# Moringa Leaf (*Moringa oleifera Lam*) Nanoparticle Supplementation on Zygote Cleavage in Goat Embryo Culture In Vitro

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## Abstract

One of the causes of failure of zygote development is oxidative stress in the culture medium. Where oxidative stress can damage the structure and function of zygotes due to free radicals Reactive Oxygen Species (ROS). Moringa oleifera Lam contains antioxidant polyphenol compounds, flavonoid flavanols namely quercetin which is the most antioxidant content in Moringa leaves which is expected to fight excess ROS. The purpose of this study was to prove that the supplementation of Moringa oleifera Lam leaf nanoparticles to increase zygote cleavage in goat embryo culture in vitro. The research subjects used goat oocytes which were divided into 4 groups consisting of 1 control group and 3 treatment groups. Each group consisted of 31 samples. Control group K1 without Moringa leaf nanoparticle supplementation, treatment group I P1 Moringa leaf nanoparticle supplementation with a dose of 0.5 µM, treatment group II P2 supplementation of Moringa leaf nanoparticles with a dose of 1.0 µM, Treatment group III P3 supplementation with Moringa leaf 2.0 µM. Embryo culture was carried out in a 5% CO2 incubator, temperature 38.5ºC for 48 hours, then observed under an inverted microscope. The results show that there are significant differences with the value of $p = 0.041$ which means $p < 0.05$. Supplementation of moringa leaf nanoparticles (Moringa oleifera Lam) can significantly increase zygote cleavage at embryo culture stage in vitro.

***Key Words:*** *Moringa oleifera Lam, Reactive Oxygen Species (ROS), Zygote Cleavage*

## Introduction

Infertility has an impact on population decline leading to zero population growth (without changes in mortality and migration)[1]. An estimated 4-6 million couples need infertility treatment to get offspring such as hormonal treatment, In vitro Fertilization (FIV)[2].
The success of In Vitro Fertilization (FIV) is not only influenced by oocytes, but also by spermatozoa which are used to fertilize and physiological conditions of the culture medium used[3].

The success of In Vitro Fertilization in couples of childbearing ages is 32.2% (Ramalingam et al., 2016). Based on the ability of zygotes to overcome cell block (developmental barriers) in the development of zygotes into phase two cells in the M16 medium at 85.09% and on the HTF medium at 83.36%. The zygote obtained from FIV in the culture medium can develop into a two-cell stage, but there are also zygotes that cannot develop and the cells undergo apoptosis, picnosis, and fragmentation[3].

One of the causes of zygote failure to overcome cell block is due to oxidative stress in the culture medium due to free radicals Reactive Oxygen Species (ROS). Endogenous antioxidant defenses in zygotes are not enough to fight the oxidative stress encountered during in vitro culture so that it can cause membrane lipid peroxidation, DNA, and protein in zygotes[4].

Modifying the condition of in vitro culture medium is one technique to increase the number of fertilization and viability of zygotes to blastocysts[5].Modifying the condition of the culture medium by the addition of exogenous antioxidants has been carried out by Gaviria et al. [6] about the addition of the antioxidant resveratrol which is present in many plant foods, especially red wine

in in vitro culture media in enhancing the development of bovine embryos from the stages of cleavage, morula and blastocyst. In addition to red wine, there are also exogenous antioxidants from plants, namely Moringa oleifera Lam leaves made in the form of nanoparticles to overcome oxidative stress[7].

Moringa oleifera Lam contains antioxidant polyphenol compounds, flavonoid flavanols namely quercetin which is the most antioxidant content in Moringa leaves. Quercetin will bind free radical species in the presence of hydroxyl groups (OH-) at C to 3, 5, 7, 3 ‘, and 4’ and catechol ring β[8].With the presence of natural antioxidants from moringa leaves, it is expected to reduce oxidative stress in the culture medium thereby increasing zygote cleavage in stage 2 cells[7].

