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The Effect of L-Arginine Supplementation on the Quality of Frozen Goat Semen

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ABSTRACT

Various attempts have been made to maintain the quality of semen by using diluents that are added with appropriate antioxidants to protect spermatozoa from *cold shock* and maintain the quality of spermatozoa, while the addition of antioxidants in the diluent is useful for suppressing free radical reactions. One of the substances that can be added to cement thinner is L-Argine. The mechanism of action of L-Argine is the same as that of an antioxidant, which is useful in inhibiting free radicals. *Nitric Oxide* contained in L-Arginine is an antioxidant so that it can inactivate superoxide produced by spermatozoa during the oxygen consumption process. Shelters were carried out 2 times a week with the artificial vaginal method, with 10 shelters during the study. The semen that will be used for research has a minimum individual motility requirement of 70% and a minimum mass motility of ++. The variables observed in this study were abnormalities, intact plasma membrane and intact acrosome cap on observations *before freezing* and *post thawing*. The results of the analysis of variance showed that L-Arginine supplementation had no significant effect ($p>0.05$) on abnormalities in the pre-freezing and post-thawing observations, L-Arginine supplementation had a significant effect ($p<0.05$) on the integrity of the plasma membrane and acrosome cap. on observation before freezing and post thawing.

Keywords: abnormalities, antioxidants, L-Argine, spermatozoa

INTRODUCTION

Boer goats have a good immune system and a fast growth rate even under unfavorable and unfavorable environmental conditions (Erasmus, 2000). Efforts to improve the genetic quality and population of Boer goats can be done through reproductive management using artificial insemination. Semen quality is one of the hygiene factors in artificial insemination, for this reason, a diluent is needed that can maintain semen quality so that semen fertility remains good. Semen quality inspection is the first step in implementing the success of artificial insemination for semen to be suitable for artificial insemination in female cattle or not. Spermatozoa in goats are usually emaciated during the freezing process. Cement also experiences a drastic increase in temperature which can increase metabolism in the form of free radicals caused by the thawing process (Rizal and Herdis, 2008). One of the obstacles is the low quality of semen due to damage to the spermatozoa head membrane during storage, one of which is caused by Reactive Oxygen Species (ROS) and oxidative stress (Romadhoni, Rachmawati and Suyadi, 2014). Reactive Oxygen Species (ROS) produced cause damage to the plasma membrane, causing a decrease in the fusion ability of spermatozoa – oocytes and high lipid peroxidation which decreases the capacity of spermatozoa to undergo acrosomal reactions for fertilization due to loss of membrane fluidity (Bucak, 2010).

Antioxidants are compounds that can fight free radicals formed as a result of oxidative metabolism. Various attempts have been made to maintain the quality of semen by using diluents that are added with appropriate antioxidants to protect spermatozoa from cold shock and maintain the quality of spermatozoa, while the addition of antioxidants in the diluent is useful for suppressing free radical reactions. One of the substances that can be added to cement thinner is L-Arginine.

The mechanism of action of L-Argine is the same as that of an antioxidant, which is useful in inhibiting free radicals. Nitric Oxide contained in L-Arginine is an antioxidant so it can inactivate superoxide produced by spermatozoa during the oxygen consumption process. Damage to the membrane caused by peroxide can be prevented by the compound present in L-arginine, namely Nitric oxide. L-Arginine is the most common amino acid which is a semi-essential amino acid that can capture Hydrogen Peroxide (H2O2) and superoxidation anion (O2-) L-Argine acts to inhibit the activity of free radicals that form NO (Nitric oxide) (Budiman, 2008).

MATERIAL AND METHOD

Location and Research

This research was conducted on on August 19th - October 21th, 2021 at the Field Laboratory of Sumber Sekar, Faculty of Animal Science, Brawijaya University.

