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Effect of Alpha Lipoic Acid (ALA) Supplementation in Goat Oocyte *In Vitro* Maturation Media on Superoxide Dismutase (SOD) and 8-Hydroxy-2'-Deoxyguanosine (8-OHdG) Levels

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Abstract

Introduction: This study explains about the effect of Alpha Lipoic Acid (ALA) supplementation in goat oocyte *In Vitro* maturation media on Superoxide Dismutase (SOD) levels and 8-Hydroxy-2'-Deoxyguanosine (8-OHdG) levels with the aim of determining the status of balanced intracellular redox status and good oocyte quality.

Objective: This laboratory experimental study contained three treatment groups: no ALA (P0), 25 µmol/L ALA supplementation (P1), and 50 µmol/L ALA supplementation (P2). SOD and 8-OHdG levels were measured using the ELISA method. SOD levels in group P0 were 1.41±0.40 pg/mL, group P1 were 2.04±0.50 pg/mL, and group P2 were 3.65±1.20 pg/mL. Data on 8-OHdG levels in the P0 group was 51.17±27.58 pg/mL, the P1 group was 11.21±5.98 pg/mL, and the P2 group was 12.66±6.01 pg/mL.

Results: The P1 group could not significantly increase the SOD level of the P0 group ($p>0.05$), while the P2 group could significantly increase the SOD level of the P0 group ($p<0.05$). Data on 8-OHdG levels in the P1 and P2 groups decreased significantly when compared to the P0 group ($p<0.05$).

Conclusion: ALA supplementation at a dose of 50 µmol/L in goat oocyte *In Vitro* maturation media can increase SOD levels and reduce 8-OHdG levels.

Keywords: Oocyte; Alpha Lipoic Acid; *In Vitro* Maturation; SOD; 8-OHdG

1. Introduction

In Vitro maturation (IVM) is the first and most important stage during *In Vitro* embryo production (IVEP) [1]. During IVM, oocytes are matured in synthesis media under a controlled laboratory setting. The maturation results are still low in oocyte quality when compared to in vivo maturation [2]. This is due to the difference in conditions between *In Vitro* maturation and in vivo maturation. One of the considerable differences between in vivo and *In Vitro* conditions in oocyte maturation is oxygen pressure. During *In Vitro* maturation, oocytes are maintained with higher oxygen concentration (O_2) compared to in vivo maturation (2-9%) [3]. The relatively high concentration of oxygen in the oocyte maturation medium *In Vitro* can disrupt the balance between Reactive Oxygen Species (ROS) and antioxidants, such an event causes a stressful condition referred to as oxidative stress [4,5]. Oxidative stress caused by excessive accumulation of intracellular ROS can cause cellular damage such as DNA damage and decreased oocyte quality [6].

One of the most well-known and proven sensitive indicators of oxidative stress damage in low quality oocytes is DNA damage [7]. Nucleic acid oxidation biomarkers are commonly used to assess this damage [8]. 8-OHdG is the most well-

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known and widely used biomarker of nucleic acid oxidation and the production of 8-OHdG by free radicals was first reported in 1984 [9]. The research results of [10], reported that 8-OHdG was negatively correlated with the number and maturity level of oocytes, fertilization rate, total number of embryos, and good embryo quality. The study also showed that 8-OHdG levels in granulosa cells were inversely related to oocyte quality.

Antioxidants with biological function are defined as substances that reduce or prevent oxidation and produce more potent reducing agents [11]. ROS can be inactivated or neutralized by a defence system consisting of enzymatic and non-enzymatic antioxidants [4]. Enzymatic antioxidants are known as natural antioxidants that are able to neutralize excessive ROS levels and prevent from damaging cellular structures [12]. Enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase [14].

