

Conference Paper

ADDING OF L-ARGININ AMINO ACID IN SKIM MILK DILUENT TO MAINTAIN QUALITY OF BUCK SPERM in COLD TEMPERATURE

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Abstract

Nowdays, the storage of buck semen in cold temperature have not satisfied yet because in buck's seminal plasma contains phospholipase enzyme which can coagulated egg yolk in diluents. The specific aim of this study was to investigate the benefits of L-Arginin amino acid in skim diluents to quality buck's spermatozoa on cold temperature. This research utilized four treatment groups, namely Controlled group (Po): skim milk diluent without L-Arginin + buck's semen; P1: skim milk diluents + L-Arginin 0,002M/ml + buck's semen; P2: skim milk diluents + L-Arginin 0,004 M/ml + buck's semen and P3: skim milk diluents + L-Arginin 0,006 M/ml + buck's semen. Then the samples stored in cold temperature (5°C). The result showed that sperm motility, viability and membrane integrity were significantly different ($p < 0,05$) among the treatments. The conclusion of this study is adding of L-Arginin Amino Acid in skim milk diluents maintain motility, viability and membrane integrity buck's sperm.

Keywords: L-Arginin, buck, cold temperature, motility, viability and membrane integrity.

1. Introduction

Frozen semen is semen freezing process at the temperature of liquid nitrogen -196°C [1]. Semen freezing is separated by slow freezing, rapid freezing and ultra rapid freezing. Freezing process causes the damaging of membrane structure and function as well as the ability of sperm to survive [2]. The purpose of the freezing process is as perfect as possible to retain some properties of biological materials, especially in viability [3]. The main obstacle of this freezing process is occurrence of cell damage. The reason partly due to the dehydration process does not occur to form intracellular ice crystals that can damage cells. It can also be due to increase the osmolarity of freezing media that lead to convert cryoprotectant into toxic resulting physical damage by the formation of extracellular ice crystals, the toxicity of concentrated electrolyte

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or the osmotic swelling [4]. The damage during the freezing steps are common in the plasma membrane and the nucleus of sperm.

Plasma membrane of sperm consist of lipid, protein and karbohidrat. A characteristic feature of biological membranes is the asymmetrical arrangement of lipids within the bilayer. The lipid composition of the plasma membrane of mammalian sperm is markedly different from, those of mammalian somatic cells so that in the process of freezing the lipid membrane of buck sperm easy stimulating then Lipid Peroxidation (LPO) occurred. Sperm cells containa high content of polyunsaturated fatty acids. The ratio of unsaturated to saturated fatty acids in small ruminant sperm membrane is also higher than in other species, making the membranes more susceptible to peroxidative damage in the precence of ROS-with a subsequent loss of membrane integrity in the acrosomal region, impaired cell function and decreased motility of the sperm [5].

L-Arginin is an amino acid which has important roles to stimulate spermatozoa motility under in vitro condition in mammals. L-Arginin also enrolls in antioxidant that produces nitric oxide from enzyme synthesis reaction so that reduces lipid peroxidation sperm membrane that occurs because free radicals when sperm interacted with oxygen directly NO^2 is a biological molecules which has important roles in spermatozoa fisiology like sperm motility, interaction between sperm and ovum, and spermatozoa. Nitric Oxyde (NO^2) enrolls in a mechanism of sperm defense to be formed in to reactive oxygen under freezing level which defense sperm motility and viability post-thawing.

Until now the process of thawing buck's semen freezing is have not satisfied, it is because the buck' seminal plasma contains egg yolk coagulating enzyme or called by phospholipase A and triglycerol lipase [6]. Therefore, researchers wanted to examine the addition of L-Arginin Amino Acid in skim milk diluents to quality buck' sperm in cold temperatures.

2. Material and Methods

2.1. Experimental Animals

Semen samples from three bucks (3-4 years of age) were use in this study. The bucks were maintained under uniform nutritional condition. Ejaculated were collected twice a week from the bucks with the aid of an artificial vagina, immediately after collection was brought to the laboratory, and semen parameters were assessed, including volume, pH, consistency, color and consentration of the semen. After bucks semen underwent microscopic and macroscopic examination (appiying prequalification that the percentages of sperm motility and viability should be 70% or above).

2.2. Semen Dilution

One hundred skim milk added with 100 cc aquadest, heated until 92-95° C and then colded until 20-27°C temperature and added Penicillin 1.000 IU/cc diluents and Streptomycin 1 mg/cc diluents too. Diluents was prepared divided into four groups. Controlled groups (PO) refers to diluents of skim milk + semen of buck's (without L-Arginin). Group I (PI) refers to diluents of skim milk + semen of buck's and L-Arginin 0,002 M/cc. Group II (PII) refers to diluents of skim milk + semen of buck's and L-Arginin 0,004 M/cc. Group III (PIII) refers to diluents of skim milk + semen of buck's and L-Arginin 0,006 M/cc All of groups colded at 5°C and then examined sperm motility, viability and membrane integrity.

