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# Supplementation of alpha lipoic acid on Kacang goat (*Capra hircus*) oocyte to the growth of GDF-9 and BMP-15

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**ABSTRACT**

Abstract. The application of assisted reproductive technology by mass-producing embryos is to maintain genetic resistance, increase productivity and livestock populations in a short time with maximum quality. Oocyte *In vitro* Maturation (IVM) is a crucial stage of *In vitro* Fertilization (IVF) implementation to increase the quantity and quality of livestock genetic trait through in *In vitro* Embryo Production (IVP). GDF-9 and BMP-15 expression are in the family of Transforming Growth Factor Beta (TGF-β) which are known to regulate oocyte and cumulus cell development in mammals. This study's objective was to investigate the effects of Alpha Lipoic Acid (ALA) on IVM media of Kacang goat (*Capra hircus*) oocytes. This research was an experimental study design consisting of 3 treatment groups: (P0) the control group, (P1) 25 µM/L ALA supplementation and (P2) 50 µM/L ALA supplementation. This study used 261 COCs which were 22 hours matured *in vitro* at 38.5°C, 98% humidity, and 5% CO2. GDF-9 and BMP-15 expression data were statistically tested with a significance of $p<0.05$. Immunocytochemistry showed the expression value of GDF-9 (2.86±1.06, 4.29±1.79 and 8.29±2.69). BMP-15 expression values (2.57±1.39, 5.71±3.72 and 11.43±1.51). The results of the study showed that supplementation with ALA on maturation media at a dose of 50 µM/L toward the maturation level and increased GDF-9 and BMP-15 expression *in vitro* maturation of Kacang goat oocyte.

## Introduction

A crucial preliminary step in assisted reproductive technology (ART) that affects the results of *in vitro* fertilization (IVF) procedure is the development of oocytes under *in vitro* maturation (IVM) procedure (Widjiati, 2020; Jose *et al*., 2022). The application of assisted reproductive technology by mass-producing embryos is to maintain genetic resistance, increase productivity and livestock populations in a short time with maximum quality (Lestari *et al*., 2019; Bahrami and Cotee, 2022).

The success of *in vitro* maturation is controlled by the quality of the oocytes as well as the maturation medium (Barakat *et al*., 2018; Obara *et al*., 2022). Oocyte maturation involved changes in the cytoplasmic structure and nuclear structure (Kirillova *et al*., 2021; Hermadi *et al*., 2023). The IVM process occurs when a surge in luteinizing hormone (LH) during pre-ovulation activates the cumulus cells to expand the cumulus-oocyte complex (COC) (Arroyo *et al*., 2020; Strączyńska *et al*., 2022). The mural granulosa and cumulus interact with each other to support nuclear maturation (Turathum *et al*., 2021; Sakaguchi *et al*., 2022). Nuclear changes are characterized by the presence of polar body I from the diplotene phase to metaphase II (MII) (Widjiati *et al*., 2022; Ozturk, 2022).

Oocyte maturation is one of the signs of the process of folliculogenesis with the granulosa cell's differentiation and proliferation (Liu *et al*., 2020; Mastrorocco *et al*., 2022; He *et al*., 2021a). Two important oocytes secrete growth factors (OSGF) which contribute to regulate the process of folliculogenesis are, growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) (Yu *et al*., 2020; Riepsamen *et al*., 2021; Elgebaly *et al*., 2022). GDF-9 and BMP-15 are polypeptide molecules from the TGF-β family that can induce cumulus cell expansion and increase oocyte maturation (Stocker *et al*., 2020). This growth factor also stimulates mRNA to stimulate FSH and LH for follicular development (Yang *et al*., 2020)

The success of IVM is lower than in vivo due to different cell physiology (Liu *et al*., 2018). Several factors contribute to the failure of IVM, one of them is the induction of apoptosis in oocytes as a result of oxidative stress brought on by an unbalanced antioxidant system and ROS generation during IVM (Mesalam *et al*., 2020). The IVM process generates more ROS than antioxidants endogenous (Cavallari *et al*., 2019). ROS must remain at normal levels and to decrease its negative effect, antioxidants are needed to neutralize high level of ROS (Ilmi *et al*., 2021). Several researchers added antioxidants to IVM media in order to neutralize ROS and improve competence in oocyte and embryo development (Rakha *et al*., 2022).