Antioxidant supplementation of oocyte IVM media is one of the most effective methods to prevent ROS accumulation [1]. It aims to protect oocytes from the detrimental effects of oxidative stress through defence of physiological levels of ROS [14,15]. Antioxidant supplementation into IVM media has been shown in several studies to improve oocyte quality and reduce their damage caused by excessive ROS exposure [16,17]. One of the ingredients that can be added to the maturation media as an exogenous antioxidant supplementation is ALA. ALA is an antioxidant that not only has an affinity for peroxyl free radicals but also has the ability to regenerate other antioxidants such as glutathione (GSH), vitamin C, and tocopherol. Then it participate in energy metabolism and cell signal transduction [18]. Based on the research results, the addition of ALA in maturation media can increase SOD expression in oocytes and parthenogensis blastocysts [19]. In this article, ALA supplementation in goat oocyte *In Vitro* maturation media as an exogenous antioxidant was further studied to determine the improvement of the oocyte maturation system *In Vitro* through increased SOD levels and decreased 8-OHdG levels.

2. Material and methods

This study was a laboratory experimental study with three treatment groups: control group without ALA (P0), treatment group 1 with 25 µmol/L ALA supplementation (P1), treatment group 2 with 50 µmol/L ALA supplementation (P2).

2.1. Medium preparation

The maturation medium was prepared by making Minimal Essential Medium (MEM) drops plus 3% Fetal Calf Serum, HCG Hormones and PMSG on disposable petridish with micropipette. The medium droplets were then covered with mineral oil, and incubated in an incubator with 5% CO_2 at 38.5^o C for 22 hours before being used for *In Vitro* maturation.

2.2. Ovary collection

Ovaries from the abattoir were brought to the laboratory, then placed in a beaker containing physiological NaCl solution and placed in a water bath at 37°C. Follicles 2-6 mm in diameter were aspirated by inserting a 10cc disposable syringe with 18 G needle containing Phosphate Buffer Saline (PBS). The aspirated fluid was allowed to stand for 5-10 minutes in a tube filled with dissection media to separate oocytes and remove residual follicular fluid. The aspirated fluid is taken out until there is almost no liquid left at the bottom of the tube, the remaining liquid in the tube is poured into a 100 mm petri dish that has been given a line. Selection and washing of cumulus oocyte complexes (CECs) that have homogeneous cytoplasm and still have at least 3 layers of cumulus cells using a dissecting microscope with a magnification of 120-240x. The results of the selection were transferred to the collection media in a 65 mm petri dish, then re-selected and the LOCs were transferred to the dissection media in a 35 mm petri dish [20].

2.3. *In Vitro* Oocyte Maturation

CECs that have been selected from the collection media are transferred into drops of maturation media. The media used was MEM media and added PMSG 0.15 IU/ml, hCG 0.15 IU/ml, FCS 3%. Each petridish contained 5 drops of maturation media (50 µl/drop and each drop contained 5 oocytes) coated with mineral oil (Lab. Cryotech, Japan) then incubated in a 5% CO2 incubator, 98% humidity, temperature 38.5˚C for 22 hours. Furthermore, observations were made on each group using a CX41 microscope (Olympus, Japan) [21].

2.4. Measurement of SOD levels 8-OHdG levels

A total of 7 CECs that have been matured *In Vitro* for 22 hours were put into an eppendorf and added 100 µl lysis buffer and vortexed for 5 minutes, then centrifuged at 300rpm for 5 minutes to separate the supernatant from the pellet. The supernatant obtained was then measured for SOD and 8-OHdG levels using the enzyme-linked immunosorbent assay (ELISA) method. SOD levels were measured using the SOD typed Colorimetric Assay Kit (E-BC-K022-M, Elabscience

Biotechnology, Wuhan, China) and according to the procedure of [22]. Measurement of 8-OHdG levels using the 8-OHdG (8-Hydroxydeoxyguanosine) ELISA Kit (Elabscience Catalog No: E-EL-0028, USA) and according to the procedures carried out by [10].

2.5. Data Analysis

The data obtained were analysed using the SPSS 23.0 software program (IBM Corp., NY, USA) to analyze the comparison between the treatment and control groups. Normality test using Shapiro-Wilk test was performed first to analyze the relationship of each variable. If the data were normally distributed, one-way ANOVA was used and followed by Duncan's test to determine differences between groups, whereas if the data were not normally distributed, Kruskal-Wallis test was used and followed by Mann-Whitney test. This study used a significance level of 0.05 with a 95% confidence level.

