% (Sciorio and Smith 2019). This is one of the factors that reduces oocyte maturity because the high level of oxygen pressure can cause free radicals during maturation. Therefore, supplementation maturation medium with antioxidants is important to prevent free radical formation. Thus, it is expected that a maturation medium supplemented with GSH makes more oocytes reach MII, which is the criterion for mature oocytes.

In this research, the maturation medium with GSH 1.5 mM resulted in a low oocyte percentage reaching MII. It is suspected that a high concentration of GSH may disrupt the redox balance caused by the elimination of excessive ROS, the presence of which is required in regulating signal transduction and modulating cellular functions (Bardaweel et al. 2018). In addition, high concentrations of antioxidants can decrease the pH of the medium (Sun et al. 2015), thus affecting oocyte maturation. In this research, changes in the pH of the medium due to high levels of GSH resulted in a low percentage of MII. The intracellular pH balance is needed because of its role in regulating the oocyte meiotic spindle stability, division and differentiation cells, embryo enzymatic activity, and formation of the blastocoel (Gatimel et al. 2020), which is very important in oocyte development until the formation of the early embryo.

Table 1. The nuclear maturation rate of sheep oocyte after the addition of GSH

Treatments	Total oocytes	Oocyte nuclear maturation status n(%)				
		GVBD	MI	A/T	MII	D
Control	88	10(12.33)	11(11.67)	5(5.50)	62(70.50)[a]	-
0.5 mM	88	5(6.96)	15(16.81)	3(2.82)	65(73.41)[ab]	-
1.0 mM	88	2(2.82)	8(9.07)	-	77(87.00)[c]	1(1.11)
1.5 mM	80	4(5.18)	13(15.58)	1(1.01)	62(78.23)[b]	-

[a, b, c] Different superscripts in the same column indicate significant differences (P<0.05). GVBD= germinal vesicle breakdown, MI= metaphase I, A/T= anaphase/telophase, MII= metaphase II, D= degeneration.