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Alpha lipoic acid (ALA) acts as an organic antioxidant. As a part of biological membranes and a cofactor of mitochondrial dehydrogenase, the antioxidant is able to scavenge ROS (Shindyapina *et al*., 2017). A study conducted by He *et al.* (2021b) stated that ALA can increase antioxidant levels in cells, significantly improving the quality of oocyte maturation and embryo development. *In vitro* studies on pre-antral follicles cultured from mouse ovaries has been improved follicular development by increasing total antioxidant capacity accompanied by decreasing ROS levels after supplementation of ALA (Talebi *et al*., 2012). ALA and the reduced form of dihydrolipoic acid (DHLA) have antioxidant potential in vivo and *in vitro* (Di Nicuolo *et al*., 2021; He *et al*., 2021b).

The synergistic relationship between GDF-9 and BMP-15 in increasing oocyte maturation and the benefits of ALA as an antioxidant. These two genes are involved in oocyte and follicle development. The purpose of this study was to determine the number of GDF-9 and BMP-15 genes that affect the maturation of oocytes and follicles in Kacang goats.

## Materials and methods

*Research design*

The study was conducted as a laboratory experiment using the ovary of the Kacang goat. COC Kacang goats were matured in an oocyte maturation medium which were divided into three treatment groups. The control group (P0) received Dulbecco's Modified Eagle Medium (DMEM) only, group one (P1) MEM + ALA 25 µM/L and group two (P2) MEM + ALA 50 µM/L.

*Ethical statement*

This research procedure has received ethical approval from the Faculty of Veterinary Medicine's Animal Ethics Committee, Airlangga University with certificate number 1.KEH.042.04.2022. Written consent prior to conducting research.

*Goat ovarian Sample*

The sample used in this study was the ovary of the Kacang goat taken from the Surabaya slaughterhouse. Ovarian samples were taken to the Faculty of Veterinary Medicine, Airlangga University's *In vitro* Fertilization sub-laboratory, Surabaya. 9 km away using a thermos containing NaCl with a temperature of 37°C.

*Oocyte aspiration and collection*

All blood in the ovary of the Kacang goat was rinsed using 0.95% NaCl. The ovaries were then aspirated using a 20G needle with a 5 ml syringe filled with 1 ml of phosphate buffered saline (PBS) with 100µ/ml gentamycin. The selected ovary is an ovary with a follicle measuring 2-6 mm. The liquid collected in the syringe was transferred into a petri dish and then oocytes were collected under a microscope with a magnification of 100-200x. The selected cumulus-oocyte complex (COC) only that have two or more layers of cumulus cells with a homogeneous cytoplasm. Oocytes were washed in a maturation medium twice. The selected COC were transferred into a petri dish containing medium Dulbecco's Modified Eagle Medium (DMEM) (Gibco®, USA) and then divided into three treatment groups.

*Alpha lipoic acid (ALA)*

The alpha lipoic acid (Chem CruzTM, Texas) supplementation was added to group 1 at a dose of 25µM/L (P1) and treatment group 2 dose 50µM/L (P2). ALA 0.1 mg and 0.05 mg were added and mixed with 10 ml MEM and 0.1 ml gentamicin sulfate (Indofarma®). PMSG and hCG 0.15 IU/ml (PG600®, Intervet, Boxmeer, Holland) and 3% FCS (FCS, F7524-Sigma-Aldrich®) are also being added until the pH reaches 7.5-7.8. The medium was then filtered using a 0.22 µm MCE membrane 25 filters, 47 mm (MF-MiliporeTM) then stored in a IVM medium container inside the refrigerator.

*In vitro maturation (IVM)*

The selected COC placed in petri dish 60 mm (Falcon ®) containing 6 maturation drops medium (50µl/drop), each drop contains 6-10 oocytes, then the drop is covered in mineral oil (Japan, Cryotech). The oocytes then matured in the incubator CO2 5% at 38.5ºC and 98% humidity for 22 hours. After 22 hours of being cultured (maturation process) COC expansion was seen in the oocytes which can be referred to as mature oocytes. The percentage of mature oocytes in each drop was P0: 60%, P1: 75%, and P2: 80%.

*Identification of GDF-9 and BMP-15 expression by Immunocytochemistry*

The COC that has been matured in each group is then placed on top of the polylisine coated object glass then covered it with a cover glass slowly. Oocytes were fixated using a fixative substance containing methanol and 100% acetic acid in a 1:3 ratio. After that oocytes were ready to be examined for GDF-9 (Santa Cruz® Catalog No. sc-514933) and BMP-15 (Catalog No. ABIN-7011715) using immunocytochemical staining.