## Material and Method

The process of making Moringa leaf nanoparticles is carried out in Nanobox Jl. Raya Serpong KM.2 Setu, Tangerang. All protocols were approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Airlangga University No. 2.KE.136.07.2019.

### Experimental design and sampling

This study uses a research design that is a pure experiment using a completely randomized design (CRD) with the Randomize Posttest-only Control Group Design approach carried out 5 times. The study was conducted in the in vitro laboratory of the Faculty of Veterinary Medicine of Airlangga University in July - October 2019. The research subjects used goat oocytes which were divided into 4 groups consisting of 1 control group and 3 treatment groups. Each group consisted of 31 samples. All data are first tested for normality using the Shapiro-Wilk test and Homogeneity test using the Levene test. Then proceed with the Kruskal Wallis test, obtained significantly different results (p <0.05) then proceed with the Mann Whitney test between 2 treatments each.

## Findings

### Zygote Cleavage

**Table 1: The result of Zygote Cleavage**

| Group | | Average±±SD |
|---|---|---|
| $K_1$ | (Control) | 6.6680±±9.13054 |
| $P_1$ | (0,5 µM) | 3.3340±±7.45505 |
| $P_2$ | (1,0 µM) | 3.3340±±7.45505 |
| $P_3$ | (2,0 µM) | 16.6700±±.00000 |

Table 1 shows that administering a 2.0 µM dose of Moringa leaf nanoparticles in embryo culture in vitro can increase zygote cleavage.

**Table 2: p value of Zygote Cleavage**

| Variable | p value | explanation |
|---|---|---|
| Zygote cleavage | .041 | There is a significant difference |

Kruskal Wallis test results showed a value of p <0.05 meaning that there were significant differences in the cleavage of zygotes at the stage of embryo culture in vitro between groups K1 P1 P2 P3.

**Table 3:The differences between groups of zygote cleavage variables**

| Group | $P_3$ | $P_2$ | $P_1$ |
|---|---|---|---|
| $K_1$ | .050 | .513 | .513 |
| $P_1$ | .014 | 1.000 | |
| $P_2$ | .014 | | |

Based on the table above, the most different groups are in the P3 group against K1, P1, and P2, namely .050, .014, .014. These results indicate that the supplementation of Moringa oleifera Lam Moringa leaf nanoparticles at a dose of 2.0 µM can significantly increase zygote cleavage.

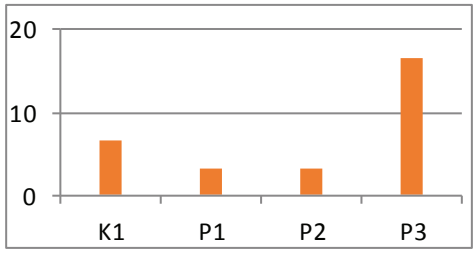


**Figure 1: Mean graph of zygote cleavage at embryo culture**

**stage in vitro**

In Figure 1, the results of the study showed that the highest zygote clavage (16.66) was found in group P3 (Treatment III Nano dose 2.0 μM).

