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The Effect of Cattle Seminal Plasma Crude Protein on the Cryopreservation of Goat Semen

Research Article

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ABSTRACT

This study aimed to improve the quality of cryopreserved goat semen for artificial insemination by the addition of cattle seminal plasma crude protein. Semen collected from cattle and centrifuged to obtain seminal plasma. Then it was purified to obtain the crude protein. The diluent used in this study was milk-yolk. There were three groups, control group without crude protein (P0), the addition of 2.5 mg crude protein/mL diluent (P1); and the addition of 5 mg crude protein/mL diluent (P2). The results showed that P1 had the highest percentage of progressive motility and viability. Meanwhile, P1 had the lowest percentage of cytochrome C expression. The lowest cytochrome C expression indicates that the mitochondria have a good function. So, by the addition of 2.5 mg crude protein/mL diluent, the quality of frozen goat semen in terms of sperm motility, viability, and cytochrome C expression in the spermatozoa could be improved.

KEY WORDS cattle seminal plasma crude protein, cytochrome C, motility, viability.

INTRODUCTION

Cryopreservation is a technique to store frozen animal or plant cells by reducing metabolic activity without affecting the cell organelles or the cell's physiological, biological, and morphological characteristics (Gazali and Tambing, 2002). It is widely used to preserve semen for artificial insemination in animal breeding. Semen cryopreservation techniques are divided into slow freezing, rapid freezing, and ultra-rapid freezing. The main principle in semen cryopreservation procedures is water removal from the cell (dehydration) prior to intracellular freezing. The absence of dehydration procedure may form intracellular ice crystals and damage the cell. However, excessive dehydration can kill the cell (Mugbal *et al*. 2018). Sub-optimal freezing procedure can damage the function and structures of cell membranes and spermatozoa viability (Lessard *et al.* 2000). The other procedure which damages the cell is increasing the osmolarity of the freezing media where the cryoprotectant becomes toxic or osmotic swelling occurs (Kassai, 1996). Damage during the freezing process can occur in the plasma membrane of the spermatozoa as well as in the nucleus. The spermatozoa plasma membrane is composed of lipids, proteins, and carbohydrates. Goat lipids have a higher content of unsaturated fatty acids compared with the other ruminants; a susceptibility to cold temperatures is associated with a higher ratio of unsaturated fatty acids compared to saturated fatty acids. As a consequence, the spermatozoa lipid membrane is easily affected during the freezing process and results in lipid peroxidation (LPO), and also forms higher reactive oxygen species (ROS) such as malondialdehyde (Bansal and Bilaspuri, 2010; Gurler *et al*. 2016).

Seminal plasma, as a medium for spermatozoa, is a complex component derived from the testes, epididymis, and the accessory glands. This mixture comprises a variety of

factors that serve an important part of spermatozoa maturation whether through hormonal signaling, enzymatic activity or modification of the spermatozoa membrane (Manjunath *et al.* 1993). Repair of the cell plasma membrane could positively affect the processes inside the cell and eventually enhance the spermatozoa quality, including the motility and the viability of spermatozoa. Research has shown that the addition of certain proteins to seminal plasma could help maintain cell viability (Beatriz *et al.* 2000). The seminal plasma is a primary antioxidant which protects the spermatozoa from an oxidative stress (Castro *et al.* 2016).

Until recently, cryopreservation techniques for goat semen until post-thawing phase have been unsatisfactory due to the susceptibility of the spermatozoa plasma membranes to cold temperatures. Therefore, this research studied about the effect of diluents of milk-yolk combined with seminal plasma crude protein (SPCP) of cattle on the spermatozoa quality and cytochrome C expression, and the function of mitochondria of goat spermatozoa after a freezing procedure. The yolk contains lecithin which preserves the integrity of the membrane of spermatozoa and prevents the cold-shock. Meanwhile, milk contains carbohydrates as the source of energy (Susilowati, 2010).

