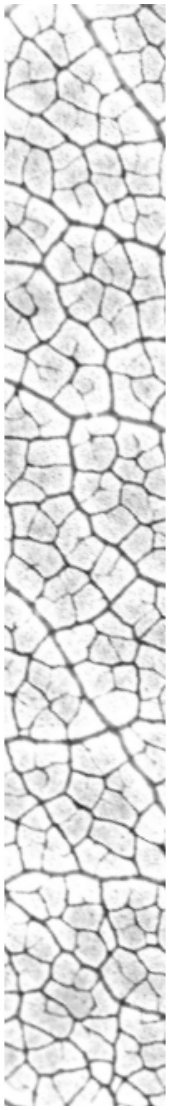

EurAsian Journal of BioSciences
Eurasia J Biosci 13, 193-197 (2019)

# Physiological study of the use of bull seminal plasma in skim milk diluent to improve quality of frozen ram semen

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**Abstract**
Ram semen freezing process still generates poor outcome due to phospholipase. Thus, an alternative to substitute ram seminal plasma is recently developed using bull seminal plasma. The aim of this study was to determine the benefits of bull seminal plasma added by ram spermatozoa diluent in the freezing process. This study used control treatment (P0); (P1) was (diluent + ram semen) + bull seminal plasma (1: 1); (P2) was (diluent + ram semen) + bull seminal plasma (1:2). The result showed that P1 generated the highest viability, motility, plasma membrane intact, and the lowest DNA fragmentation, compared to second treatment and control. Statistical analysis of viability, motility, plasma membrane intact and DNA fragmentation were significantly different ($p<0.05$) among treatments. It can be concluded that additional of seminal plasma of bull can maintain motility, viability, plasma membrane intact and decrease the of DNA fragmentation in ram spermatozoa after-thawing.

**Keywords:** after-thawing, bull seminal plasma, DNA fragmentation of spermatozoa, ram semen, freezing process

Susilowati S, Wurlina, Anom Adnyana IDP, Mustofa I, Hariadi M (2019) Physiological study of the use of bull seminal plasma in skim milk diluent to improve quality of frozen ram semen. Eurasia J Biosci 13: 193-197.



## INTRODUCTION

Frozen semen has been widely used in artificial insemination of animals. It is a preserved semen after diluted with some diluters and stored in freezing temperature of liquid nitrogen -196°C (Direktorat Jenderal Produksi Peternakan 2007). The purpose of semen storage in freezing temperature is to retain as much as possible some biological properties of sperm, especially in viability and motility. However, some damage on membrane structure and function that decrease spermatozoa viability, take place during freezing processes (Lessard et al. 2001). The reason is partly due to the dehydration process that exhibits the formation of intracellular ice crystals. It can also be due to the increase of osmolarity of freezing media that lead to convert cryoprotectant into toxic. The toxicity of concentrated electrolyte or the osmotic swelling results in physical damage by the formation of extracellular ice crystals (Al-Jiffri and Alsharif 2017). Damage during freezing processes is common in the plasma membrane and the nucleus of spermatozoa. Damage in the core of spermatozoa can even cause gene mutations.

Seminal plasma of semen consists of various components that regulate the specific biochemical function of spermatozoa. Seminal plasma is mainly composed of water and some organic and inorganic substances. Seminal plasma also contains decapacitation factor (DF), which coats the surface of spermatozoa during ejaculation (Riadi 2004). DF will bind to spermatozoa surface and activate intracellular calcium-ATPase to maintain the intracellular calcium concentrations to remain low (Al-Jiffri and Alsharif 2017). Factors contained in seminal plasma may affect the viability, motility and membrane integrity of cold spermatozoa (Barrios et al. 2000). Seminal plasma has the capacity to increase the percentage of the motility spermatozoa during cooling down, freezing and thawing (Purdy and Graham 2004). Repairs to the plasma membrane of sperm cell have a positive influence on biochemical processes in the cell and ultimately improve the quality of spermatozoa such as motility and viability. Biomolecular research proved that some additional protein can improve the fertilization process, as well as sustain the life of the cell.