Immunocytochemistry is a technique that uses chromogenic detection. Antibodies bind to specific antigens or proteins in the cell. The chromogen substrate change into a colored precipitate at the reaction site by enzyme-conjugated antibodies. The oocytes were then observed under a light microscope (H600L, Nikon) connected to a digital camera (DS Fi2 300) using 100x and 400x magnifications. Data was collected from the number of mature oocyte of each treatment.

The expression of GDF-9 and BMP-15 was calculated semi-quantitatively with the modified Remmele Scale Index (IRS), which is the results of multiplying the percentage of immunoreactive cells (positive cells) by the color intensity score generated on the cell (Hendarto *et al*., 2019).

*Statistical Analysis*

The data were derived from the number of mature oocyte after 22 hours maturation in each treatment. The data were analyzed using the Kolmogorov–Smirnov normality test first. Followed by One-Way ANOVA and Duncan's test to see which groups have significant differences ($p < 0.05$) (Leavy, 2017).

## Results

The results of GDF-9 expression by immunocytochemical staining were seen in the dark color of the immunoreactive cells which showed the effect in each group P0, P1, and P2 (Figure 1). All oocytes that were expanding in each drop were stained. This staining indicates the expression of the GDF-9. In contrast to the green color of oocytes, which indicates a lack of antigen-antibody binding and the appearance of the fundamental color Methyl green, the brown color seen in cumulus and oocytes indicates the presence of antigen-antibody binding, which causes GDF-9 to be expressed.

Table 1 shows the results of the GDF-9 expression value for each group P0, P1, and P2. Data from GDF-9 expression calculations between the control group (P0) and P1 did not show a significant difference. However, the P0 and P1 groups when compared to the P2 group showed significantly significant differences ($p<0.05$). The treated group of oocytes supplemented with 25 µM/L ALA expressed a darker color than oocytes supplemented with 50 µM/L.

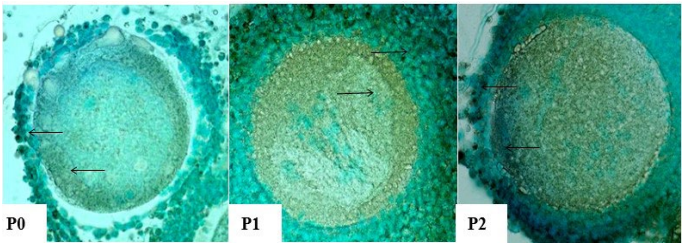


Fig. 1. Representative the result of Growth Differentiation Factor-9 (GDF-9) immunocytochemical staining in goat oocytes after *in vitro* supplementation with alpha lipoic acid (ALA) at 400x magnification. (P0): without ALA supplementation, (P1): ALA supplementation 25µM/L, (P2): ALA supplementation 50µM/L. Arrows indicate the presence of cells that are immunoreactive GDF-9 in COC immunohistochemical staining of Kacang goat.

Table 1. GDF-9 expression in Kacang goat oocytes after *in vitro* maturation

| Group | Amount total oocyte | No. of maturated oocyte | GDF-9 expression (Mean±SD) |
|---|---|---|---|
| P0 | 60 | 25 | 2.86±1.069[a] |
| P1 | 44 | 35 | 4.29±1.799[a] |
| P2 | 36 | 27 | 8.29±2.690[b] |

Note: [a, b, c] Mean values with different superscripts within the same column are significant p<0.05. P0: control group (wthout ALA supplementation), P1: ALA supplementation 25µM/L; and P2: ALA supplementation 50µM/L.

Immunocytochemical staining of BMP-15 proved a significant effect of ALA implementation of expression BMP-15. There was a significant difference between all treatment group (P0, P1, and P2) (p<0.05). Table 2 shows the BMP-15 expression increased from P2 to the control group (P0). Figure 2 shows the result of BMP-15 immunocytochemical staining. All oocytes that were expanding in each drop were stained. This staining indicates the expression of the BMP-15. BMP-15 expression in Kacang goats is determined by the immunoreactive cells' dark color in the immunocytochemical staining results, which show different colors. Table 2 also shows a significant increase in BMP-15 expression from treatment group 0, treatment 1 and treatment 2. ALA treatment group 50µM/L is the highest group with a value of 11.29. Therefore, it can be reported that ALA administration significantly affects the expression of BMP-15.