MATERIALS AND METHODS

Seminal plasma crude protein collection from simmental semen

Semen was collected from three healthy male simmental bulls with good semen production by using artificial vagina equipped with a scaled glass tube. The artificial vagina was prepared by installing both sheath and sterilized collection tube while the space between outer and inner sheath were filled with warm water (45 ˚C) to form the desired temperature, approximately 42-43 ˚C. In addition, one-third of the front sheath was smeared with vaseline. After the artificial vagina was ready to use, the male simmental was stimulated by seeing a female simmental then the semen was collected. It was conducted for two times in a week. The volume obtained was about 7.26 mL. The semen was centrifuged to separate the seminal plasma and spermatozoa. Then the seminal plasma was purified to obtain the crude protein and added with phosphate buffer saline (PBS) and pnenylmethenesulfonyl fluoride (PMSF). After homogenizing for 5-10 minutes at 4 ˚C, it was re-homogenized and centrifuged at 6000 rpm for 10 minutes. The supernatant was separated and added with absolute ethanol 1:1 and precipitated overnight. The ethanol was removed and the pellet was added with Tris-HCl with a volume of 1-2 times of the pellet (Aulani'am, 2005).

The crude protein would be used as an additional material in the diluent of goat semen.

Goat semen collection

The research was carried out in Indonesia which is a tropical country. The activity of bulbourethral gland which produces a specific enzyme does not depend on the season. The semen was collected from eighteen goats. Eighteen goats with the body weight of 45 kg and age of 3-4 years were used for semen collection in this study. Semen collection was carried out for 2 times in a week. The semen was divided into 3 groups, P0, P1, and P2. Goat semen was collected using an artificial vagina and examined macroscopically and microscopically prior to further treatment. Some parameters of fresh goat semen: yellowish white color, distinctive odor, thick consistency, pH 6.8, volume of 1.1 ± 0.45 mL, concentration of 3850 × 106 spz/mm^3, mass motility +++, progressive individual motility of 91 ± 2.25%, viability of spermatozoa of 93 ± 5.35%, abnormal head and spermatozoa tails of 5.90 ± 0.2%, spermatozoa membrane integrity of 86.50 ± 3.55%, and resistance number of 3000 (resistance to 1% NaCl). Under normal circumstances, the processed goat semen should have a percentage of progressive motility and viability of approximately ≥ 70%.

Production of milk-yolk diluent

A total of 10 g fat-free powdered milk was dissolved in 100 mL distilled water and heated to the temperature of 92–95 ˚C for 10 minutes. Then, the milk was cooled down until it reached 37 ˚C. A chicken egg was cleaned and its yolk was separated out from the egg white. A total of 5 mL yolk was added into milk and diluted until it reached a progressive volume of 100 mL. Then, 1000 IU/mL penicillin and 1 mg/mL streptomycin were added into the mixture. The diluent was divided into diluent A and B.

Two diluents were prepared: (A) with the addition of goat semen; (B) with the addition of 16% glycerol (Susilowati *et al.* 2010). Three treatment groups were developed: (P0) as control group, consisted of diluent and goat semen (without seminal plasma crude protein); (P1) diluent, goat semen, and crude protein (2.5 mg/mL diluent); (P2) diluent, goat semen, and crude protein (5 mg/mL diluent). The doses of 2.5 mg and 5 mg were according to the preliminary research. All mixtures were stored at a temperature of 5 ˚C (equilibration time) for one hour, filling and sealing straw. Filling is a process to fill the straw with the mixture of semen and diluent. The straw used was a mini straw in a size of 0.25 mL. Semen should contain at least 25 million of live spermatozoa. After sealing process, the straws filled with semen were stored in cool top at -140 ˚C for 9 minutes.

Then they were put into the goblet and soaked into liquid nitrogen with a temperature of -196 ˚C. Then the thawing process was conducted at a temperature of 37 ˚C for 30 seconds and the thawed treatment samples were examined for spermatozoa motility, viability, and cytochrome C expression.

Examination of sperm motility

A total of 10 µL semen suspension was added 10 µL of physiological NaCl. The mixture was homogenized and placed on a slide for examination. Motile spermatozoa that moved forward were examined using a microscope with 400x magnification.

The progressive sperm motility was determined by rating visually the spermatozoa with moving to spermatozoa without moving and expressed as percentage (%). The motility was categorized into 4 criteria: moving forward, moving backward, moving around or in place, and not moving (Susilowati *et al.* 2010).

