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The Effect of Alpha Lipoic Acid (ALA) Supplementation on the in Vitro Maturation Media of Goat Oocyte on Tumor Alpha Necrosis Factor (TNF-α) Expression and Malondialdehyde (MDA) Levels

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ABSTRACT

This study aimed to determine the effect of Alpha Lipoic Acid (ALA) supplementation on in vitro maturation media of goat oocytes on Tumor Necrosis Factor Alpha (TNF-α) expression and Malondialdehyde (MDA) levels. This laboratory experimental study consisted of three treatment groups: control group (G0), 25 µmol/L ALA supplementation (G1), and 50 µmol/L ALA supplementation (G2). A total of 366 goats Cumulus Oocyte Complex (COCs) were collected, then selected into 216 COCs, and matured in vitro for 22 hours in a 5% CO2 incubator, 98% humidity, 38.50C temperature. After that, TNF-α expression was identified using Immunocytochemistry staining with the addition of TNF-α antibody and calculated using the Remmele Scale Index. Measurement of MDA levels using the ELISA method. The data obtained were analyzed using Kruskal-Wallis, One Way ANOVA, and Duncan's in the SPSS 24 software program ($p<0.05$). The expression value of TNF-α G0 was 0.75±1.39, G1 was 5.56±3.05, and G2 was 2.00±1.80. MDA levels of G0 were 26.52 ±2.92, G1 was 46.44 ±4.87, and G2 was 30.41±5.67. TNF-α expression data and MDA levels G0 and G2 didn't show a significant difference, while G0 and G2 compared to G1 showed a significant difference ($p<0.05$). ALA supplementation of 25 µmol/L increased TNF-α expression or MDA levels and 50 µmol/L on in vitro maturation media of goat oocytes could reduce TNF-α expression or MDA levels than 25 µmol/L.

Keywords: alpha lipoic acid, in vitro maturation, MDA, TNF-α, reproductive health

INTRODUCTION

In vitro maturation of oocytes is considered an important first step in successful embryo engineering technology[1,2]. According to Widjiati *et al.* (2020) oocyte maturation rate can support embryonic development after in vitro fertilization (IVF)[3]. Oocyte maturation is characterized by changes in the morphology of the cell nucleus, namely the change in the oocyte from the diplotene stage to the metaphase II stage so that it is ready for fertilization[4]. Oocytes during in vitro maturation undergo the development of the meiotic cycle and structural changes in the cytoplasm that are necessary for successful fertilization and further embryonic development[5]. This causes the success rate of embryonic development from oocytes that are matured in vitro to be lower than that of oocytes that are matured in vivo[6].

One that is quite different between in vivo and in vitro conditions in oocyte maturation is oxygen tension. During in vitro maturation, oocytes are maintained with a higher concentration of oxygen (O_2) (21%) compared to in vivo maturation (2-9%)[7].

Relatively high oxygen concentrations (hyperoxic) in the oocyte maturation environment in vitro can increase Reactive Oxygen Species (ROS), so these conditions will disrupt the balance between ROS and antioxidants that cause oxidative stress[8]. Oxidative stress is involved in various biological events, such as amino acid and nucleic acid oxidation, apoptosis, necrosis, and lipid peroxidation in cell membranes and organelle membranes[9]. The plasma membrane of mammalian oocytes is a rich source of unsaturated fatty acids and is susceptible to lipid peroxidation associated with ROS[10]. The level of lipid peroxidation can be determined by measuring the levels of Malondialdehyde (MDA), which is a stable lipid peroxidation product[11,12].

Oocyte maturation is a complex coordinated event in ovarian follicular development[13]. During the growth and differentiation process of ovarian follicles, some cytokines become paracrine regulators in mediating communication between oocytes, granulosa, and theca cells[14,15].