2.3. Semen Evaluation

Progresive sperm motility was assessed using a phase contrast microscope. The motility of sperm was analyzed by mixing the semen gently and placing a 10 µl drop diluents of semen on a slide covered with a glass cover slip from five selected representative fields. Samples were selected randomly from 3 fields, for a total of 100 cells. Individual sperm were recorded as being viable or dead [7].

Eosin negrosin staining was used to evaluate sperm viability as cescribed by Susilowati [7] Semen was placed on glass slide and this samples was mixed with one drop of eosin-negrosin solution. The mixture was smeared on the the glass slide and allowed to air dry. One hundred of sperm were evaluated in at least five different fields in each smear under a light microscope. Eosin penetrates non viable cells, which appear red and negrosin offers a dark background for facilitating the detection of viable, non stained cells [7].

Membran integrity was determined using the hypo-osmotic swelling test (HOST) described by Susilowati [7]. A total of 100 µl of semen was mixed with one mL of hypoosmotic solution (containing 13,51 g of fructose and 7,35 g of sodium citrate in 1000 mL of distilled water. The mixture was incubated at room temperature for 30 min. Following incubation, 15 µl of the sample was placed on a slide, covered with a cover slip and observed under a light microscope. The sperm were categorized according to the presence or absence of a swollen tail. At least 100 sperm were observed and the results were recorded as percentages. The membrane integrity after HOST was classified into two groups, normal sperm that displayed coiled tails and abnormal sperm without coiled tails.

TABLE 1: Macroscopic and Microscopic Examinations Buck Semen.

Indicator	Character
Volume (cc)	1,5±0,45
Consistency	Viscous
Color	Milky white
Odor	Typicaly
pH	7
Mass Movement	+++
Individual Movement (%)	Progresive (90±6,50)
Concentration	3975x10 ⁶ spz/ml
Membrane integrity (%)	87±6,55

2.4. Statistical Analysis

All data were expressed as the mean values \pm SEM. The statistical significances of the effects of sperm quality (motility, viability and membrane integrity) were determined by Anova, P-values $\leq 0,05$ were considered to be significantly different.

3. Result

Fresh buck semen used in this research had undergone macroscopic and microscopic examinations. Macroscopic examination included examinations on buck semen volume, color, odor, consistency and pH. Meanwhile, microscopic examinations investigated spermatozoa mass motility, individual motility, concentration, and viability. The result of microscopic and macroscopic examinations was presented on Table 1.

The research shows that buck's semen stored has milky white colour, typical odor, viscous consistency, pH = 7, volume 1,5±0,45 ml, concentration 3975x10⁶, mass motility +++ (the motion formed big and many waves), individual motility generally semen volume will increase based on their age, body weight, changing of condition, reproduction organ health, and frequency of semen collection. Colour, consistency, and spermatozoa concentration has correlation with others. The percentages of sperm motility, viability, membrane integrity after adding L-Arginin Amino Acid on the cold temperature were show in table 1, 2 and 3. Sperm motility, viability and membrane integrity were significantly different ($p < 0,05$).

TABLE 2: Percentage of buck’s sperm viability in the skim milk diluents + L-Arginin Amino Acid which stored in cold temperature (5°C) and evaluated everyday.

Treatment	1 st day (%)	2 nd day (%)	3 rd day (%)	4 th day (%)	5 th day (%)
Po	71,67±2,73 ^c	64,50±5,32 ^c	57,00±5,40 ^c	48,00±6,23 ^c	41,17±4,87 ^c
PI	75,50±3,45 ^b	70,67±4,32 ^b	64,00±4,19 ^b	55,67±5,28 ^b	46,33±8,04 ^b
PII	75,50±2,17 ^b	70,50±3,15 ^b	64,67±4,59 ^b	58,00±2,28 ^b	50,00±3,03 ^b
PIII	80,58±7,24 ^a	72,54±8,02 ^a	75,50±3,56 ^a	69,00±3,92 ^a	59,50±3,21 ^a

Note: Value in the same row with different superscripts indicate significant difference at (p<0,05)

