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Additional Seminal Plasma Crude Protein to Preserve DNA Integrity of Goat Spermatozoa on Freezing Process

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Abstract : Recently, freezing post-thawing goat semen has not provided satisfying result due to low spermatozoa quality. It may be caused by triglycerol lipase enzyme content inside goat semen plasma which may reduce spermatozoa motility inside milk diluter.

This study aims to investigate the DNA integrity of goat's spermatozoa inside milk diluter after being supplemented from crude protein plasma seminalis of goat on freezing process.This research utilized three treatment groups, namely Controlled group (P0): goat semen thawed into skim milk without crude protein seminal plasma supplementation; P1: crude protein plasma seminalis supplemented into thawed goat semen inside skim milk diluter in composition 1:1 (one part of crude protein seminal plasma was added into one part of thawed goat semen); and P2: crude protein plasma seminalis supplemented into thawed goat semen inside skim diluter in composition 1:2 (two part of crude protein seminal plasma was added into one part thawed goat semen). Then the third group performed freezing and post-thawing checked against motility, viability and DNA integrity of spermatozoa.

Observations of semen without addition of crude protein seminal plasma diluter produce the percentage of motility, viability lowest and DNA integrity showed the highest percentage. Anova test for motility, viability and DNA integrity spermatozoa are significant differences ($p < 0,05$) between P0, P1 and P2. Duncant Multiple Range Test observation in group P1 generates the percentage of motility, viability were highest and the lowest percentage of spermatozoa DNA integrity.

The conclusion of this study is addition of crude protein seminal plasma of goat's can maintain motility, viability and can increase of DNA integrity in goat's spermatozoa in skim milk diluter postthawing.

Keywords : Crude protein seminal plasma of goat, post-thawing, motility, viability and DNA integrity.

Introduction

Freezing refers to a technique to store cells (either animal or plants) and genetic materials (including semen and oocytes) in frozen condition by reducing its metabolic activities without affecting celluler organelles thus its physiological, biological and morphological functions are well preserved. The most important principle on freezing spermatozoa ishow to dehydrate the cell properly before it freezes. Without dehydration, big ice crystals will be formed inside cell which may damage the cells. Meanwhile, excessive dehydration may dry the cells resulting dead cellas[9].

Fozen semen refers to a process to freze semen at – 196oC temperatures using liquid nitrogen medium[6]. Freezing process may damage spermatozoa functions and membrane structure. The process may also lowerits survivability[13]. The objective of freezing process is to preserve some characteristics of biological materials as much as possible, particularly its viability[17]. The main issue on freezing process is damage cells caused by imperfect dehydration resulting intracellular ice crystals which damage the cells. Another issue lies on increasing osmolarity of the medium used which causes cryoprotectant substance becomes toxic eventually resulting in physical damages, such as extracellular ice crystals, thick electrolyte toxicity and osmotic swelling. Damages during freezing process commonly occur on plasma membrane or spermatozoa nuclei.

Seminal plasma consists of various specific biochemical components which regulate the function of spermatozoa.Seminal plasma was composed of water and several organic and anorganic substances[15]. Seminal plasma also contains decapacitation factors (DF) covering the surface of spermatozoa during ejaculation. Decapacitation factors bind the surface of spermatozoa and activating intracellular ATP – ase calcium to maintain the concentration of intracellular calcium concentration low[2].These condition might affect the viability, motility and integrity of spermatozoa membrane in cold condition[4]. Seminal plasma supplementation on sheep semen during freezing process may also improve the motility, viability and integrity of the acrosom[3]. According to[10], seminal plasma can also increase the percentage of motile spermatozoa during cooling down process, freezing process and post thawing process. Mammals' seminal plasma may function either to inhibit or stimulate the function of spermatozoa. Its main component (i.e. protein) covers the surface of spermatozoa right after ejaculation and during passing female reproductive tract[3]. Improvement on cellular plasma membrane positively affects intracellular biochemical processes wich in turn improve the quality of spermatozoa, including its motility and viability. Biomolecular researches prove the several proteins supplementation does not only improve fertilization process, but also preserves the life of cells.

Recently, post-thawing goat semen freezing process has not provided satisfying results because goat seminal plasma contains phospholipase A enzyme which function is assumed to coagulate egg-yolk in thawing substance. This enzyme is secreted by bulbourethral glands. Goat seminal plasma also contains triglycerol lipase secreted by bulbourethral glands which becomes responsive to inhibit goat spermatozoa when interacting with skim milk diluter substance[12]. Goat spermatozoa plasma membrane as also vulnerable to low temperature due to low cholesterol contents[5]. Plasma membrane intactness is vital in assuring spermatozoa viability and succesfull fertilization. Besides its function in protecting cellular organelles from mechanichal damage, plasma membrane also function as filter to maintain extracellular and intracellular ion content on intracellular substance circulation[14]. Therefore, the writers intended to anlysze the effects of goat seminal plasma protein supplementation on goat semen diluter substance during freezing process.