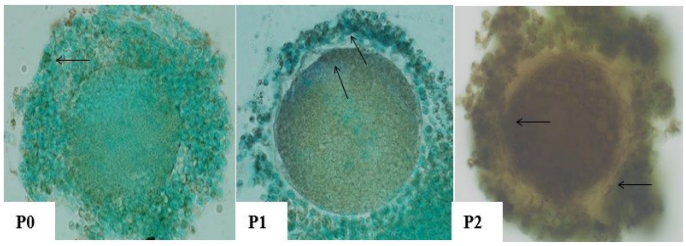


Fig. 2. Representative the result of bone Morphogenetic Protein-15 (BMP-15) immunocytochemical staining in goat oocytes after *in vitro* supplementation with alpha lipoic acid (ALA) at 400x magnification. (P0): without ALA supplementation, (P1): ALA supplementation 25µM/L, (P2): ALA supplementation 50µM/L. Arrows indicate the presence of cells that are immunoreactive BMP-15 in COC immunohistochemical staining of Kacang goat.

Table 2. BMP-15 expression in Kacang goat oocytes after *in vitro* maturation.

| Group | Amount total oocyte | No. of maturated oocyte | BMP-15 expression (Mean±SD) |
|---|---|---|---|
| P0 | 52 | 31 | 2.57±1.397[a] |
| P1 | 34 | 20 | 5.71±3.729[b] |
| P2 | 32 | 28 | 11.29±1.512[c] |

Note: [a, b, c] Mean values with different superscripts within the same column are significant p<0.05. P0: control group (without ALA supplementation), P1: ALA supplementation 25µM/L; and P2: ALA supplementation 50µM/L.

Figure 3 above shows that P0 treatment without ALA showed GDF-9 levels calculated based on the IRS method of 2.86, P1 treatment with 25 µM/L ALA of 4.29 and P2 50 µM/L ALA treatment of 8.29. BMP-15 levels on Fig. 3, also showed a significant increase between treatments. Each treatment was tested using a significance level of 5% (p<0.05), so that when the statistical test was found (p<0.05) it was said to be significant, whereas if (p>0.05) it was said to be insignificant.

Data on GDF-9 expression levels between the control group P0 and P1 did not show a significant difference, while the P0 and P1 groups when compared to the P2 group showed significantly significant differences (p<0.05). Data on BMP-15 expression levels between each group P0, P1 and P2 showed significant differences (p<0.05). group P2 expressed the highest BMP-15, namely 11.29 when compared to groups P0 and P1, respectively 2.57 and 2.57.

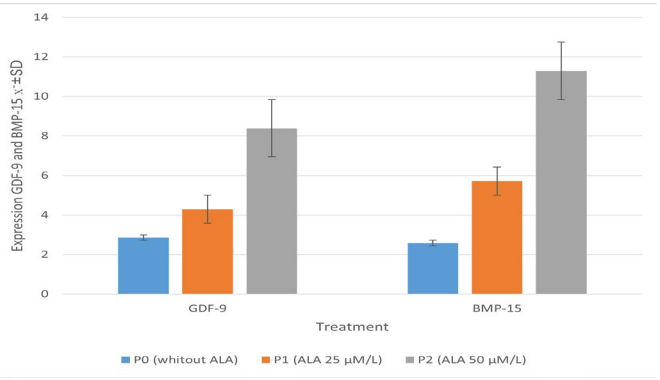


Fig. 3. Expression levels of GDF-9 and BMP-15 in Kacang goat oocytes on the IRS scale after *in vitro* maturation for 22 hours in 3 different treatment groups.

## Discussion

Alpha Lipoic Acid (ALA) as an antioxidant has been known as both lipid and water soluble (Attia *et al.*, 2020). The beneficial effects of ALA are related to cell growth and cell metabolism (Zeng *et al.*, 2023). In addition, ALA works synergistically with other antioxidants like GSH, vitamin C, and vitamin E in regenerating and activating antioxidants (Tripathi *et al.*, 2023). Alpha Lipoic Acid (ALA) has potential in the reproduction field, especially in IVF. The optimal concentration of ALA added to goat oocyte maturation media has the potential to increase blastocyst maturation and embryo development (Zhang *et al.*, 2022; Azari *et al.*, 2017). The therapeutic effect of ALA in an animal model using mice increases levels of in vivo natural antioxidants in the body (Liu *et al.*, 2018; Rezaie, 2020; Yang *et al.*, 2020).