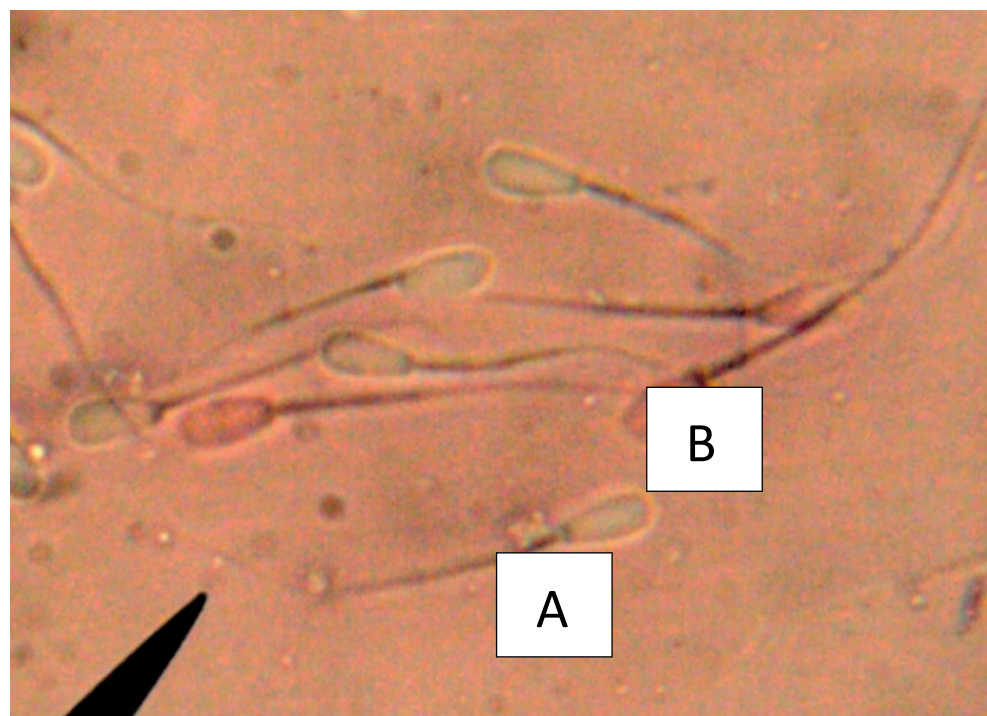


Figure 1: Live sperm (A. uncoloured head) and dead (B. coloured head).

4. Discussion

In this study motility, viability and membrane integrity after adding L-Arginin Amino Acid in skim milk in group III was higher compare with another group. Viability of sperm was conducted utilized through eosin negrosin coloration which was based on coloration permeability into spermatozoa. The mechanism of this process was the living spermatozoa are indicated by membrane integrity, although their environment is colorless (in this case reddish) the head of living sperm will remain colorless or transparent due to normal plasma membrane integrity, meanwhile dead sperm (due to damaged plasma membrane) could not control the permeating coloration substance resulting the head to appear reddish Plasma membrane didn’t function in protecting

TABLE 3: Percentage of buck's sperm motility in the skim milk diluents + L-Arginin Amino Acid which stored in cold temperature (5°C) and evaluated everyday.

Treatment	1 st day (%)	2 nd day (%)	3 rd day (%)	4 th day (%)	(5 th day (%)
Po	61,50±3,08 ^d	56,17±4,42 ^c	50,50±5,24 ^c	44,17±6,08 ^c	36,83±6,08 ^c
PI	67,33±3,45 ^c	56,83±4,35 ^c	51,83±7,22 ^c	49,50±5,24 ^c	39,33±6,50 ^c
PII	73,50±2,17 ^b	69,50±3,50 ^b	62,17±4,75 ^b	55,33±3,23 ^b	46,17±2,64 ^b
PIII	78,00±2,28 ^a	75,17±2,28 ^a	75,50±3,56 ^a	65,67±3,50 ^a	57,33±3,72 ^a

Note: Value in the same row with different superscripts indicate significant difference at (p<0,05)

TABLE 4: Percentage of buck's sperm membrane integrity in the skim milk diluents + L-Arginin Amino Acid which stored in cold temperature (5°C) and evaluated everyday.

Treatment	1 st day (%)	2 nd day (%)	3 rd day (%)	4 th day (%)	(5 th day (%)
Po	31,00±2,83 ^c	28,33±2,16 ^c	25,07±2,87 ^c	22,83±2,79 ^c	17,83±2,63 ^c
PI	32,00±2,10 ^c	35,53±3,31 ^b	31,33±2,25 ^b	22,67±2,80 ^c	18,67±1,63 ^c
PII	38,67±2,16 ^{ab}	36,50±3,27 ^b	31,50±3,33 ^b	25,17±4,17 ^b	22,83±3,92 ^b
PIII	40,80±4,38 ^a	37,17±2,93 ^a	34,50±4,23 ^a	28,33±6,08 ^a	25,50±5,57 ^a

Note: Value in the same row with different superscripts indicate significant difference at (p<0,05)

