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Calcium Ionophore Supplementation in *in Vitro* Fertilization Medium Towards Fertilization Rates of Kacang Goats (*Capra hircus*)

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ABSTRACT

In vitro fertilization is assisted reproduction technology that can overcome infertility problems. This method can produce embryos with high quality in large quantities. In its application, the success rate of IVF is still relatively low. This is due to the poor quality of oocytes and spermatozoa, which can cause IVF failure. The absence of oocyte activation during fertilization also results in failure of fertilization. Calcium ionophore is known to be able to increase the oocyte activation process. This study aims to prove that Calcium ionophore can increase the rate of fertilization in goat oocytes *in vitro*. This study was divided into two groups: the control group was fertilized oocytes using a medium without Calcium ionophore supplementation, the treatment group was fertilized oocytes using a medium supplemented with Calcium ionophore at a dose of 5.2 µl/ml. Fertilization rates are observed based on the formation of zygote or two pronuclear (2PN) in the cytoplasm of oocytes and observed under an inverted microscope. The data obtained were analyzed using the T test. The results of the study after being analyzed statistically showed that there were significant differences between the control group and the treatment group ($P<0.05$). Fertilization rates in the treatment group (93.12±13.7) showed higher results than the control group (65.96±28.36). Based on these results it can be concluded that calcium ionophore supplementation at a dose of 5.2 µl/ml in the fertilization medium can increase oocyte activation thereby increasing the rate of oocyte fertilization of the goat nuts.

Key words *: Food production, Infertility, Oocyte activation, Medium, Supplementation.*

Introduction

In the field of reproduction, infertility is a problem that is still commonly found in both humans and animals. According to the World Health Organization (WHO) in 2012, there were around 50-80 million couples experiencing infertility in the world. Efforts that can be made in tackling infertility are assisted reproductive technologies that are now increasingly developing. One of the assisted reproductive technologies that are now popular for tackling the problem of infertility is *In vitro* Fertilization (IVF). IVF is a very profitable method, because besides being able to overcome infertility problems, this method can produce embryos with high quality in large numbers (Amin, 2000). Kacang goat is a local Indonesian goat whose existence began to be marginalized because of its small shape different

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from outside goats like Boer goats. Although Kacang goat is an Indonesian germplasm, people are less interested because of the small body shape of the goat so that the number of carcasses is less. The success of IVF methods in conventional goats is still relatively low. With IVF technology, it is expected to be able to increase the population of Indonesian Kacang goats.

The success rate of IVF methods in conventional goats is still relatively low. Research conducted by Boediono *et al.* (2000) states that the success rate of *in vitro* fertilization carried out in goats only reached 40.91%. In addition, oocyte failure after fertilization to reach the blastocyst phase of *in vitro* fertilization is high, approximately until 60% (Gilchrist and Thompson, 2007). One of the factors that mostly support the success of IVF is the quality of spermatozoa and oocytes used. The quality of oocytes can be seen by the presence or absence of cumulus cells surrounding the oocytes, which act as chemo attractant spermatozoa to reach the oocytes so the fertilization occurs.

Oocytes with intact cumulus cells provide essential factors during the process of maturation, play a role asin maintaining oocytes, during the stages of meiotic division and have the potential to be fertilized. Besides those spermatozoa also have an important role in the success of *in vitro* fertilization. During the fertilization process, spermatozoa make a very important contribution to the process of division and development of the embryo by providing oocyte activation factors, centrosome components, and paternal chromosomes (Dogan *et al.,* 2015). Oocytes activation is one of the factors that influence the success of fertilization. Oocyte activation can occur with complex interactions triggered by the entry of spermatozoa into the oocyte. An early indicator of oocyte activation is characterized by repeated increase of intracellular calcium concentrations (Karabulut *et al.,* 2018).