3. Results and discussion

The results of maturation of goat oocytes *In Vitro* for 22 hours in maturation media without ALA supplementation (P0), 25 µmol/L ALA supplementation (P1), 50 µmol/L ALA supplementation (P2), then measured SOD levels and 8-OHdG levels using the ELISA method (Table 1). Data on SOD levels and 8-OHdG levels that have been obtained were then analyzed for normality using Shapiro-Wilk did not show normal distribution, so the Kruskal-Wallis nonparametric test was continued and continued with the Mann-Whitney test ($p<0.05$).

Table 1 SOD levels and 8-OHdG levels in goat oocytes after *In Vitro* maturation

Group	SOD levels (pg/mL) ($\bar{X}$ ± SD)	8-OHdG levels (pg/mL) ($\bar{X}$ ± SD)
P0	1.41^{a}±0.40	51.17^{a}±27.58
P1	2.04^{ab}±0.50	11.21^{b}±5.98
P2	3.65^{b}±1.20	12.66^{b}±6.01

Notes: Different superscripts (^{a,b}) in the same column indicate significant differences between treatments ($p<0.05$). P0: the control group without ALA supplementation; P1: treatment group 1 with 25 µmol/L ALA supplementation; P2: treatment group 2 with 50 µmol/L ALA supplementation.

Table 1. shows the measurement results of SOD levels and 8-OHdG levels of goat oocytes matured *In Vitro* for 22 hours. SOD levels in the P0: 1.41 ± 0.40 pg/mL, P1: 2.04 ± 0.50 pg/mL, and P2: 3.65 ± 1.20 pg/mL. Data on SOD levels in group P0 with group P1 did not show significant differences ($p>0.05$) and showed significant differences when compared with group P2 ($p<0.05$). Data on SOD levels in group P1 compared to groups P0 and P2 showed no significant difference ($p>0.05$).

The measurement results of 8-OHdG levels of goat oocytes matured *In Vitro* for 22 hours are also shown in Table 1. Data on 8-OHdG levels in the P0: 51.17 ± 27.58 pg/mL, P1: 11.21 ± 5.98 pg/mL, and P2: 12.66 ± 6.01 pg/mL. Data on 8-OHdG levels of group P0 with group P1 and group P2 showed significant differences ($p<0.05$), while data on 8-OHdG levels of group P1 with group P2 did not show significant differences ($p>0.05$). The bar chart of SOD levels and 8-OHDG levels of goat oocytes matured *In Vitro* in three different treatment groups can be seen in figure 1.

This study describes the effect of ALA supplementation in goat oocyte *In Vitro* maturation media on SOD and 8-OHdG levels with the aim of determining the status of balanced intracellular redox status and good oocyte quality. Analysis of oocyte oxidative stress conditions during IVM showed significant differences in 8-OHdG levels between the control group without ALA supplementation and the treatment group supplemented with ALA. The level of 8-OHdG in the control group (P0) without ALA supplementation increased significantly and decreased significantly in treatment group 1 (P1) supplemented with 25 µmol/L ALA. A significant decrease in 8-OHdG levels also occurred in treatment group 2 supplemented with 50 µmol/L ALA and was not significantly different from treatment group 1 (P1). This finding may be that oocytes experience oxidative stress during the *In Vitro* maturation process and agrees with previous findings by (10) that an increase in 8-OHdG levels during IVM affects the number of maturing oocytes.

The increased level of 8-OHdG in the control group without ALA supplementation indicates that during IVM the oocytes produce ROS. This may be due to several factors that can regulate ROS generation such as variations in cellular metabolic reactions, oxygen concentration, light, oocyte handling, and physicochemical parameters that have a negative impact on oocyte physiology. Increased levels of ROS in oocytes beyond the physiological range can induce oxidative stress and lead to decreased oocyte quality [4, 23, 24, 25].