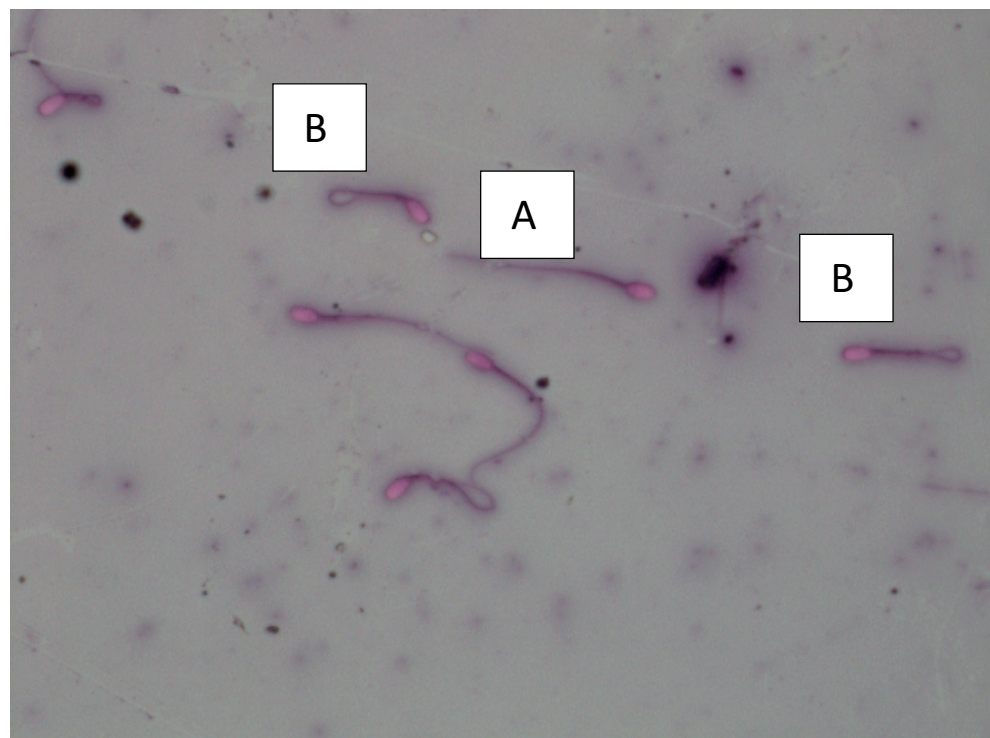


Figure 2: Damage membrane sperm (A. straight sperm tail) dan intact membrane sperm (B. round spermatozoa tail).

cellular organelles from mechanical damages, it also functioned as a filter which circulated and preserved intracellular substances on metabolism process [6]. Sperm motility correlated to spermatozoa ability to fertilize ovum. Spermatozoa movement was enhanced by energy (in form of Adenosin Triphosphat/ATP) produced by mitochondria and dynein motor (spermatozoa flagellum cytoskeleton whose movement was regulated by Ca^{2+} and cyclic Adenosine Monophosphate (cAMP) [11]. The energy was the result of oxidative Phosphorylation of enzymatic processes happened in mitochondria. The enzymes which took part in oxidative phosphorylation process lied on mitochondria inner surface. Oxidative phosphorylation process produced free energy (which was converted into ATP molecules and Reactive Oxygen Species (ROS) compounds. As long as stored, motility of sperm would be decreased. This condition correlated in increasing Ca^{2+} in the cells so cAMP also decreased, beside causing by lactic acid form. As many as lactic acid formed would be decreasing pH., so metabolic process would be disturbed. Metabolic decreasing causing ATP formed decreasing and then sperm motility would be decreasing [6].

L-Arginin is amino acid which can stimulate sperm motility in the in vitro condition and also have role in cellular immunity defense [8]. L-Arginin such protect spermatozoa against lipid peroxidation through increased production of nitric oxide used against reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and anion superoxide (O_2^-) [9]. Antioxidants work by giving an electron to the free radical compounds. Antioxidants are able to activate the growth of the oxidation reaction, by preventing the formation of free radicals. Antioxidants can inhibit oxidation reactions by binding free radicals, that damage cells can be inhibited [10]. L-Arginin is secondary antioxidants which works by cutting the oxidation chain reaction of free radical or by catching free radicals, as a result of free radicals cannot react with cellular component. L-Arginin increases glycolysis spermatozoa so the process can produce energy in the form of energy the movement, so can remain motile and simultaneously maintain viability in the process.

5. Conclusion

Adding of L-Arginin Amino Acid can maintain progressive motility, viability and integrity membrane of buck's sperm in skim milk diluents on cold temperature.

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