Calcium ionophore (CaI) A23187 is a compound commonly used to increase intracellular calcium (Ca^{2+}). CaI A23187 is a chemical compound that acts as a divalent cation ionophore, which allows these ions to pass through cell membranes that are generally inaccessible (Nalley and Hine, 2015). This allows the administration of CaI A23187 to increase intracellular Ca^{2+} levels in oocytes. An increase in intra-cellular calcium is an initiator mechanism of oocyte activation that can be observed after a complex interaction which triggered by the entry of sperm cells into the oocyte during the fertilization process (Karabulut *et al.,* 2018). During fertilization, the endoplasmic reticulum in the oocyte releases Ca^{2+} ions which are known as important triggers for the development of oocytes into embryos (Hardy 2002). Increased Ca^{2+} levels in the cytoplasm of the oocyte will initiate pronucleus formation, which is a sign that the oocyte has been fertilized.

An increase in intracellular calcium levels can be increased by administering CaI A23187. Therefore, it is necessary to conduct research on supplementation of CaI A23187 in *in vitro* fertilization medium so that intracellular Ca^{2+} levels in oocytes can be increased.

Materials and Methods

This research went through several stages, i.eoocyte collection, oocyte maturation, spermatozoa preparation, *in vitro* fertilization and observation of fertilization rates. This research has obtained ethical eligibility with the number: 1.KE.061.04.2019 from the Faculty of Veterinary Medicine, UniversitasAirlangga.

Oocyte Collection

Kacang goat ovaries as a source of oocytes were obtained from Surabaya Abattoir as many as 54 ovaries. The ovaries are cleaned from the mesovary, then washed with 0.9% physiological NaCl to clean the remaining fat and blood attached to the ovaries and stored in 0.9% physiological NaCl which has been added with 50 µg/ml gentamycin sulfate, at a temperature of 30-35 ^{o}C. The ovaries were brought to the laboratory to be washed again thoroughly using 0.9% physiological NaCl which had been added with 50 µg/ml gentamycin sulfate. Furthermore, the oocyte is taken out from follicles with a follicular surface diameter of 3-5 mm, by aspirating ovarian follicular fluid using a 10 ml disposable syringe that has been filled with aspiration media namely Dulbeccos Modified Eagle Medium (DMEM-Sigma-Aldrich, Inc, St. Louis, Missouri United States). The aspiration follicular fluid is accommodated in a sterile tube, waited 5-10 minutes until sediment of oocyte show up. After 10 minutes the supernatant is removed and the sediment is poured in a petri dish and added with DMEM medium. The oocyte collection is then carried under inverted microscope. Oocytes from the collection are washed with DMEM medium until clean. The clean oocytes are selected and only oocytes in grade A, where it

coated with more than 3 layers of cumulus cells used for *in vitro* maturation were choosen.

Maturation of Oocytes

The maturation medium used was DMEM added with Pregnant Mare Serum Gonadotrophin (Folligon, PT Intervet Indonesia, Jakarta Indonesia) 50 µg / ml medium. The maturation medium is prepared 20 hours before being used in a warm way that is incubated in a 5% CO_2 incubator at 38.5 °C. Medium maturation in the form of medium drops with a size of 50 µl and covered with mineral oil (Sigma-Aldrich, Inc, St. Louis, Missouri United States). Grade A oocytes that have been selected after oocyte collection, are then washed using the maturation medium before being inserted into the drops of the maturation medium. In 1 drop, 50 µl maturation medium is filled with 5-6 oocytes. Then drop the maturation medium which has been filled with oocytes incubated in incubator of 5% CO_2 at 38.5 °C for 22 hours.

Preparation and Capacitation of Spermatozoa

Rosset medium in the form of large pits connected by bridges to small pits is prepared 20 hours before being used for spermatozoa preparation. The Rosset medium was incubated in a 5% CO_2 incubator at 38.5°C to warm. The spermatozoa used for *in vitro* fertilization are derived from frozen semen of the goat nuts obtained from the Insemination Center for Animal Husbandry Education at the Faculty of Veterinary Medicine, UniversitasAirlangga, Surabaya. Spermatozoa preparation and spermatozoa capacitation were performed two hours before *In vitro* Fertilization. The frozen semen was thawed at 35°C, removed from the straw and put in a centrifuge tube. The centrifuge tube that has been filled with cement plus DMEM medium with a ratio of 1: 1, then centrifuged at a speed of 2500 g for 10 minutes. The supernatant is removed using a carefully sterilized Pasteur pipette. After that, spermatozoa pellets were dropped 150 µl into a large well on a prepared rosset medium. Medium form of rosset that already contains spermatozoa is left for 30 minutes to give the spermatozoa an opportunity to move towards a small well. After 30 minutes the motile spermatozoa will move towards small wells.