Examination of sperm viability

Fresh semen was dropped on the object glass, stained with eosin-negrosin, homogenized, made into thin tissue section, and dried quickly above a flame. Samples were then examined with a microscope at 400x magnification. The percentage of dead and live spermatozoa out of 100 spermatozoa was calculated with three repetitions.

The evaluation protocol for sperm viability was as follows: live sperm was marked with a transparent head, while the dead sperm was marked with a damaged plasma membrane and an increased permeability, and thus the stain entered the cell and the head appeared reddish (Susilowati *et al.* 2010).

Examination of cytochrome C expression

The object glass was coated with L-lycine and then dried on the hot plate. One drop of cement is placed on the glass of the object left at room temperature for 5 minutes and then fixed by using absolute methanol solution and acid glacial (3:1) for 15 minutes. Each preparation was washed with PBS two times for 5 minutes then immersed in 3% H_2O_2 for 30 minutes.

Each preparation was washed with PBS three times for 2 minutes. After that it was proteolytic digested using 0.025% trypsin in incubator with temperature of 37 ˚C for 10 minutes. Then it was washed with PBS three times for 2 minutes.

Then it was added with 5% primary antibody and incubated for 60 minutes and washed with PBS 2 times for 5 minutes. Biotinylated goat anti-polyvalent was added and incubated for 10 minutes at room temperature. The preparation then rinsed with PBS two times for 5 minutes, added with streptavidin peroxidase, and incubated for 30 minutes at room temperature.

It was painted with 1 drop (40 µL) DAB plus chromogen in 2 mL DAB plus substrate then incubated for 5-15 minutes. The preparation was rinsed with PBS two times for 5 minutes and then with aquadest for 5 minutes.

Cytosolic cytochrome C expression was observed through immunositochemistry technique by using ultra vision detection system. Spermatozoa expressing cytosolic cytochrome C were stained brown in the middle to end part of their tail (Figure 1).

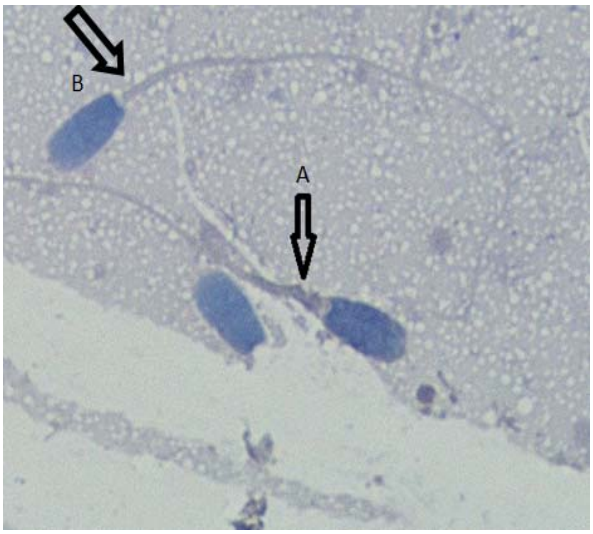

Figure 1 A: ekspresi cytochrom C nampak pada bagian leher spermatozoa
B: ekspresi cytochrom C tidak tampak pada bagian leher spermatozoa

The total number of spermatozoa expressing cytosolic cytochrome C was the total of three different fields of view at 400x magnification, with 100 spermatozoa examined per field.

Data analysis

Motility, viability, and cytochrome C expression data were analyzed using an F-test ($P<0.05$) to see the differences between group experiments. The analysis was continued by using BNT test if there were any differences (Santoso and Fandy, 2001).

RESULTS AND DISCUSSION

Seminal plasma crude protein collection from simmental semen

The collected semen from simmental cattle was yellowish, with a distinctive odor, thick consistency, pH in the range of 6 to 7, volume of 7.26 ± 1.72 mL, spermatozoa concentration of 1040.106 ± 226.55/mL, mass motility +++ (the movement forming a large and plentiful waves), motility of 85.83 ± 2.89%, and viability of 95.65 ± 2.36% (Table 1).