2. Material and Methods

2.1. Experimental animals

A total of three male goats were use d for semen collectionin this study. The average body weight was 45 kg and the average age was 3-4 years.Semen was collected from the goats two weeks after start of adaptation to the location with the aid of an artificial vagina. Finally, the collected semen was brought to the laboratory to separate spermatozoa from its plasma protein. Plasma protein was supplemented into goat semen diluter substance.

After goat semen underwent microscopic and macroscopic examination (applying prequalification that the percentages of spermatozoa motility and viability should be 70% or above), the semen was centrifuged at 4^{o}C and 1800 rpm for 10 minutes to separate spermatozoa and plasma protein. The plasma protein was sonicated at 4^{o}C temperatures based on pattern 60 secons sonication and 30 seconds resting (conducted three times) to fractionate protein. The supernatants were removed from the refrigerator and ready to use.

One hundred mL water was added into grams skim milk 10% and being heated at 92-95^{o}C. The suspension was cooled down until reaching 20-27^{o}C. Penicillin 1000 IU/mL and Streptomycin 1 mg/mL. The suspension was use as diluter substance. The diluter substance was divided into two groups : diluter A and diluter B. Diluter B was supplemented by glycerol. After the diluter substances were ready, the qualified goat semen (semen with motility and viability percentages 70% or above) was mixed with goat plasma protein and added into skim milk diluter. Controlled group refers to diluter A + goat semen. Group I (P1) refers to diluter A

+ goat semen + goat plasma protein (one part of plasma protein was added into one part of goat semen). Group 2 refers to diluter A + goat semen + goat plasma protein (two parts of goat plasma protein were added into one part of goat semen). Diluter B was added gradually into diluter A with four times frequency in 15 minute intervals. The suspension was rested for an hour to equilibrate. Then the third groups performed in freezing and thawing and then examined sperm motility, viability and DNA integrity..

2.2. Sperm viability

Eosin-negrosin staining was used to evaluate sperm viability as described by[18]. After thawing one drops of semen was placed on a tempered glass slide and this samples was mixed with one drop of eosin-negrosin solution. The mixture was smeared on the glass slide and allowed to air dry. One hundred spermatozoa 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[18].

2.3. Assesment of motility

The motility of spermatozoa was analyzed by mixing the semen gently and placing a 10 uL drop of diluted semen on a warm slide covered with a glass cover slip (18x18 mm) from five selected representative fields. Samples were selected randomly from 10 fields, for a total of 200 cells. Individual sperm were recorded as being viable or dead [18].

2.4. Assesment of sperm DNA fragmentation (DNA Integrity)

DNA fragmentation was assessed by a terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay. Briefly, all elements were fixed in 2% formaldehyde in 1 x PBS solution (pH 7,4; Gibeo) for at least one hour. Each sample was placed into one well of a multiwall plate (4 mm diameter). After 2-3 hours, each well waswashed with cold methanol. Before incubation with TUNEL solution, each well was washed again with 1 x PBS. For each sample, one extra well was incubated with DNA (1U/mL; Sigma) for 30 minutes at 37^{o}C as a positive control, and in another well, the TUNEL "enzyme" solution was omitted as a negative control. The total sample was incubated in TUNEL solution for one hour at 37^{o}C. All samples were finally washed with 1 x PBS (three times, 5 min each) and mounted in Vectashield *H-1000* medium (Vector Laboratories). A total of 400 spermatozoa were counted by fluorescence microscopy for each fraction. DNA fragmentation in spermatozoa characterized by the head has black spots while the greenish fragmentation are not experienced.

2.5. Statistical analysis

All data were expressed as the mean values ± S.E.M. The statistical significances of the effects of DNA integrity were determined by ANOVA (S-PLUS Statistical Program, Insighful Corporation Seattle, WA,USA); P-values <0.05 were considered to be significantly different.

3. Result

Fresh goat semen used in this research had undergone macroscopic and microscopic examinations. Macroscopic examination included examinations on goat 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 below:

Indicator	**Character**
Volume (cc)	1,7
Consistency	Thick
Color	Beige
Odor	Typical
pH	7.00
Mass Movement	+++
Individual Movement (%)	Progresive (92±6,50)
Concentration	2,320x10^{6} spz/ml

Goats ejaculated semen volume is not same between the stud and stud type itself. In general, the volume of semen will increase influenced by age, great body, changes in circumstances, reproductive health and semen collection frequency[16]. Color, consistency and concentration of spermatozoa have a close relationship with each other. The more dilute the semen will lower the sperm concentration and semen paler color. While the consistency of semen depens on the ratio of spermatozoa and seminal plasma (Evans dan Maxwell, 1987). Degree of acidity (pH) greatly affects the survival of spermatozoa. When the high or low pH will cause the sperm to die. The degree of acidity (pH) semen is likely influenced by the concentration of lactic acid produced in the final process of metabolism. Spermatozoa metabolism in anaerobic conditions will produce lactic acid that accumulate and increasing or decreasing pH of semen[19].