***In vitro* fertilization**

Fertilization medium is prepared 20 hours before fertilization *in vitro*. In this study, prepared 2 Petri dish*in vitro* fertilization medium consisted of 1 petri dish *in vitro* fertilization medium for the control group (P0): DMEM medium without supplementation, and 1 more petri dish for the treatment group (P): DMEM medium that was given CaI A23187 at a dose of 5.2 µl/ml. before use incubated in incubator of 5%CO_2 temperature 38.5°C. Oocytes from *in vitro* maturation were transferred to the fertilization medium which had previously been added with spermatozoa at a dose of 500,000-1,000,000 spermatozoa. After the spermatozoa and oocytes are mixed in the Fertilization medium then reinserted in a 5% of CO_2 incubator at 38.5 °C for 24 hours to give the spermatozoa time to fertilize the oocytes.

Observation of Fertilization Rates

Observation of fertilization rates were done 24 hours after the *in vitro* fertilization process and observe based on the formation of two pronuclear (2 PN) or zygote (Widjiati *et al.,* 2012). Oocytes that are successfully fertilized are characterized by the formation of two pronuclear (2 PN) in the cytoplasm of the oocyte which can be observed 24 hours after fertilization. Observation of the number of zygotes formed using inverted microscope Nikon Diaphot 300 with magnification of 40 times.

Statistical analysis

Data obtained from the number of fertilized and non-fertilized oocytes between the control group and the treatment group were analyzed using the statistical T Test. They were considered to be significantly different if $P< 0.05$.

Results and Discussion

The study compared fertilization rates performed on two fertilization mediums, namely the control group (P0) using the medium without supplementation and the treatment group using the medium with calcium ionophore supplementation at a dose of 5.2 µl / ml with each group performed 9 replications. The number of oocytes used as a source of gametes in this study is limited, because the number of goat'sKacang ovaries obtained by the abattoir is little related to the goat Kacang population which is starting to decline. Observation of oocyte number of goat nuts can be observed 24 hours after *in vitro* fertilization culture. Fertilization rates can be determined by observing the formation of 2PN in the oocyte cytoplasm, which can be observed under an

inverted Nikon Diaphot 300 type microscope with a magnification of 40 times. Based on data obtained using the Mann Whitney U test on SPSS, the results of the research from the two research groups showed significantly different results (P<0.05). The results are shown in Table 1 and Fig. 1.

In the treatment group, the oocytes were fertilized using CaI A23187 supplementation medium showed a higher percentage of fertilization rates than the oocyte group which was fertilized with the medium without supplementation (Table 1). Based on the table above shows that the control group was significantly different from the treatment group (*P*<0.05), this shows that CaA A23187 supplementation on the fertilization medium was proven to increase the rate of fertilization by the method of *in vitro* fertilization. In the treatment group, ie oocytes were fertilized using CaI A23187 supplementation medium showed a higher percentage of fertilization rates than the oocyte group which was fertilized with medium without supplementation (Table 1). Based on the table above shows that the control group was significantly different from the treatment group (P<0.05), this showed that supplementation of CaI A23187 in the fertilization medium was proven to be able to increase the rate of fertilization by *in vitro* fertilization methods.