Table 1 Characteristic of simmental fresh semen

Parameter	Mean ± SD
Volume (mL)	7.26±1.72
Consistency	Medium to thick
Color	Milk white to yellow
Odor	Distinctive
pH	6-7
Mass motility	+++
Motility (%)	85.83±2.89
Viability (%)	95.65±2.36
Abnormality (%)	6.69±1.86
Concentration (million/mL)	1040±226.55
Seminal plasma crude protein (mg/mL)	45.8–196

Effect of seminal plasma crude protein (SPCP) addition on the motility (%) of goat spermatozoa

The results about the mean (±standard deviation) and F-test of the percentage of sperm motility after freezing on each experiment are presented in Table 2.

Table 2 The percentage of sperm motility after freezing procedure

Treatment	Mean of spermatozoa motility (%) ± standard deviation
P0	33.89±2.32^{b}
P1	42.75±2.67^{a}
P2	35.53±3.20^{b}

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

The results show that there are no differences in the sperm motility between P0 group and P2 group. Meanwhile, there was a significant difference between P1 group with group control (P0) and P2 group with the percentage of 42.75 ± 2.67%. BNT test showed that P1 (2.5 mg crude protein) had the highest total motility.

Effect of seminal plasma crude protein (SPCP) addition on the viability (%) of goat spermatozoa

The results about the mean (±standard deviation) and F-test of the percentage of sperm motility after freezing on each experiment are presented in Table 3.

Table 3 The percentage of spermatozoa viability after freezing procedure

Treatment	Mean of spermatozoa viability (%) ± standard deviation
P0	50.87±2.92^{b}
P1	59.81±2.71^{a}
P2	52.20±3.28^{b}

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

From the analysis, there were no significant differences in the spermatozoa viability of P0 with a P2 group (P>0.05). In contrary, the P1 group had significant differences with the P2 group, showed by the highest percentage of P1 group (59.81±2.71%). BNT test showed that P1 (2.5 mg crude protein) had the highest viability.

Effect of seminal plasma crude protein (SPCP) addition on the cytochrome C expression (%) of goat spermatozoa

The results of SPCP addition into the cytochrome C expression of goat spermatozoa are presented in Table 4. From the analysis, there were no significant differences in the cytochrom C expression of P0 with a P2 group (P>0.05). In contrary, the P1 group had significant differences with the P2 group, showed by the lowest percentage of P1 group (59.81±2.71%).

The analysis demonstrates that the order percentage of cytochrome C expression started from the lowest to the highest was in P1, followed by P2, and P0. The lower cytochrome C expression indicates that the mitochondrial is still in a good function.

Table 4 The cytochrome C expression (%) of goat spermatozoa after freezing procedure

Treatment	Mean of cytochrome C expression (%) ± standard deviation
P0	7.14±0.53^{a}
P1	3.23±0.73^{b}
P2	4.76±0.36^{b}

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

BNT test showed that P1 (2.5 mg crude protein) had the lowest cytochrome C expression.

In general, the volume of semen should increase according to age, body size, change in condition, reproductive organ health, and semen storage frequency. The color, consistency, and concentration of spermatozoa are strongly related to each other. The resistance of the semen is also important to be examined. It will show the ability of the spermatozoa to be not affected by 1% NaCl. NaCl is a hypotonic solution which can diffuse into spermatozoa and will cause the spermatozoa dead (Susilowati *et al*. 2010).

A dilution can lower the spermatozoa concentration and make it pale. Meanwhile, semen consistency depends on the ratio of spermatozoa and plasma semen (Evans and Maxwell, 1987). The more base or acidic pH can reduce the spermatozoa viability.

The acidity of the semen may be caused by the lactic acid produced during the final process of metabolism. According to Toelihere (1985), spermatozoa metabolism in an anaerobic state will accumulate the lactic acid which could increase or decrease the semen pH. The semen that used in this study meet the requirements for seminal plasma crude protein isolation. In addition, Toelihere (1985) also explained that a good quality plasma can be obtain from a semen which has a minimum percentage of motility and viability of 70% and the minimum spermatozoa concentration of 600 million per millimeter semen.