The cellular transport of ALA as an antioxidant occurs through several systems (Sharma *et al.*, 2020). Fatty acid chain transport systems, vitamin transport with $Na^+$ ions, and monocarboxylate transport with $H^+$ ions (Metzler-Zebeli *et al.*, 2022). Through this transport system and its fat- and water-soluble feature, ALA can easily penetrate the cell monolayers and mitochondria. ALA is oxidized to lipoic acid (LA) and reduced to dihydrolipoic acid (DHLA) via the thioredoxin reductase (TRx) enzyme which is driven by Nicotinamide adenine dinucleotide phosphate (NADPH) (Theodosis-Nobelos *et al.*, 2023). Glutathione (GSH) plays a role in the ALA antioxidant system, which functions as a ROS scavenger and as a redox buffer to maintain cellular redox balance (Zhang and McCullough, 2016).

Previous research on ALA supplementation at a dose of 10 µM/L in oocyte maturation media was able to increase the level of GSH levels in bovine oocytes (Zhang *et al.*, 2013; Hassan *et al.*, 2017). The results of the study support earlier findings. The P2 group supplemented with ALA with a concentration of 50 µM/L on maturation media showed an average expression of growth differentiation factor-9 (GDF-9) with an average of 8.29. This is in line with the research by Azami *et al.* (2019) which stated that oocyte maturation can be evaluated through GSH levels which will increase together with GDF-9.

The value of the P2 group showed the highest expression compared to the P0 and P1 treatment groups. However, there was no significant difference in the mean GDF-9 between the group that was not supplemented with ALA and the group that was supplemented with 25 µM/L ALA. This shows that ALA at a dose of 25 µM/L has not been able to provide a protective effect from ROS. This situation is approximately due to the nature of ALA which is quickly absorbed but didn't last long in circulation, so that not all ALA reaches target cells (Prathima *et al.*, 2017).

The results of BMP-15 expression for treatment group 2 (P2) supplemented with 50 µM/L ALA were higher than in treatment group 1 (P1) supplemented with 25µM/L, the results showed a significant difference. This may be because ALA supplementation with a concentration of 50 µM/L can regenerate GSH and SOD enzymes intracellularly, causing GSH and SOD levels to increase which can reduce ROS levels (Hassan *et al.*, 2017).

Growth Differentiation factor-9 (GDF-9) and Bone Morphogenetic Protein-15 (BMP-15) are members of the family of TGFβ that are pre-

dominantly secreted by oocytes and play an important role in the development of ovarian folliculogenesis (Hendarto *et al*., 2019). Infertility has been linked to studies on female mice which deprived of the growth factor GDF-9, and showed impairment of follicular development after primary follicle growth. GDF-9 is a determining factor for the growth, maturation, and survival of follicles through inhibition of the apoptotic process in granulosa cells and follicular atresia (Orisaka *et al*., 2021).

The antioxidant action of ALA against ROS, supports GDF-9 and BMP-15 to maintain its physiological state in the oocyte (Yang *et al*., 2020). This is consistent with the average results of ALA supplementation on GDF-9 and BMP-15 expression which shows in increase GDF-9 and BMP-15 expression and according to the representative immunocytochemical results of the presence of brownish color indicates GDF-9 and BMP-15 expression in oocytes and cumulus cells (Tables 1,2 and Figure 1,2). Based on research conducted by Hendarto *et al*. (2019), the addition of the antioxidant curcumin can increase growth factor expression by reducing inflammation in oocyte-cell interactions granulosa will increase as a result of the expression of GDF-9 and Kit-L brought on by the anti-inflammatory effects of curcumin.

## Conclusion

This study concluded that ALA supplementation in IVM media had a positive effect on increasing the expression of GDF-9 and BMP-15 because the antioxidant effect of ALA could increase the interaction of cumulus cells and Kacang goat oocytes. Higher ALA dose (50 μM/L) leads to higher expression of GDF-9 and BMP-15 to support oocyte maturation. P0 showed mostly green colour, P1 showed a little bit brown and P2 showed mostly bbrown colour in oocyte nucleus.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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