In vitro fertilization culture involves the fusion of mature oocytes and spermatozoa that are cultured in a medium. Mature oocytes can be obtained by maturation *in vitro* with medium maturation. After the vitro maturation process, the mature oocytes are characterized by the expansion of the accumulation cells, which can be observed under an inverted microscope (Fig.1A). The mature oocytes are transferred in a fertilization medium which is then reunited with the spermatozoa which are capacited. Capacitated spermatozoa move towards the oocyte and will bind to the oocyte pellucida zone. After binding to the oocyte pellucida zone, spermatozoa will penetrate the pellucida zone which will then occur fusion between spermatozoa and oocytes. The fusion process between spermatozoa and oocytes is called the fertilization process (Fig. 1B). The process of fertilization *in vitro* can be declared successful if in the cytoplasmic the oocyte forms 2 pronucleus which can be observed 24 hours after the culture of fertilization under microscopic inverted (Fig. 1C).

In vitro Fertilization (IVF) is a reproductive biotechnology that is commonly used to overcome infertility problems that have been widely applied both to humans and animals. IVF is a method of fertilization that is carried out outside the parent body in a medium. This method is a beneficial method because it can improve the genetic quality of the embryos produced and overcome problems related to infertility. The occurrence of fertilization has a close relationship with oocyte activation. Oocyte activation is initiated by repeated oscillations or intracellular Ca^{2+} levels. This oscillation is initiated by the release of Ca^{2+} from inositol 1,4,5-trisphosphate (InsP3) which plays a role in increasing Ca^{2+} concentration by activating the InsP3 Receptor (InsP3R) which causes rapid calcium release and results in increased Ca^{2+} levels in the cytoplasm (Dutta, 2000). Increased intracellular Ca^{2+} levels contribute to the resumption of meiotic division and the formation of pronuclei in cytoplasmic oocytes, whichis a sign that the oocyte has been fertilized (Nomikos *et al*., 2017).

Oocyte activation is the transition from oocytes to zygotes where a series of intracellular Ca^{2+} oscillations will occur initiated by the fusion between spermatozoa and oocytes. Calcium oscillations play an important role in fertilization, and calcium ions act as secondary messengers in different metabolic pathways and cellular processes during oocyte acti-

Table 1. Mean, standard deviation and percentage of effect of post fertilization CaI A23187 supplementation on *in vitro* fertilization rates.

Observation	Group of Treatment		P
	P0 (Without CaI Supplementation)	P1 (With CaI Supplementation)	
Fertilizes Oocytes	$65,9^{b}$±28,36	$93,1^{a}$ ± 13,70	0.028*
Infertilized Oocytes	$34,04^{a}$±26,74	$6,88^{a}$ ± 13,70	0,244

* p <0.05 indicates there is a significant difference
Information:
P0 = Control group without CaI supplementation 23187
P1 = Treatment group (With CaI supplementation 23187)

vation. Upon oocyte activation, calcium ions are released from intracellular storage in the endoplasmic reticulum; free in the cytosol, which functions as an intracellular messenger and acts as a process modulator in the early stages of fertilization and embryo development (Hojnik and Kovacic, 2019). Under normal circumstances, the presence of sperm in the cytoplasm of the oocyte will evoke characteristic patterns of intracellular calcium oscillation, where extrusion of polar bodies will occur, exocytosis of cortical granules, cytoskeletal rearrangement, recruitment of maternal mRNAs, and formation of pronucleus (Yeste *et al.,* 2017).

In the development of embryos in the IVF method, oocytes are placed in a medium that is made to resemble the conditions in the female reproductive tract and contain nutrients needed for each development. The composition of nutrients in the culture medium is made close to the composition of nutrients, electrolytes, and macromolecules that are in the female reproductive tract (Widjiati *et al.* 2012). In the process of fertilization, spermatozoa must pass through cumulus cells, the pelucida zone, and the vitellin membrane. Entry of spermatozoa into the egg cell involves attaching the head of spermatozoa cells to the surface of the vitellin membrane is a very important period because this is when oocyte activation occurs. Oocyte activation is initiated by an increase in intracellular Ca^{2+} levels which is a signal that contributes to the resumption of meiosis and early embryonic development (Karabulut *et al.* 2018).