Received: November 2018
Accepted: March 2019
Printed: May 2019

The chance of sperm damage during the freezing process and after thawing, are even higher in ram semen. This might be due to the presence of egg yolk coagulating enzymes in ram seminal plasma, called phospholipase A and triglycerol lipase. In addition, the plasma membrane of ram spermatozoa is more susceptible to cold stress caused by its low levels of cholesterol in the plasma membrane (Carmen et al. 2005). The integrity of plasma membrane is absolutely necessary to ensure the sperm survival and the success in the fertilization of the ovum. Plasma membrane also acts as a filter that regulates in the exchange of substances while maintaining the levels of intracellular ions inside and outside the cell. Therefore, researchers wanted to examine the effects of addition of bull seminal plasma in milk diluent into the freezing process.

## MATERIALS AND METHODS

This experiment was done using randomized block design with three treatments and six replications. The treatments were a different level of bull seminal plasma proteins addition on ram semen dilution for preservation. Diluent composed of skim milk (10%) in 100ml water. The heating procedure was applied and added with penicillin plus 1000 IU and streptomycin 1 mg /ml. The diluent was then divided into two parts, namely diluent A (50 ml) and diluent B (50 ml). Diluent B was further added with 5 ml glycerol. Control treatment (T0) was ram semen + diluent A (ratio 1:1 volume base), T1 = T0 + bull seminal plasma proteins (ratio 1:1 volume base), T2 = T0 + bull seminal plasma proteins (ratio 1:2 volume base). Ram semen in all treatments were mixed with diluent B at ratio 1:2 volume base. Diluent B was added in four steps an interval of 15 minutes. The mixture was let for an hour to allow the achievement of equilibration stage. After the equilibration stage was achieved, sample of semen on each treatment was taken for sperm motility, viability, and plasma membrane intact observation. Then the rest of semen on each treatment were stored in liquid nitrogen for one week. The frozen semen was then incubated at a temperature of 37 °C for 30 sec for thawing process. Post-thawing semen was observed for sperm motility, viability, plasma membrane intact, and DNA fragmentation.

Analysis for sperm motility was done by use 10 ml of a semen sample on each treatment was added with 10 ml physiological NaCl solution and homogenized. One drop of the prepared semen sample was put on object glass, covered with a cover glass and observed for sperm motility using a light microscope of 400x magnification. A total of 100 sperm in several sites of observation were counted and divided into four criteria based on their movement, i.e. moving forward, moving backward, moving or rotating in place and not moving (Beatriz et al. 2000). The same procedure was done for all other semen samples.

Viability analysis was done using one drop of a semen sample was put on an object glass, then one drop of eosin-nigrosin was added on the semen sample and mixed thoroughly using glass stick. Then a staining of the prepared semen sample was quickly made using another object glass and quickly air dried. Dry staining was then observed using a light microscope of 400x magnification and a total of 300 sperm were observed from three different sites of observation. The counted sperm were divided into two groups based on their staining: unstained sperm as viable or live sperm and stained sperm as dead sperm. The same procedure was done for all other semen samples.

Sperm plasma membrane intact was observed using hypoosmotic swelling test. The hypoosmotic solution was prepared using 7.35 g Na-citrate. 2 $H_2O$ and 13.52 g fructose dissolved in 1000 ml distilled water. A total of 0.1 ml semen sample was added with 1 ml hypoosmotic solution then incubated at 37 °C for 30 min. Then, observation using a light microscope of 400x magnification was done. Sperm with plasma membrane intact was indicated by a circle and bulging tail, which showed that the plasma membrane still functions properly for water absorption in a hypotonic environment. Damaged sperm plasma membrane was indicated by a straight tail.

DNA fragmentation was analyzed using Apo-BrdU-IHC situ DNA Fragmentation Assay Kit Bio Vion with the following procedure: semen was dropped on an object glass, covered with a cover glass and soaked with methanol for 24 hours. After soaking, the object glass was taken and washed with phosphate buffer saline (PBS) followed by sequence incubation at room temperature using proteinase K for 20 minutes, using 3% $H_2O_2$ for 5 minutes, using reaction buffer for 10-30 minutes, and finally using labeling reaction mixture at 37°C for 60-90 minutes followed by blocking buffer drops evenly on the surface of the object for 10 minutes, dripping with antibody solution for 60-90 minutes in dark atmosphere minutes, etching with blocking buffer, dropping with conjugate solution and incubated at room temperature for 30 minutes. After each incubation and before the next incubation, washing the sample with PBS was done. Finally etching substrate for peroxidase (DAB-Diamino Benzidine) for 15 min at room temperature, then washed with $H_2O$ and dried. After that, it was done counterstain with Methyl Green at room temperature for 3 minutes and soak briefly twice in absolute alcohol after it dried. After observation under inverted microscope with a magnification of 400x and 1000 x. DNA fragmentation in spermatozoa was indicated by blackhead spots when fragmentation did not take place the color was greenish.