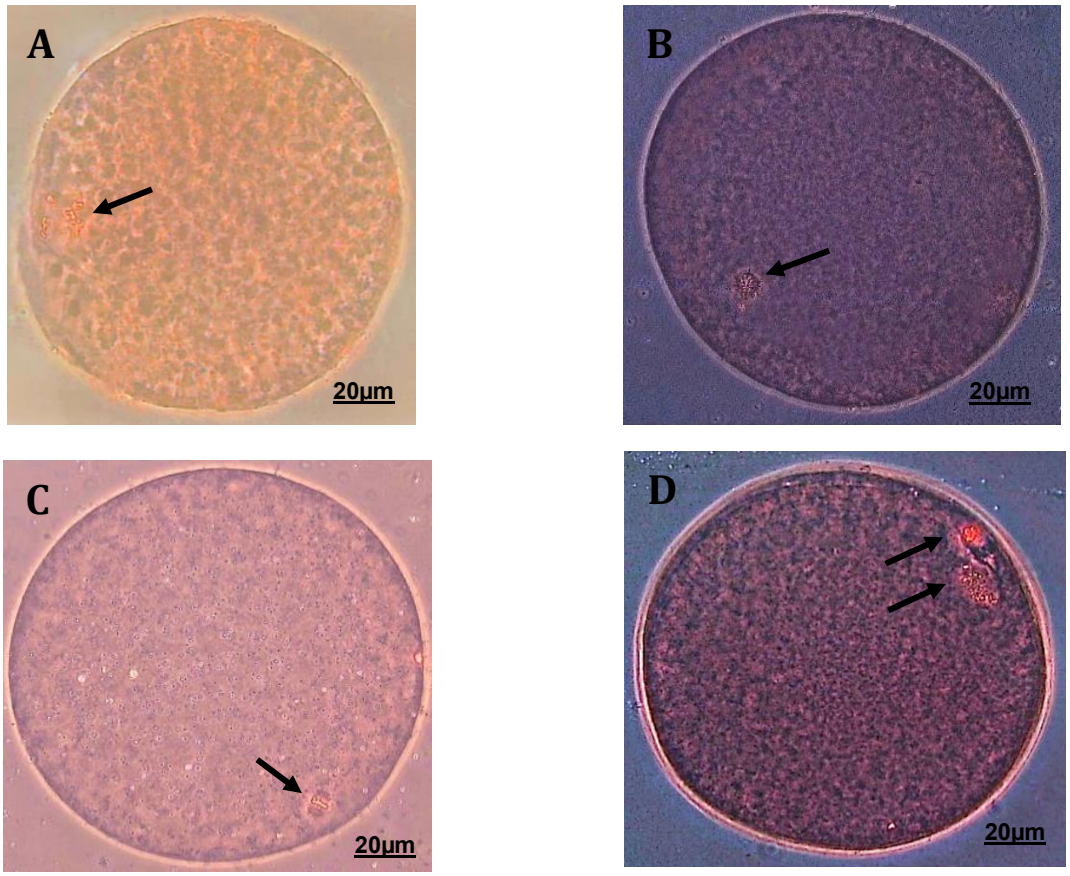


Figure 1. Maturation stages of sheep oocyte nuclei after in vitro maturation. A: germinal vesicle breakdown (GVBD), B: metaphase I (MI), C: anaphase (A), D: metaphase II (MII). The arrow indicates the maturation status of the oocyte nucleus.

Pronucleus formation competence of sheep oocytes after GSH addition

The formation of two pronuclei (2PN) is categorized as normal fertilization (Kemper et al. 2023). Oocytes with one or more than two pronuclei (polyspermy) are indicated as abnormal fertilization. The GSH concentration used in this research was the optimum doses obtained from the first research. This research showed that GSH in the maturation medium (56.43%) had a significantly higher normal fertilization rate (P < 0.05) compared the group of GSH in the combination of maturation and fertilization medium (18.45%). This indicates that the supplementation of GSH in the maturation medium more significant, increasing the normal fertilization rates of oocytes.

Table 2. Fertilization rate of sheep oocytes after GSH addition

Treatments		Total	Total Fertilization	Pronuclear formation n(%)			
IVM	IVF	Oocytes (n)	n(%)	1PN	2PN	>2PN	D
+	-	35	20(58.21)[b]	7(20.60)	19(56.43)[b]	1(1.79)	-
-	+	37	11(33.27)[ab]	7(15.49)	9(29.18)[ab]	2(4.08)	-
+	+	35	5(18.45)[a]	5(17.02)	5(18.45)[a]	-	-

[a, b]Different superscripts in the same column indicate significant differences (P<0.05). 1PN= one pronucleus, 2PN= two pronuclei, >2PN= polyspermi, D= degeneration.