In a culture medium in IVF, conditions of the female reproductive tract cannot be fully replicated *in vitro*, but the ability of the medium used in the *in vitro* fertilization method can be improved by adding various substances and compounds to the culture medium. Modification of *in vitro* culture conditions is one technique that can be used to increase the rate of fertilization and viability of blastocysts (Widjiati *et al.,* 2017). The addition of supplements to the culture medium aims to increase the success rate of IVF depending on the function of substances and compounds used.

Calcium ionophore (CaI) A23187 is a compound commonly used in research to increase intracellular

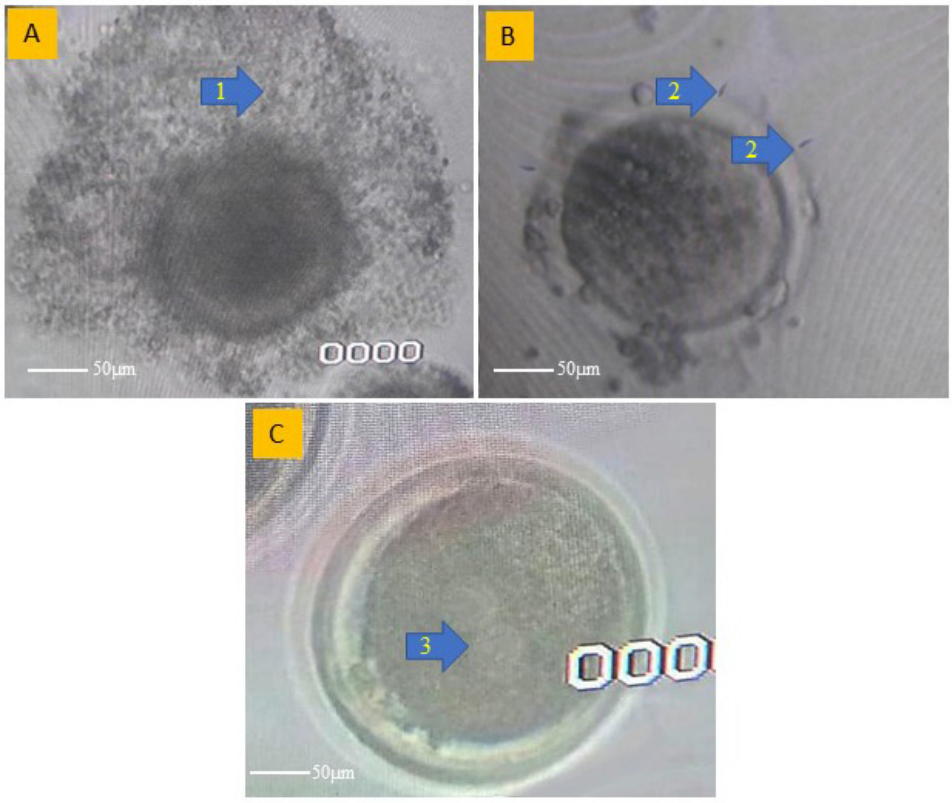

Fig. 1. (A) Mature oocytes characterized by expansion of cumulus cells (1), (B) Spermatozoa that penetrate the oocyte pelucida zone walls (2), (C) and fertilized shell oocytes which are characterized by the presence and 2 pronucleus (3) observed under an inverted microscope at 40x magnification

calcium. This substance has been shown to be able to activate cells by inducing Ca^{2+} signaling, intracellular Ca^{2+} mobilization, and activating Ca^{2+} entry (Dedkova *et al*. 2000). In the field of *in vitro*, CaI A23187 is often used in the method of intra cytoplasmic sperm injection (ICSI) which is an IVF method by injecting spermatozoa on the cytoplasm of oocytes using a micromanipulator. In the ICSI method, CaI A23187 plays a role in activation after spermatozoa injection (Eftekhar *et al*., 2012). The study compared the use of fertilization medium without supplementation (control group) and medium supplemented with calcium ionophore (treatment group). Supplementation was carried out using Dulbeccos Modified Eagle's Medium (DMEM) as a base medium supplemented with CaI A23187 at a dose of 5.2 µl/ml. In the treatment group, the mature oocytes were cultured on supplementation medium and then incubated in CO_2 incubators for 24 hours. DMEM media was a basal medium consisting of vitamins, amino acids, salts, glucose, and pH indicators. This media does not contain protein or growth agents. This media requires complete supplementation such as Fetal Bovine Serum (FBS) (Rashid and Coombs, 2019).