One of the cytokines contained in oocytes is Tumor Necrosis Factor Alpha (TNF-α) and Tumor Necrosis Factor Receptor (TNFR) localized to granulosa cells and theca cells[16]. These cytokines mediate various biological responses including inflammation, infection, injury, and cell apoptosis[5]. Based on the research report of Ma *et al.* (2010)[17] an increase in TNF-α expression significantly reduced the rate of oocyte maturation. Recently, Kong *et al.* (2018)[18] showed that aging mice cumulus cells secrete TNF-α to accelerate oocyte aging by interacting with TNFR. In vitro, culture media manipulation is needed to increase oocyte maturation ability[19]. The use of a basic maturation medium (Minimal Essential Medium) with the addition of supplementation of various substances and compounds has been widely studied to study and improve oocyte maturation in vitro[20]. One of the ingredients that can be added to the maturation media as a supplement to exogenous antioxidants is Alpha Lipoic Acid (ALA). Alpha Lipoic Acid (ALA) is an antioxidant that not only has an affinity for peroxyl free radicals but also can regenerate other antioxidants such as glutathione (GSH), vitamin C, and tocopherols and participates in energy metabolism and cell signal transduction[21]. Besides having antioxidant properties, ALA also has anti-inflammatory properties[22].

The survival environment of oocytes created in vitro has always been a challenge for in vitro maturation research[23]. Therefore, research on the effect of Alpha Lipoic Acid (ALA) supplementation on in vitro maturation media of goat oocytes on the expression of Tumor Necrosis Factor Alpha (TNF-α) and levels of Malondialdehyde (MDA) still needs to be done to determine the improvement of the oocyte maturation system in vitro.

METHODS

This research was conducted from May to July 2022 and has been awarded an Ethical Appropriate certificate by the Animal Ethics Commission, Faculty of Veterinary Medicine, Universitas Airlangga with certificate no. 1.KEH.042.04.2022. This study is an experimental laboratory study with three treatment groups, namely the control group without ALA (G0), treatment group 1 with ALA 25 μmol/L (G1), treatment group 2 with ALA 50 μmol/L (G2)[24, 25].

Ovarian collection

The ovaries from the Slaughterhouse were brought to the laboratory and then put in a thermos containing 0.95% physiological NaCl solution at 37°C. After arriving at the laboratory, the ovaries were sterilized and washed with 0.95% physiological NaCl solution + 100 μl of gentamicin. Ovarian follicles with a diameter of 2-6 mm were aspirated by inserting a 10-cc disposable syringe with an 18 G needle containing Phosphate Buffer Saline (PBS). The aspirated fluid was left for 5-10 minutes in a tube filled with dissection media to separate the oocytes and remove the remaining follicular fluid. The aspirated liquid was taken until it was almost gone, there was a little part left at the bottom of the tube, and the remaining liquid in the tube was poured into a 100 mm petri dish that had been given a guideline. Selection and washing of cumulus-oocyte complex (COC) which has homogeneous cytoplasm and still has at least 3 layers of cumulus cells using a dissecting microscope with a magnification of 120-240x. The selection results were transferred to a 65 mm petri dish, then re-selected and the COC was transferred to a 35 mm petri dish containing the dissection media (MEM, GibcoBRL®).

Oocyte Maturation in Vitro

The cumulus-oocyte complex (COC) that had been selected from the collection medium was transferred into drops of maturation media. The medium used was MEM media and added with 0.15 IU/ml Pregnant Mare Serum Gonadotropin (PMSG) (PG600®, Canada), 0.15 IU/ml Hormone Chorionic Gonadotropin (hCG) (PG600®, Canada), and 3% Fetal Calf Serum (FCS) (F7524-Sigma-Aldrich®). Each Petri dish contained 5 drops of maturation media (50 μl/drop and each drop contained 5 oocytes) coated with mineral oil (Cryotech Lab, Japan) and then incubated in a 5% CO_2 incubator, 98% humidity, 38.5˚C for 22 o'clock. Furthermore, observations were made in each group using a CX41 microscope (Olympus, Japan)[26].

Identification of TNF-α Expression by Immunocytochemistry

Oocytes that have been maturated for 22 hours are placed on an object glass then aspirate the remaining fluid around the oocyte and apply Vaseline on four sides around the oocyte. The next step is to slowly cover the oocyte with a cover glass so that the oocyte is not compressed or damaged and then put it in a fixative solution containing 100% acetic acid (glacial) and absolute methanol in a ratio of 1:3 for 24 hours before immunocytochemistry (ICC) staining is performed. After fixation, the next stage was Immunocytochemistry (ICC) staining according to the procedure performed by Hoffman *et al.* (2008)[27]. Observation of TNF-α expression in cumulus-oocyte complex (COC) was performed under an Olympus® CX-41 microscope. The results of the observation of TNF-α expression were assessed semi-quantitatively based on the modified Remmele Scale Index method[28].