Research Material

The collection of goat semen is carried out 2 times a week using the artificial vaginal method. Before the male shelter was carried out, false mounting was carried out 3-5 times using female goats placed in a clamping cage to increase libido so that an erection occurred perfectly. The holder holds the penis with the left hand and directs it into the artificial vagina so that ejaculation occurs inside the artificial vagina. An artificial vagina that has been prepared according to body temperature and has been given Vaseline at the end of the rubber, using an angle of 45^{o} and at the end there is a test tube that has been covered with dark material so that the cement produced is not exposed to direct sunlight (Susilawati, 2011). The semen that will be used for research has a minimum individual motility requirement of 70% and minimum mass motility of ++. The semen was diluted using Tris aminomethane Egg Yolk with the addition of L-Arginine.

Research Procedure

Freezing of semen begins with gradual dilution using Tris aminomethane + egg yolk + L-Arginine. After dilution, the cement was equilibrated in a refrigerator at 5^{o}C for 2 hours. Continued filling straw (filling and sealing) by sucking as much as 0.25 ml/straw. Pre-freezing of cement was done manually using a box Styrofoam (modified) by inserting a cement straw into Styrofoam to be evaporated with liquid nitrogen for 30 minutes. Next to the freezing stage, put the cement straws into liquid nitrogen at a temperature of -196^{o}C. Evaluation of the quality of the cement after freezing (post thawing) was carried out by thawing the cement straws using warm water at 37^{o}C for 15 seconds.

Research Methods

The research used a laboratory experimental method with 4 treatments with 10 replications and a laboratory experiment using a Completely Randomized Design (CRD). The treatments of this study were as follows: P0: 0mM L-Arginine, P1: 5mM L-Arginine, P2: 6mM L-Arginine, P3: 7mM L-Arginine.

Research Variables

The variables observed on this research were abnormalities in the observation before freezing and post thawing, the plasma membrane in the observation before freezing and post thawing, the acrosome cap intact on the observation before freezing and post thawing.

Data Analysis

The data were analyzed with a variety analysis (ANOVA) and continued with the Duncan Multiple Range Test if there were differences.

RESULT AND DISCUSSION

Percentage of Spermatozoa During Frozen Storage Spermatozoa

Abnormalities are classified into two, namely primary and secondary abnormalities. Primary abnormalities occur because of a failure in the process. spermatogenesis in the seminiferous tubules. Primary abnormalities can be due to heredity and bad environmental influences. The features of the primary abnormality include a large head (macrocephalus) or a small head (microcephalus), a short, broad head, and a double tail. The secondary abnormalities occurred during the storage or cryopreservation of spermatozoa and were most likely due to the treatment during staining in the process of making the smear preparations. The secondary abnormalities included a folded tail, the presence of proximal or distal cytoplasmic granules, and the acrosome sheath detached from the head without any tail, and the severed tail. Abnormalities observed in this study are secondary abnormalities that can be seen from the tail of the spermatozoa coiled or bent (Afiati et al., 2015). The percentage of spermatozoa abnormalities during frozen storage is shown in Table 1.

The results obtained from the percentage of post-thawing observations showed the highest abnormality was in the P0 treatment (0 mM L-Arginine) 5.56 ± 2.16, while the lowest abnormality was in the P2 treatment (6 mM L-Arginine) 3.73 ± 1.49. According to Parera, et al. (2009) that spermatozoa abnormalities with a value of 8-10% do not have a significant effect on fertility, but if it is more than 25% then the decline in fertility cannot be anticipated. According to Chenoweth (2005). The impact of increasing spermatozoa abnormalities is the influence of membrane structure and metabolism resulting from a high increase in ROS (Thilagavathi et al., 2012). This can have an impact on motility and decreased ability of spermatozoa to fertilize an egg caused by a high level of abnormality. Antioxidant levels decrease during the thawing process due to cement dilution and excess ROS molecules (Tuncer et al., 2010).

The antioxidant is one of the compounds that can inhibit quality degradation in the cement storage process, by suppressing free radical reactions. Free radicals are molecules that have lost one electron from their lone pair or are the result of the homolytic separation of a covalent bond. As a result of homolytic splitting, a molecule will split into free radicals which have unpaired electrons. Electrons need a partner to balance their spin values, so that the radical molecule becomes unstable and easily reacts with other molecules, forming new radicals (Harmita 2014). Another cause of abnormalities, according to Wiratri et al. (2014) states that the treatment of semen after ejaculation such as handling fresh semen, mixing semen with diluent and at the time of making smear preparations can cause an increase in spermatozoa abnormalities.