The motility of spermatozoa is an important indicator to determine the semen quality. With motility, spermatozoa will be able to reach the infundibulum for the penetration process into the ovum cells. The definition of progressive individual motility is the ability of spermatozoa to move forward. This ability is needed by the spermatozoa while they are in the reproductive track in order to reach the fertilization site, especially when penetrating the pellucid layer (O'Flaherty *et al.* 2006). P1 (2.5 mg crude protein) had the highest percentage of the motility and viability and also had the lowest percentage of cytochrome C expression for the post-thawing goat spermatozoa.

It indicates that the optimal dose of crude protein addition to increase the autophosphorylation of spermatozoa associated with the increasing motility was 2.5 mg. The increasing phosphorylation of tyrosine kinase would increase hyperactivation of spermatozoa motility that is necessary for the penetration process into the pellucid zone of ovum cells. It was confirmed by Naz and Rajesh (2004), who proposed that the phosphorylation of tyrosine proteins in spermatozoa is very important for motility hyperactivation and zona pellucida reaction.

The freezing procedure results in membrane damage, so there should be an excessive water release from inside the spermatozoa. Thus eventually increases the concentration of intracellular electrolytes and results in the formation of ice crystals. If this happens continuously, the spermatozoa lose viability. The damage caused by freezing procedure, including freezing and thawing continuously, mostly occurs in the plasma membrane or mitochondria, and in the worst case could damage the nucleus (Blesbois, 2007; Wach-Gygax *et al.* 2017).

In cold-stressed spermatozoa, an initiation of apoptosis (nucleus damage) occurs because of an intrinsic signal that is generated when the cells experience a stress. The intrinsic or mitochondrial pathway is started with the permeability of the outer membrane of mitochondria that is mediated by the permeability transition pore (PT pore). The opening of PT pores results in the entry of water into the mitochondrial matrix so the intermembrane space swells and the outer membrane damaged.

This damage results in the leak of pro-apoptogenic proteins, such as cytochrome C, apoptosis inducing factor (AIF) and endonuclease G (Crompton *et al.* 1998; Li *et al.* 2001).

The cytochrome C system is in mitochondria. Mitochondria have an important function as the powerhouse that provides energy for the cell. Cytochrome C is a heme protein that acts as an electron carrier in the phosphorylation of mitochondrial oxidation. This protein would leak out from the mitochondria after a change of electrochemical potential in the membrane, including protein from inside and the outside the membrane. The potential changes cause the opening of a non-specific channel in the permeable membrane (Lumongga, 2008). This pathway could be activated through other lethal stimulatory response, such as DNA damage (Ustuner *et al.* 2015), oxidative stress, and hypoxia (Kumar *et al.* 2005). Cytochrome C along with Apaf-1 and procaspase 9 forms a complex called an apoptosome. Inside the apoptosome, procaspase 9 is activated into caspase 9 enzyme which cleaves procaspase 3 to caspase 3, one of the so-called executioner caspases (Peterson *et al.* 2010). The apoptosome complex can trigger the apoptosis (Wang and El-Dairy, 2004).

The P1 (2.5 mg crude protein) was the optimum dose to improve the strength of plasma membrane in order to protect the spermatozoa from cold temperature, in the freezing and liquefaction procedures. In this condition, spermatozoa that would be used for insemination into the female reproductive tract are able to maintain its viability. In the previous experiment, Roncoletta *et al.* (2000) stated that additions of 40% seminal plasma protein have the ability to maintain spermatozoa viability during the freezing procedure. This characteristic is associated with the ability to participate in the modification of spermatozoa membrane permeability. The ability of cattle seminal plasma protein in protecting the spermatozoa membrane is very high compared with the plasma protein of other animals (Sinha *et al.* 1996). Besides, seminal plasma protein could stabilize the protein elements in the lipid membrane (Castro *et al.* 2016).

According to Bergeron *et al.* (2005), the seminal plasma protein of a goat has a negative effect on the freezing process of goat semen because some proteins in seminal plasma could cause cholesterol and phospholipid release from the spermatozoa membrane. Goat seminal plasma also has a phospholipase enzyme called egg yolk coagulating enzyme (EYCE) that works as an antifreezing factor (Gazali and Tambing, 2002).

CONCLUSION

The addition of seminal plasma crude protein with the dose of 2.5 mg into the goat diluent can improve spermatozoa

motility and viability and decrease cytochrome C expression during and after thawing.

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