Malondialdehyde (MDA) Measurement

A total of 7 cumulus-oocyte complexes (COC) that had been matured in vitro for 22 hours were added with 100 μl of lysis buffer and vortexed for 5 minutes, then centrifuged at 300 rpm for 5 minutes to separate the supernatant from pellets. The supernatant obtained was then measured for MDA using the enzyme-linked immunosorbent assay (ELISA) method (Cat. No. E0156Ra, Elabscience, Texas, USA) and according to the procedure performed by Jusup *et al.* (2021)[29].

Data Analysis

The data obtained were analyzed using SPSS 23.0 software program (IBM Corp., NY, USA) to analyze the comparison between the treatment and control groups. The normality test using the Shapiro-Wilk test was carried out first to analyze the relationship between each variable. If the data is normally distributed, then one-way ANOVA is used and continued with Duncan's test to determine the differences between groups, whereas if the data is not normally distributed, it is continued with the Kruskal-Wallis test. This study uses a significance level of 0.05.

RESULTS AND DISCUSSION

This study used oocytes from goat ovaries which were grouped into 3 groups based on treatment with Alpha Lipoic Acid (ALA) supplementation on in vitro maturation media consisting of G0 without ALA supplementation, G1 with ALA supplementation 25 μmol/L, G2 with ALA supplementation 50 μmol/ L. The results of oocyte maturation in vitro for 22 hours and then identification of TNF-α expression in oocytes using immunocytochemistry staining with the addition of TNF-α antibody. The results of observations of TNF-α expression in oocytes tested for normality with Shapiro-Wilk did not show a normal distribution, so the Kruskal-Wallis nonparametric test was continued ($p<0.05$).

TABLE 1: TNF-α expression and MDA levels in goat oocytes after in vitro maturation.

Groups	TNF-α Expression (x±SD)	MDA Levels (μM) (x±SD)
G0	0.75^{a} ±1.39	26.52^{a} ±2.92
G1	5.56^{b} ±3.05	46.44^{b} ±4.87
G2	2.00^{a} ±1.80	30.41^{a} ±5.67

Note: Different superscripts (a,b) in the same column show significant differences between treatments ($p<0.05$). G0 was the control group without ALA supplementation, G1 was treatment group 1 with 25 μmol/L ALA supplementation, and G2 was treatment group 2 with 50 μmol/L ALA supplementation.

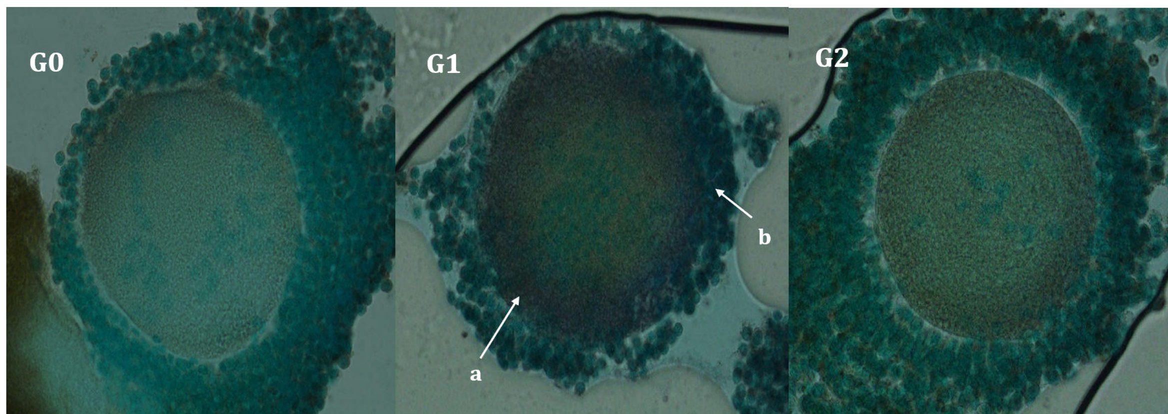

FIGURE 1: Representative results of TNF-α immunocytochemistry staining of goat oocytes after maturation in vitro in maturation media supplemented with Alpha Lipoic Acid (ALA) with 400X magnification. a) brownish color in oocytes, b) brownish color in cumulus. G0 is the control group without ALA supplementation, G1 is treatment group 1 with 25 μmol/L ALA supplementation, and G2 is treatment group 2 with 50 μmol/L ALA supplementation.