Data analysis for motility, viability, plasma membrane intact and DNA fragmentation of sperm was done by ANOVA, and if there was significant difference, then

**Table 1.** Macroscopic and microscopic characteristics of Bull and Ram semen

| Indicator | Character | |
|---|---|---|
| | Bull | Ram |
| Volume (ml) | 5.5 | 1.2± 0.50 |
| Consistency | Thick | Thick |
| Color | Beige | Beige |
| Odor | Typical | Typical |
| pH | 6.6 | 7.00 |
| Mass Movement | +++ | +++ |
| Individual Movement (%) | Progressive (P) | Progressive (92 ± 7,45) |
| Motility | 82% | 80% |
| Concentration (million) sel spz/mm$^3$ | 2.320x10$^6$ | 3.860x10$^6$ |
| Viability (%) | | 93 ± 7.50 |
| Plasma membrane intact (%) | | 87 ± 7.40 |

Notes: +++ (high)

**Table 2.** Quality of ram semen in freezing process

| Treatment | Motility (%) | Viability (%) | Plasma membrane intact (%) |
|---|---|---|---|
| P0 | 53 ± 1.45 c | 56 ± 1.65 c | 54 ± 2.35 c |
| P1 | 73 ± 1.75 a | 75 ± 1.50 a | 74 ± 2.15 a |
| P2 | 63 ± 1.75 b | 66 ± 1.65 b | 65 ± 2.25 b |

Notes: Different annotation showed a significant difference ($p < 0.05$)

data analysis was continued with followed by Duncan Multiple Range Test (Ersoy et al. 2015).

## RESULTS AND DISCUSSION

### Characteristics of Simental Bull Fresh Semen

The result was shown that both Simental bull and ram fresh semen used in this experiment were normal. The volume of ejaculated semen by bulls varies between bulls and by same bull itself (**Table 1**). In general, the volume of ejaculated semen will increase as influenced by age, great body, increase testis size or circumstances, reproductive health, and frequency of semen collection (Santoso and Fandy 2001). However, color, consistency, and concentration of bull semen were closely similar to each other. More dilute or lower consistency of semen is generally followed by the lower sperm concentration in the semen and lighter color. While consistency of semen depends on the ratio of spermatozoa and seminal plasma (Salisbury and Van Demark 1985). Degrees of acidity (pH) greatly affects the survival of spermatozoa. Normal semen pH is 6.5 – 7.0 in which higher or lower pH causes sperm death (Santoso and Fandy 2001). pH is likely influenced by the concentration of lactic acid produced in the final process of metabolism. Spermatozoa metabolism in anaerobic conditions produces accumulation of lactic acid that decrease pH of semen (Evans and Maxwell 1987).

Acidity or pH of semen is usually acid in the presence of toxic substance secreted during cell death, that enables cells to undergo DNA fragmentation (Barrios et al. 2000). The addition of seminal plasma can reduce sperm DNA damage in dilution process of ram spermatozoa. The seminal plasma of bull plays its role as an antioxidant. However, it remains unclear whether compounds prevent excessive oxidant or the chain reaction. In conclusion, the addition of bull seminal plasma improved sperm motility, viability, plasma membrane intact and reduced DNA fragmentation of post-thawing ram semen.

### Macroscopic and Microscopic Examination of Fresh Ram Semen Prior to the Freezing Process

Ram semen collected and used in this experiment was yellowish white, distinctive smell, thick consistency, pH ±7, volume 1.2 ± 0.5 ml, concentration of 3,860 x 10$^6$ cell spz/mm$^3$, +++ mass motility (movement formed a large wave and many), progressive motility of individual movement sperm (move forward) 92 ± 7.4%, a mean viability of 93 ± 7.5% and 87 ± 7.4% plasma membrane intact. Thus, based on the characteristics, ram semen used in this experiment was normal and met the requirements for further use in this study.