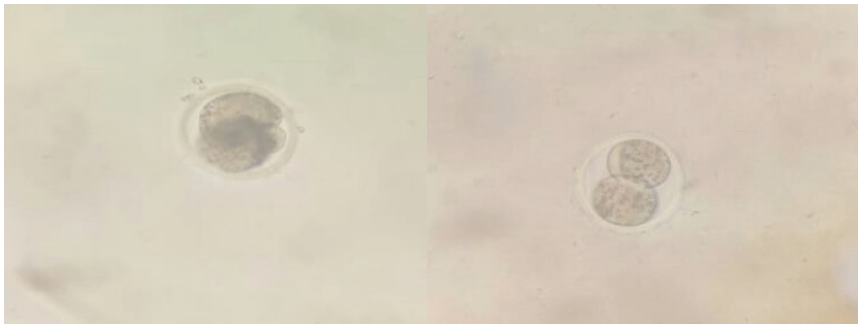

**Figure 2: Results of the cleavage of zygote 2-cell using an inverted microscope**

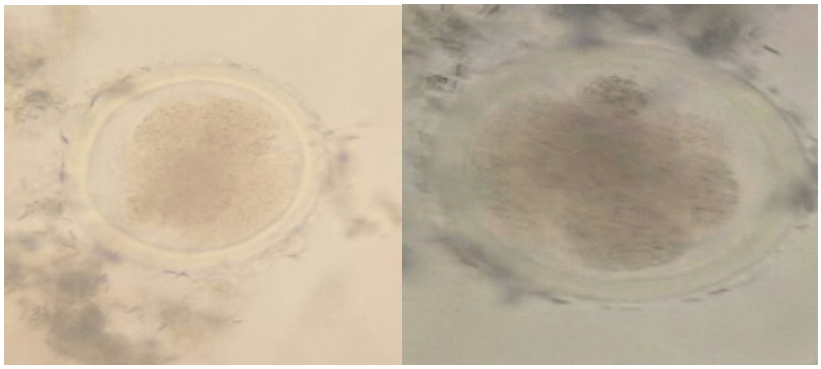

**Figure 3: The results of the cleavage of zygote 4 cells and 8 cells using an inverted microscope**

## Discussion

At the time of embryo culture in vitro can trigger the occurrence of Reactive Oxygen Species (ROS) due to external conditions or in vitro culture medium. ROS can come directly from gametes and embryos, or from their environment[9]. Although ROS can regulate cell function and activate key signaling pathways, increased ROS can cause the formation of highly radial hydroxyl which can increase lipid peroxidation, increase DNA oxidation and damage DNA nuclei[10], increase protein oxidation[11].

In the female reproductive system such as the ovary there are endogenous antioxidants which naturally protect the embryo from oxidative damage, but in the production of embryos in vitro, the embryo lacks endogenous antioxidants because it continues to be exposed to stressors that are too high[6]. To determine the ability of the zygote through the observation of developmental barriers (cell block), which is based on the ability of the developing embryo to reach the stage of two cells[12]. In addition, ROS levels exceed basal levels, nuclear factor-kappa B subunit 1 (NFkB) is induced. NFkB is activated by ROS during embryo development, so that it can result in cell death in the embryo[13].

Moringa oleifera Lam leaves contain a source of vitamin A, C, B calcium, protein, and potassium[14]. In addition, it contains special plant pigments with strong antioxidants [15]. The most antioxidant in moringa leaves is quercetin, where the system works by binding to free radical species in the presence of hydroxyl groups (OH-) at C to 3, 5, 7, 3 ‘, and 4’ and catechol ring β[8]. Quercetin is a powerful antioxidant whose strength is 4-5 times higher compared to vitamin C and vitamin E which are known as potential vitamins[16]. Quercetin as an antioxidant can prevent oxidation through hydrogenation and complex formation and prevent auto-oxidation[8].

This is consistent with Uswatun’s research[17] that Antioxidants are needed to fight excess ROS in zygote cells so as to prevent zygote cell death and can develop to the next stage. The right concentration of antioxidants can contribute to the generation of high-quality embryos[13].

In this research, Moringa oliefera Lam

containing exogenous antioxidants is made in the form of nanoparticles to more quickly penetrate the space between the intended cells and to specific cells[18]. Exogenous antioxidants can be added to a culture medium to protect the embryo from oxidative stress (OS) during in vitro culture (IVC) via the lipophilic cation conjugation pathway by penetrating the zygote cell membrane lipid bilayer[19].