Based on table 1, the TNF-α expression value in the G0 group was 0.75±1.39, the G1 group was 5.56±3.05 and the G2 group was 2.00±1.80. The calculated TNF-α expression data in the control group (G0) and the G2 group did not show a significant difference, while G0 and G2 compared to G1 showed a significant difference ($p<0.05$). The results of immunocytochemistry staining showing TNF-α expression can be seen in Figure 1 with a color change in oocytes and cumulus cells to brown, whereas if it does not show TNF-α expression it will be greenish. The brown color seen in oocytes and cumulus cells indicates the presence of antigen-antibody binding, causing TNF-α to be expressed, while the green color in cumulus cells indicates there is no binding between antigen-antibody so that what is visible is the color of counterstaining methylene green (Figure 1).

The results of measuring levels of Malondialdehyde (MDA) in goat oocytes after 22 hours of in vitro maturation in maturation media supplemented with Alpha Lipoic Acid (ALA) were tested using the ELISA method (Table 1). The data on MDA levels that had been obtained were then analyzed for normality using Shapiro-Wilk showing normal distribution so that it could be continued with the One-Way ANOVA test and Duncan's test to show significant differences in values between groups, $p<0.05$. MDA levels in the control group (G0) were 26.52±2.92, treatment group 1 (G1) was 46.44±4.87, and treatment group 2 (G2) was 30.41±5.67. Data on MDA levels in the G0 group and the G2 group did not show a significant difference, while G0 and G2 compared to G1 showed a significant difference ($p<0.05$).

ROS production in oocytes increases during in Vitro Maturation (IVM). This is 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 hurt oocyte physiology[30]. Increased levels of ROS in oocytes that exceed the physiological range can cause oxidative stress and cause a decrease in oocyte quality[31]. Overproduction of ROS can lead to oxidative stress conditions associated with inflammatory conditions [32,33].

Oxidative stress can also be induced by the release of reactive oxygen species (ROS) from inflammatory cells[34]. Inflammatory cell-derived ROS have been reported to up-regulate the TNF-TNFR inflammatory pathway[35] and activate intracellular signaling cascades to increase proinflammatory gene expression[36,37]. Consequently, inflammation and oxidative stress are pathophysiological phenomena that are highly interrelated and inextricably linked[34]. This study proves that the results of the observation of TNF-α expression and the measurement of malondialdehyde (MDA) levels have the same pattern, meaning that they have a synergistic relationship (Table 1). ROS-mediated inflammation will induce the activation of Nuclear Factor Kappa B (NF-κB), Activator Protein-1 (AP-1), and Mitogen-Activated Protein Kinases (MAPK)[38]. ROS are also believed to phosphorylate Kappa B inhibitors (IκB) resulting in inflammatory factors such as NF-κB degradation, release, and activation. At the same time, NF-κB facilitates the production of inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α)[39]. TNF-α and TNFR1 transcription was increased in bovine cumulus cells from in vitro matured cumulus-oocyte complex (COC) compared to immature cumulus-oocyte complex (COC)[40]. TNF-α expression in cumulus cells and TNF-R in oocytes have been reported in several mammalian species[18]. The results of this study proved that TNF-α expression was found in cumulus cells and goat oocytes (Figure 1).

Based on several previous research reports, ALA supplemented in culture medium acts as an antioxidant by increasing the synthesis of glutathione (GSH), Total Antioxidant Capacity (TAC), and enzymatic antioxidants such as SOD and GPX4[25,41,42,43]. However, ALA supplemented in the maturation medium in this study may act as a pro-oxidant[44], thereby causing increased ROS production that mediates the inflammatory pathway.