Table 1. Results of the percentage of spermatozoa abnormalities during frozen storage

Treatment	Observation	
	Before Freezing	Post Thawing
0mM L-Arginine	2.33 ± 1.47	5.56 ± 2.16
5mM L-Arginine	2.14 ± 0.70	4.92 ± 1.67
6mM L-Arginine	2.05 ± 1.35	3.73 ± 1.49
7mM L-Arginine	2.19 ± 0.77	4.36 ± 1.27

Percentage of Plasma Membrane Integrity of Spermatozoa During Frozen Storage

The plasma membrane is the first protection of spermatozoa against the influence of seminal plasma or diluents. Changes in the structure of the diluent or seminal plasma can affect the integrity of the spermatozoa membrane during storage (Setiadi et al., 2006). According to Hartono (2008) the plasma membrane functions to protect cell organelles and regulates osmotic pressure during the metabolic process. Lipid peroxidation that occurs in spermatozoa causes damage to the plasma membrane of spermatozoa, resulting in a disturbance in the balance of osmotic pressure inside and outside the cell. A significant decrease in viability value during post thawing is suspected that spermatozoa were stored at a temperature (-196˚C) to allow changes during the freezing process and contact between cement and air during cement processing causes free radicals. The results of the description based on the average value ± standard deviation of the plasma membrane post thawing showed the highest plasma membrane in treatment P2 (6 mM L-Arginine) 70.62 ± 5.86, while the lowest plasma membrane in treatment P1 (5 mM L-Arginine) 64.24 ± 4.88. This happens because when stored at low temperatures the spermatozoa undergo metabolism so that the longer the storage time, the lower the nutrients for the spermatozoa, the high peroxidation of unsaturated fatty acids in and in the cell wall, so that peroxidative damage can occur which affects membrane integrity and fertility spermatozoa.

Antioxidants play a role in preventing damage to the plasma membrane of spermatozoa caused by cold stress and provide protection against changes caused by clotting and prevent lipid peroxidation. Antioxidants are nucleophilic compounds, where they extinguish or suppress free radical reactions and they can end the reaction cycle (Feradis, 2009). This is why the integrity of the plasma membrane must be maintained intact to maintain spermatozoa viability, motility, and ability to fertilize. This is because the plasma membrane functions as a continuous cell barrier, which protects cell organelles from mechanical damage and regulates the traffic in and out of nutrients and ions needed in metabolic processes. Damage to the plasma membrane results in the disruption of metabolic processes and physiological processes, resulting in the death of spermatozoa (Lubis et al., 2013).

In the post-thawing observations in Table 5. It shows that treatment P2 (6 mM L-Arginine) 70.62 ± 5.86 had a better percentage than treatment P1 (5 mM L-Arginine) 64.24±4.88 and P3 65.31 ± 5.15 decreased according to Wahjuningsih et al. (2019) The antioxidant level decreases during cement processing because the excess production of ROS molecules increases functional damage and structural integrity, therefore antioxidant

supplementation in cement diluents is required. The addition of L-Arginine to the addition of P2 (6 mM L-Arginine) was able to prevent lipid peroxidation and maintain the quality of the integrity of the plasma membrane of spermatozoa during frozen storage. This is comparable to Lukman et al. (2014) in their research using the antioxidant-tocopherol which stated that excessive doses of antioxidants in semen caused a percentage reduction in the progressive motility of spermatozoa. The assumption is that giving too much -tocopherol to cement diluent will cause antioxidant saturation, -tocopherol is lipophilic (fat-soluble) and -tocopherol acts as an exogenous chain breaker to prevent lipid peroxidation and hydrogen atom transfer. Excessive doses of -tocopherol as an antioxidant interfere with the function of ROS in spermatozoa resistance and influence the activity of a-tocopherol in diluents that affect spermatozoa motility due to apoptosis.