There is research conducted by Barakat et al.[14] about Moringa oleifera found that with a dose of Moringa oliefera extract 100 µg / ml effectively increases the level of maturation of sheep oocytes and can act as a promoter to induce mRNA gene expression (Cyclin B and CDK2 for cell cleavage control), and synthesis of essential proteins, eg. MPF (Maturation Promoting Factor). MPF is a complex protein consisting of two subunits including a catalytic subunit (P34 CDK2 kinase) and a regulator subunit (Cyclin B). While MAPK (Mitogen Activated Protein Kinase) activates cyclin (cell cycle protein). The abundance of specific transcripts in mRNA pools is important for the first embryonic cell cleavage[14].

Where MAPK (Mitogen Activated Protein Kinase) namely Cyclin B (cell cycle protein) is activated by serine protein and MPF (Maturation Promoting Factor) namely CDK2 is activated by threonine protein. The binding between Cyclin and CDK triggers mitosis by activating cyclin binding CDK to Cyclin CDK Complex so that it triggers cells to pass the G2 check point to the M phase (Mitosis) in the cell cycle[20].

Determination of the 0.5 µM and 1.0 µM doses of the researchers refers to the study of Gaviria et al.[6] Supplementation of medium culture with low levels of resveratrol improves embryo quality[21]. Low doses cause a significant decrease in cleavage age and blasocyst rate, which does not produce a fast-developing blastocyst percentage.

In pharmacodynamic tests, it is only fitting that the greater the dose used, the greater the effect will be on the dose-response effect. The therapeutic effect is a function of dose and time, such charts illustrate the dose-response relationship that is not time-dependent. In this situation the maximum effect intensity (ceiling effect) can be caused[22]. In this condition, it can also be concluded that the dose of moringa leaf nanoparticles which can cause an effect that is an increase in zygote cleavage is at a dose of> 2.0 µM while the doses of 0.5 µM and 1.0 µM do not have pharmacological effects in this case do not support embryonic cell cleavage.

Osman et al (2015) reported that the dose of Moringa leaves was given incorrectly and could cause toxicity to the cells themselves[23]. These effects can be mutually supportive but can also be contradictory. The variety of active chemicals found in plants will work in synergy to produce the expected therapeutic effects and these effects can be lost or can cause adverse effects when the chemicals are not in accordance with the target cell needs[24].

The form of the reproductive system of living things for each species is different so that the ability to reproduce is also different. The lust cycle will affect the time for holding in vitro fertilization and affect the success of fertilization. The process of fertilization in goats is around 20-24 hours while in cattle it is 36 hours. So that the positive effect of antioxidants on embryonic development in vitro is shown in cows on embryonic development to blastocysts because cow embryos can tolerate low antioxidant concentrations in order to produce beneficial effects[23]. In addition, goat spermatozoa have phospholipase A2 in plasma causing early acrosome reactions and result in spermatozoa cells being damaged more quickly than cattle spermatozoa. As a result, the failure of in vitro embryo production among goats is higher than cattle[25].

So, resveratol with a dose of 0.5 µM and 1.0 µM has been able to increase the development of cow embryos due to the adequacy of energy and antioxidants while a dose of 0.5 µM and 1.0 µM in moringa oliefera produces energy and antioxidants needed are still lacking so that it can trigger embryonic cell damage.

Antioxidants can cause prooxidant activity under certain conditions, such as at high doses or when there are metal ions. Prooxidant activity is highly dependent on its concentration at high doses (> 50 µM) can potentiate superoxide radicals (O2-) in isolated mitochondria and cells that are cultured, reduce cell survival and cause damage to DNA, proteins, lipids while too low doses can induce cytotoxicity, DNA strand breaks and apoptosis[26].

## Conclusion

In the number of doses between Moringa leaf extract and nanoparticles is not much different in increasing zygote cleavage in vitro because there has been no research on organic nanoparticles against zygote cleavage in vitro so in this study the amount of Moringa

leaf nanoparticles dose is based on previous research journals on natural antioxidant extracts in plant.

**Conflict of Interest:** The authors declare that there is no conflict of interest.

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