Table 2. Quality and goat's spermatozoa DNA fragmentation examination in skim milk diluter added with crude protein seminal plasma post-thawing

Treatment Groups	Motility (%)	Viability (%)	DNA fragmentation (%)
P0 : Treatment Control (Without added crude protein seminal plasma	80,55±5,45^{c}	83,65±12,10^{c}	1,78±0,38^{c}
P1: one part of diluter substance and goat semen : one part of crude protein seminal plasma	81,55±4,65^{a}	88,90±6,75^{a}	0,95±0,48^{a}
P1: one part of diluter substance and goat semen : two part of crude protein seminal plasma	72,55±4,50^{b}	77,50±8,80^{b}	1,15±0,56^{b}

Note : Letters in superscript indicated significant differences ($p<0,05$).

In table 2 it can be seen that the addition of crude protein seminal plasma in goat's semen diluter produce significant differences in the pattern of motility, viability and sperm DNA fragmentation. Observations motility, viability of sperm in the goat in milk diluter without the addition of crude protein seminal plasma produce the lowest percentage, but the percentage of DNA fragmentation showed in highest result. Anova test for motility, viability and DNA fragmentation of sperm showed significant differences ($p<0,05$) between control treatment, firt treatment and second treatment. Duncan's Multiple Range Test observation in firt treatment produces percentage motility, viability of sperm highest and lowest of DNA fragmentation.

Discussion

Motility was one of spermatozoa quality criteria. Motility correlated to spermatozoa ability to fertilize ovum. Spermatozoa movement was enhanced by energy (in form of Adenosine Triphosphate /ATP) produced by mitokondria and dynein motor (spermatozoa flagellum cytoskeleton whose movement was regulated by Ca^{2+} and cyclic Adenosine Monophospate (cAMP) (Yanagimachi, 1994). The energy was the result of oxidative phosphorilation of enzymatic processes happened in mitokondria. The enzymes which took part in oxidative phosphorylation process lied on mitokondria inner surface. Oxidative phosphorilation process produced free energy (which was converted into ATP molecules and Reactive Oxygen Species (ROS) compounds. The result showed that the addition of crude protein seminal plasma in the first treatment produce the highest percentage of motility,it is because several possible mechanisms of crude protein seminal plasma in maintaining sperm motility is through energy metabolism as indicated by an increase in the take up glucose, increased production of lactic acid, increased activity of pyruvate dehydrogenase and the other is the possibility to have an antioxidant effect.

Viability was one of spermatozoa quality parameters. Spermatozoa viability examination 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 intact plasma membrane, although their environment is colorless (in this case reddish) the head of living spermatozoa will remain colorless or transparent due to normal plasma membrane permeability, meanwhile, dead spermatozoa (due to damaged plasma membrane) could not control the permeating coloration substance, resulting the head to appear reddish. Spermatozoa viability required intact plasma membrane. Plasma membrane didn't only function in protecting cellular organelles from mechanical damages, it also functioned as a filter which circulated and

preserved intracellular substances on metabolism process[8]. The addition of crude protein seminal plasma improve viability and prevent cold shock membrane damage of goat's spermatozoa. The proportion of polyunsaturated fatty acids in the sperm membrane influences lipid fluidity. A lower ratio of polyunsaturated fatty acid has been found in the sperm membrane to be most readily disrupted by cold shock.

DNA fragmentation is a sign of programmed cell death in response to certain stimuli that occur in physiological processes. DNA fragmentation can occur via two pathways, namely extrinsic pathway and the intrinsic pathway. In the intrinsic pathway of apoptosis initiation starts from cytosolic calcium levels are high and biochemical products derived from oxidative stress. When cells can not cope with the biochemical products will give a signal to the mitochondria which began with the opening of the mitochondrial outer membrane, followed by matrix swelling and loss of mitochondrial membrane potential cause cytochrome c release. Cytochrome c release will induce a series of events that leads to the activation of the intracellular protease caspase group needs. Caspase 3 binds DNA endonuclease the DNA-ase will migrate towards the nucleus and initiate DNA break[11]. There are two important components that cause the release of cytochrome c are Mitochondrial permeability Transition Pore (MPTP) and proapoptotik protein Bax.MPTP opening is influenced by several factors such as the accumulation of calcium, oxidants and mitochondrial transmembrane potential is low. MPTP is actually too small to be bypassed by cytochrome c (13 kDa), but merging with Bax proteins form a special channel for cytochrome c. Bax merging with MPTP determine the viability of a cell[1].

The results showed that the number of sperm DNA damage between treatments were significant differences ($p < 0.05$). The degree of acidity (pH) semen usually acid can cause cells to undergo DNA fragmentation, this is because the toxic substance secreted during cell death (Ramukhiti *et al*, 2011). The addition of seminal plasma can reduce the number of sperm DNA damage in the process of dilution of ram's spermatozoa. This is according to[7] that the seminal plasma of bull's possibility of acting as an antioxidant, but if the road being gathered compounds prevent excessive oxidant or prevent the chain reaction continues unclear.

Conclusion

The addition of goat's seminal plasmacrude protein can maintaining the motility, viability and reduce DNA fragmentation of goat's spermatozoa in skim milk diluents post thawing

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