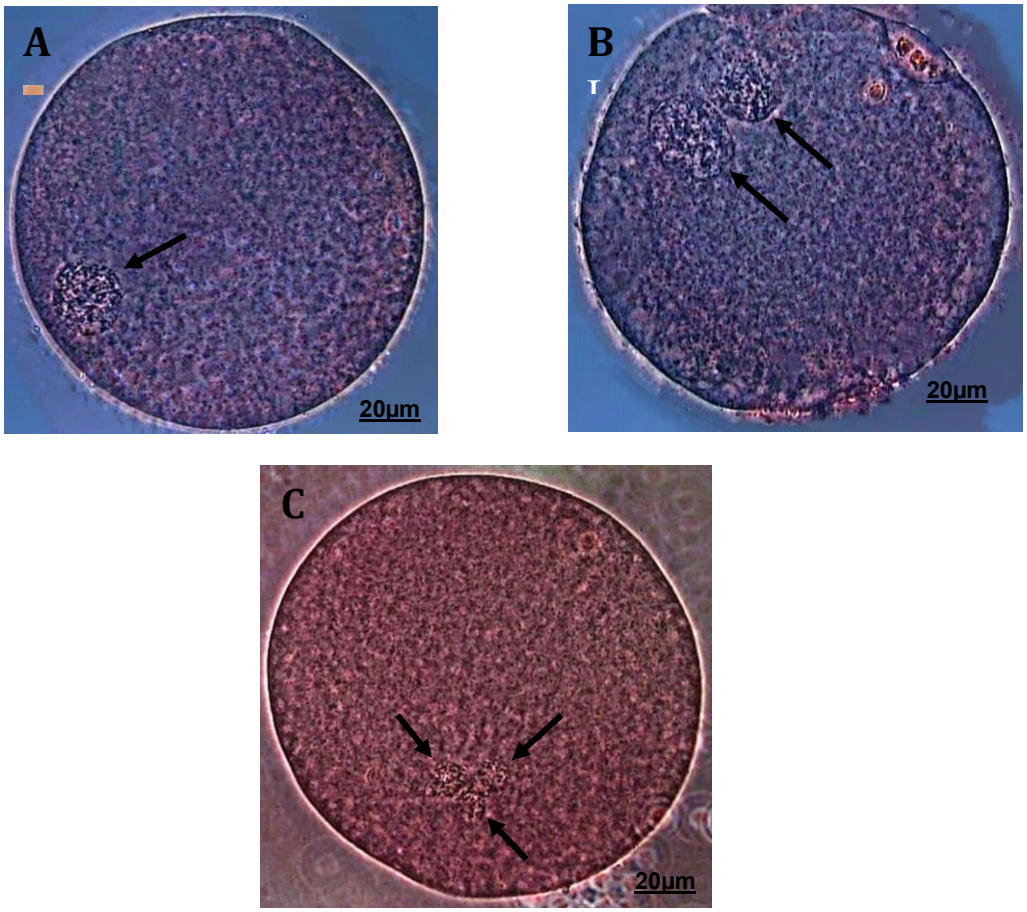


Figure 2. Pronucleus (PN) formation in sheep oocytes after in vitro fertilization. A: oocyte with one pronucleus (1PN), B: oocyte with two pronuclei (2PN), C: Polyspermy (>2PN). The arrow indicates the fertilization rate of the oocyte.

In the maturation process, glutathione intracellular synthesis occurs and plays a role in oocyte cytoplasmic maturation (Luberda 2005; Liu et al. 2023). Oocyte cytoplasmic maturation is an important component for successfully forming two pronuclei during the in vitro fertilization stage. This aligns with Fan and Sun (2019) that cytoplasmic maturation plays a role in preparing oocytes for fertilization and competence in embryo development at a subsequent stage. In addition, the importance of GSH synthesis during maturation is related to the ability of oocytes to maintain meiotic spindle morphology so that oocytes can support male pronucleus formation (Ufer and Wang 2011; Gulo et al. 2020). Furthermore, penetration of spermatozoa into oocytes is easy because GSH reduced disulfide bonds found in zona pellucida (Takeo and Nakagata 2011). During spermiogenesis, histone is replaced by protamines, forming very tight disulfide bonds; therefore, when interacting with the oocyte, the disulfide

bonds between protamine molecules must be broken so that decondensation of the sperm nucleus can occur (Chapman and Michael 2003). GSH plays a role in reduced protamine disulfide bonds, thus supporting the decondensation of sperm chromatin in fertilized oocytes (Unnikrishnan et al. 2021; Itahashi et al. 2022). Thus, it is suspected that the supplementation of GSH in the maturation medium is a primary factor in improving the competence of cytoplasmic maturation of oocytes and fertilization rate to support develop of the early embryo.

GSH supplementation in the fertilization medium resulted in no significant difference ($P > 0.05$) than the other groups. GSH synthesis occurs during oocyte maturation and plays a main role in the preparation of oocyte maturity to receive sperm (Ufer and Wang 2011). However, the concentration of GSH slowly decreases as the early cleavage of the embryo continues (Ufer and Wang 2011). Therefore, relying only on GSH in the IVF medium did not save the oocytes from free radicals during maturation. Furthermore, Luberda (2005) states that GSH is implicated in the protection of gamete cells from free radical damage. In addition, the synthesis of GSH requires precursors, such as cysteine, as the primary raw material. However, these components are absent in the fertilization medium used (Suzuki et al. 2000), so the role of GSH in the fertilization medium does not cause an increase in pronucleus formation.

GSH supplementation in the combined maturation and fertilization medium decreased significantly ($P < 0.05$) the normal fertilization after IVF. High concentrations of GSH in the medium may disrupt redox balance due to the excessive elimination of ROS. ROS requires regulators of various cellular signaling pathways and cellular functions (Reczek and Chandel 2016; Lennicke and Cocheme 2021). Furthermore, Kozlov et al. (2024) stated that excessive antioxidant neutralization leads to reductive stress, which disrupts cell signaling and affects intracellular messenger-mediated pathways. High concentrations of antioxidants can also lower the pH of the medium (Sun et al. 2015), leading to decreased fertilization rates when oocytes are incubated in both maturation and fertilization medium supplemented with GSH. Several studies have shown that the role of exogenous GSH during IVC is able to increase the developmental potential and quality of bovine IVF embryos, due to the ability of GSH to maintain redox balance (Sun et al. 2015). Glutathione synthesis also has several precursors, one of which is N-acetylcysteine (NAC). NAC increases GSH levels and reduces ROS accumulation, thereby increasing the rate of oocyte and blastocyst division (Wang et al. 2025).

CONCLUSION

Supplementation of Glutathione in the maturation medium was more effective in increasing the competence of the sheep oocytes to mature and resulted in improved fertilization rates. More research is needed about the early development of embryos in oocytes supplemented with GSH in the maturation medium.

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