In this study, IVF culture with supplementation medium was administered using doses with low concentrations and was the optimum dose according to the research conducted by Maitra (2018) which was 5.2 µl / ml. Giving calcium ionophore with excessive doses can cause oocyte damage because prolonged exposure to CaI A23187 can cause toxins (Swann, 2018). In addition, increased levels of Ca^{2+} with high concentrations can affect embryonic development. This is consistent with research conducted by Nalley and Hine (2015) which states that post-exposed CaI A23187 embryos with high concentrations indicate lower cleavage rates.

The results showed that oocytes cultured by IVF using a supplemented medium CaI A23187 showed a higher fertilization rate (93.12 ± 13.7) compared to oocytes cultured using a medium without supplementation that is (65.96 ± 28.36). This result is higher than research conducted by Taylor *et al.* (2008) where the number of oocyte fertilization reached (53.2 ± 19.8) in humans. Research conducted by Eftekhar *et al*. (2012) using CaI A23187 at a dose of 5 µL given after ICSI showed fertilization rates reaching 72.5%. The success of fertilization with the method of *in vitro* fertilization shows varied results, which depend on several influential factors. These influential factors are the quality of the oocytes and spermatozoa used, and also the factors in the culture medium which include temperature, pH, osmotic pressure, and CO_2 levels.

This shows that oocytes fertilized using a medium supplemented with calcium ionophore have been shown to increase fertilization rates. Ionophore calcium can increase intracellular calcium levels in oocytes both internally and externally, which internally CaI A23187 works in internal storage through channels in the endoplasmic reticulum and externally depends on extracellular Ca^{2+} entry (Ebneretal, 2014). Repeated increases in intracellular Ca^{2+} levels are signals that initiate oocyte activation and pronucleus formation. In mammals, repeated increases of Ca^{2+} in oocytes that occur during fertilization are signals that play a role in early embryonic development, and without the presence of Ca^{2+} signals during fertilization can cause failure in embryo development (Ducibella *et al*., 2006). The conclusions from this study shows that these supplementation of Calcium Ionophore (CaI) A23187 in the vitro fertilization medium at a dose of 5.2 µl/ml can increase oocyte activation so as to increase the oocyte fertilization rate of goat nuts

Conflict of Interest

Authors declare that they have no conflict of interest.

Acknowledgments

The authors express sincere thanks to the Ministry of Research, Technology and Higher Education of the Republic of Indonesia for funding research and Dean Faculty of Veterinary Medicine for providing all necessary facilities and fund for conducting research work.

Authors' Contribution

AYN designed the study, interpreted the data, and drafted the manuscript. TIR, SPM, IM, MM and VFH were involved in collection data and also contributed in manuscript preparation. AYN, EML and WW took part in preparing and critical checking of this manuscript.

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Dear Mr. Epy Muhammad Luqman,

Your Paper **EEC-F-3677- Calcium Ionophore Supplementation in *In vitro* Fertilization Medium towards Fertilization Rates of Kacang Goats (*Capra hircus*),** has been evaluated and can be published. We may inform you that we have policy of page charges, the cost of your paper shall be **US$ 300.00 (including taxes)** which includes one galley proof PDF, one e-reprint, putting the abstract on website. The paper shall come in **2022 (2)** of the International Journal - Ecology, Environment and Conservation.

PDF proof for this issue shall be available in April, 2022. Final issue shall be available around **June, 2022.**

The Journal is covered in **Web of Science** (**ISI, Thomson Reuters, U.S.A.**) **NAAS Score– 5.41.** The Journal is in the **UGC CARE** list.

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Best regards

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