In addition, the use of the type and dose of antioxidants in the maturation medium may not reduce oxidative stress in a specific (target) manner, thus producing any effect or producing harmful effects[45,46, 47]. This explanation can be considered valid because the antioxidant mechanism pathway is very complex and interrelated. For example, SOD can catalyze O2− but also produces another ROS such as H_2O_2[48].

Alpha Lipoic Acid (ALA) is soluble in lipids and water and is easily transported to cells where it is reduced to Dihydrolipoic Acid (DHLA). Alpha Lipoic Acid (ALA), which enters the cell, is then reduced by cytosolic enzymes such as GSH-reductase, thioredoxin reductase, and also mitochondrial enzyme E3 (dihydrolipoyl dehydrogenase). Mitochondrial enzyme E3 reduces ALA to DHLA by involving Nicotinamide Adenine Dinucleotide Phosphate (NADPH). Alpha Lipoic Acid (ALA) is also a substrate for the NADPH-dependent enzyme GSH-reductase[49,50].

In addition to acting as antioxidants, in vitro and in vivo studies have shown that reduced forms of ALA and DHLA also act as pro-oxidants (Akatay, 2006)44. There are some reports describing the potential pro-oxidant effects of ALA and DHLA[51]. The formation of thiol radicals and disulfide radical anions from DHLA means that these compounds can act as pro-oxidants[52]. Most of the pro-oxidant effects induced by thiol compounds have been attributed to the formation of reactive species, including superoxide, H2O2, and hydroxyl radicals[53].

The results of TNF-α expression and goat oocyte MDA levels in treatment group 2 (G2) were lower than those in treatment group 1 (G1) and showed significant differences. This may be because treatment group 2 (G2) supplemented with 50 µmol/L ALA in goat oocyte maturation media increased intracellular GSH regeneration and caused excess GSH which could reduce basal ROS and contribute to decreased activation of the proinflammatory NF-κB signaling pathway and reductive stress[54].

TNF-α expression and goat oocyte MDA levels in the control group (G0) compared to treatment group 2 (G2) did not show significant differences. This may be because supplementation of extracellular antioxidants in vitro maturation media did not provide a beneficial effect and perhaps cumulus cells isolated oocytes from the extracellular medium. The results of this study are linear with the research report conducted by Alvarez *et al.* (2015)[55] that the addition of extracellular scavengers could not reduce ROS levels in vitro matured pig oocytes and could not increase nuclear or cytoplasmic maturation. The results of this study showed that TNF-α expression and MDA levels of goat oocytes in treatment group 1 (G1) supplemented with ALA 25 µmol/L were higher than the control group (G0) without ALA supplementation and treatment group 2 (G2) supplemented with ALA 50 µmol/L and showed a significant difference. This may be due to the selection of a pharmacological dose of ALA that is not appropriate or the dose of ALA used is too large so it can cause cellular dysfunction by changing the redox balance after interacting with physiological concentrations of ROS (Martin *et al*., 2002). Based on several research reports, the majority of ALA doses used in experimental studies conducted in vitro ranged from 5 µmol/L to 10 µmol/L[56]. The use of ALA doses of 25 µmol/L and 50 µmol/L in this study was based on Azam *et al.* (2017) and He *et al.* (2021)[24, 25], so that excessive antioxidant supplementation can lead to a dangerous condition called the antioxidant paradox. The explanation for the occurrence of the antioxidant paradox according to Biswas (2016) is as follows; (i) failure to select antioxidant therapies that specifically target oxidative stress and inflammation, (ii) failure to use both antioxidant and anti-inflammatory agents at the same time, and (iii) use of nonselective agents that downregulate multiple oxidative and inflammatory pathways by causing side effects. others and may be responsible for the failure of preclinical or clinical trials of antioxidants [57].

CONCLUSION

The conclusion that can be drawn from this study is that supplementation of Alpha Lipoic Acid (ALA) at doses of 25 µmol/L increased TNF-α expression or MDA levels and 50 µmol/L on in vitro maturation media of goat oocytes could reduce the expression of Tumor Necrosis Factor (TNF-α) and levels of Malondialdehyde (MDA) than 25 µmol/L.

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