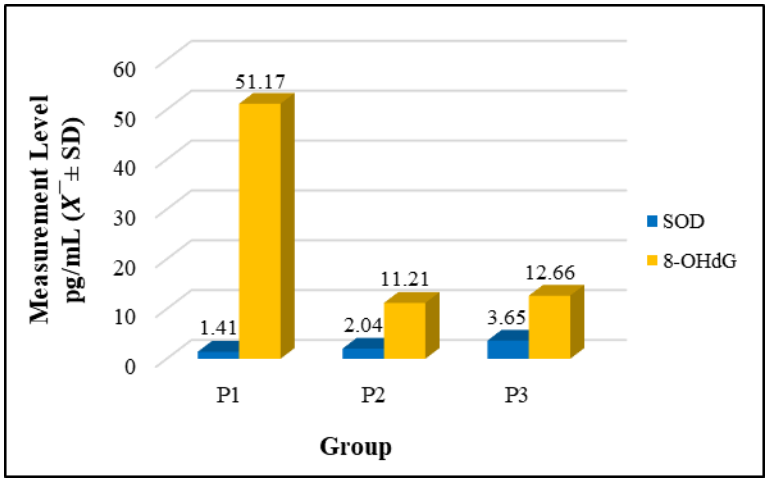

Figure 1 Bar chart of SOD levels and 8-OHDG levels of *In Vitro*-matured goat oocytes in three different treatment groups

ROS are a group of molecules consisting of free radicals such as hydroxyl (HO^-), peroxynitrite ($ONOO^-$), superoxide (O^{2-}), and non-radicals such as hydrogen peroxide (H_2O_2) [26, 27]. The hydroxyl radical (HO^-) interacts with the nucleobases in the DNA strand, such as guanine, which results in the C8-hydroxyguanine (8-OHGua) production or when interacting with its nucleoside (guanosine) forms deoxyguanosine (8-hydroxy-2-deoxyguanosine). ROS attacks the 8th carbon atom of guanine in the DNA to create 8-OHdG (which is an oxidised derivation of deoxyguanosine) [10].

A group of enzymatic and non-enzymatic intracellular antioxidants repeatedly neutralize intracellular ROS (1). SOD is one of the enzymatic antioxidants. This enzyme presents three isoforms, the cytosolic Cu, Zn-SOD (SOD1), the mitochondrial Mn-SOD (SOD2) and the extracellular SOD3 [28]. SOD converts O^{2-} into H_2 O_2 via its mitochondrial, manganese-dependent superoxide dismutase (Mn-SOD) and cytoplasmic, copper, zinc superoxide dismutase (Cu/ZnSOD) [29].

Based on several previous research reports, ALA supplemented in the maturation medium acts as an antioxidant by increasing gluthathione (GSH) synthesis, Total Antioxidant Capacity (TAC) and enzymatic antioxidants such as SOD and GPX4. The results of this study indicate that SOD levels in P1 supplemented with 25 µmol/L ALA has not been able to significantly increase the SOD levels of the control group P0 which was not supplemented with ALA, while 50 µmol/L ALA supplementation in P2 was able to significantly increase SOD levels in the control group P0 without ALA supplementation. This finding is in accordance with the results of research by (19) and (30) that ALA-supplemented treatment groups increased oocyte intracellular SOD synthesis [19, 30, 31, 32]

Our current study provides evidence that oxidative stress that occurs during *In Vitro* maturation of goat oocytes increases 8-OHdG levels. Increased levels of 8-OHdG during *In Vitro* maturation can be reduced by exogenous antioxidants such as ALA by the mechanism of increasing endogenous antioxidants that are enzymatic SOD. Therefore supplementation of exogenous antioxidants can reducing 8-OHdG levels.

4. Conclusion

The conclusion of this study, the supplementation of ALA at a dose of 50 µmol/L in goat oocyte in vitro maturation media can increase SOD levels and reduce 8-OHdG levels.

Compliance with ethical standards

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Disclosure of Conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

This animal study received ethical approval from the Ethical Committee at the Faculty of Medicine, Universitas Airlangga (No. 1.KEH.042.04.2022). All research procedures were conducted in strict accordance with ethical guidelines and relevant regulations.

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Author's short biography

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	**Authors Name:** Widjiati Widjiati Prof. Widjiati, a professor at Universitas Airlangga (UNAIR) delivered a embryo transfer program (TE) as a solution for breeding cattle in Indonesia. Prof. Widjiati said that it is time to think of other assisted reproductive technologies besides artificial insemination to overcome the slow growth of the cattle population. Prof. Widjiati studied Bachelor at Universitas Airlangga, Master at IPB-Bogor, and Doctoral at Universitas Brawijaya Indonesia.