Table 2. Percentage results of spermatozoa plasma membrane integrity during frozen storage

Treatment	Observation	
	Before Freezing	Post Thawing
0mM L-Arginine	78.24 ± 4.19 [ab]	66.16 ± 4.83 [ab]
5mM L-Arginine	75.16 ± 2.13 [a]	64.24 ± 4.88 [a]
6mM L-Arginine	81.12 ± 3.10 [b]	70.62 ± 5.86 [b]
7mM L-Arginine	77.73 ± 6.44 [ab]	65.31 ± 5.15 [a]

Percentage of Intact Spermatozoa Acrosome Caps During Frozen Storage

Shah et al. (2017) stated that spermatozoa with intact acrosome integrity were marked with purple color on the head and damaged acrosomes were marked with a light purple color. In the acrosomal hood, there are hyaluronidase, acrosin and other enzymes that can lyse the protective layer of the egg, therefore the intact acrosomal hood plays an important role for spermatozoa in the fertilization process. The membrane on the head serves to penetrate the egg in the fertilization process. The membrane behind the acrosome functions to make the first contact and become one with the ulema of the egg during the fertilization process (Susilowati, 2007). According to Melisa (2016), the intact acrosomal hood is a sheath found on the head of the spermatozoa which functions to protect the release of genetic material and enzymes from the head of the spermatozoa.

The results of the description based on the average value ± standard deviation of the intact acrosomal hood post thawing showed the highest intact acrosomal hood in the P2 treatment (6 mM L-Arginine) 71.39 ± 5.28, while the intact acrosomal hood. the lowest was in the P1 treatment (5 mM L-Arginine) 64.79 ± 3.49. According to Cahyani et al. (2020) the acrosomal hood serves to protect the enzymes in it so that the fertilization process can run well, therefore observation of the intact acrosomal hood is needed to ensure success in fertilizing the egg. The factor causing the low TAU in this study is thought to be due to physical factors due to shaking during the cement mixing process with the diluent medium, this will adversely affect the integrity of the acrosomal hood due to the collision of spermatozoa with the test tube wall. This is also comparable to Susilowati (2010) who stated that chemical influences in the diluent medium, as well as mechanical influences such as friction with diluent particles or with tube walls, can reduce the quality of the intact acrosome hood. In addition, the primary abnormality or originating from a failure in the process of spermatogenesis in the form of the Golgi apparatus of spermatids that do not form the acrosomal hood can result in damage to the intact acrosomal hood. According to Effendy et al. (2015), the metabolic process of sperm cells produces lipid peroxidative reactions when reacted with free radicals. Lipid peroxidation can change the structure of sperm cells and damage the lipoprotein sheath, causing the protective coat of the sperm head to rupture and resulting in damage to the intact acrosomal cap of the spermatozoa. *reactive oxygen species* Excessive(ROS) will affect membrane lipids, especially polyunsaturated fatty acids, resulting in lipid peroxidation which will disrupt the plasma membrane and the acrosome cap of spermatozoa (Susilowati, 2007). In the observation of intact acrosome hood in *post thawing* treatment P2 (6 mM L-Arginine) 71.39 ± 5.28 had a high percentage compared to other treatments. L-Arginine functions in research as a potent antioxidant that can suppress free radical reactions due to cooling, freezing and storage of cement. Tetool et al. (2017) explained that antioxidants in diluents are useful for maintaining cell metabolism and overcoming the rate of cell membrane damage due to fat peroxidation.

Table 3. Results of percentage of intact spermatozoa acrosome caps during frozen storage

Treatment	Post Thawing
0mM L-Arginine	64.98 ± 5.86 [a]
5mM L-Arginine	64.79 ± 3.49 [a]
6mM L-Arginine	71.39 ± 5.28 [b]
7mM L-Arginine	66.72 ± 5.88 [ab]

CONCLUSION

Addition of L-Arginine in storage post-thawing semen is P2 with 6 mM L-Arginine which can maintain the lowest percentage of spermatozoa abnormalities 3.73 ± 1.49, the highest percentage of plasma membrane integrity is 70.62 ± 5.86, and the highest percentage of intact acrosomal hood is 71.39 ± 5.28. This is due to the presence of antioxidants and sufficient levels of L-arginine so that no toxicity occurs.

CONFLICT OF INTEREST

There is no financial, personal, and organizational conflict of interest related to the material discussed in this article.

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