The results showed that the addition of bull seminal plasma in ram semen diluents increased significantly sperm progressive motility, viability and plasma membrane intact. The value of those parameters in P1 and P2 were much higher than those in P0. While those values in P1 were significantly higher than those in P2 (**Table 2**). Although not significantly different, the same pattern of sperm quality was also shown by the same treated ram semen post-thawing. The addition of seminal plasma improved the viability and prevent cold shock membrane damage of ram spermatozoa. The proportion of polyunsaturated fatty acids in the sperm membrane influences lipid fluidity. A lower ratio of polyunsaturated to saturated fatty acids has been found in the sperm membrane to be most readily disrupted by cold shock. Conversely, control treatment (P0) showed the highest DNA fragmentation of sperm as compared to P2 and P1 (**Table 3**).

**Table 3.** Quality of ram semen in Post-thawing process

| Treatment | Motility (%) | Viability (%) | Membrane plasma intact (%) | DNA fragmentation (%) |
|---|---|---|---|---|
| P0 | 35 ± 1.25$^{c}$ | 40 ± 1.75$^{c}$ | 36 ± 1.25$^{c}$ | 35 ±1.75$^{c}$ |
| P1 | 43 ± 2.35$^{a}$ | 45 ± 2.00$^{a}$ | 44 ± 1.50$^{a}$ | 24 ± 2.45$^{a}$ |
| P2 | 40 ± 2.25$^{b}$ | 41 ± 2.45$^{b}$ | 40 ± 2.15$^{b}$ | 30 ± 1.80$^{b}$ |

Notes: Different annotation showed a significant difference ($p < 0.05$)

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. The intrinsic pathway is initiated with elevated cytosolic calcium and biochemical products derived from oxidative stress. Imbalance in biochemical products will give a signal to the mitochondria that later open mitochondrial outer membrane, followed by matrix swelling and loss of mitochondrial membrane potential, resulting in cytochrome c release. Cytochrome c release will induce a series of events that leads to the activation of the intracellular protease caspase group. Caspase 3 binds to DNA endonuclease that migrates towards the nucleus and initiates DNA breakdown (Solmaz and Houshang 2013). There are two important components that cause the release of Cytochrome C, namely Mitochondrial Permeability Transition Pore (MPTP) and proapoptotic protein Bax. MPTP opening is influenced by several factors such as the accumulation of calcium, oxidants, and low mitochondrial transmembrane potential. 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 (Kleinsmith 2006, Kola et al. 2015).

Motility is one of the criteria of semen quality that strongly correlates with the ability of sperm to fertilize the ovum. The results showed that the addition of bull seminal plasma proteins in the first treatment, produce the highest percentage of motility, in the absence of phospholipase A and comparison in accordance with ram seminal plasma levels replaces. Some research proved that in the seminal plasma are proteins that have a positive role in the process of capacitation and acrosome reaction of spermatozoa. In the bull with the addition of Insulin-Like Growth Factor Complex-I present in seminal plasma can increase the percentage of motile spermatozoa. Several possible mechanisms of seminal plasma proteins in maintaining sperm motility is through energy metabolism as indicated by an increase in glucose uptake, increased production of lactic acid, increased activity of pyruvate dehydrogenase, and the possibility to have an antioxidant effect (Susilowati 2007).

## CONCLUSION

Additional of seminal plasma of bull can maintain motility, viability, plasma membrane intact and decrease the of DNA fragmentation in ram spermatozoa after-thawing.

## ACKNOWLEDGEMENT

Authors thank Brawijaya University for facilitated this research.

## REFERENCES

Al-Jiffri OH, Alsharif FM (2017) Levels of circulating adipokines and their relation with glycemic control and insulin resistance in Saudi patients with non-alcoholic fatty liver disease. European Journal of General Medicine, 14(4): 99–102. https://doi.org/10.29333/ejgm/81739

Barrios B, Pérez-Pé R, Gallego M, Tato A, Osada J, Muiño-Blanco T, Cebrián-Pérez JA (2000) Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod., 63(5): 1531-7. https://doi.org/10.1095/biolreprod63.5.1531

Beatriz B, Rosaura P, Margarita G, Agustín T, Jesús O, Teresa MB, José ACP (2000) Seminal plasma protein revert the cold shock damage on ram sperm membrane. Biol. Reprod., 63: 1531-7. https://doi.org/10.1095/biolreprod63.5.1531

Carmen C, Rosaura P, Adriana C, Mario O, Lucia C, Margarita G, Teresa MB, Jose ACP (2005) Remodelling of lipid rafts during in vitro capacitation and acrosome reactionof ram spermatozoa. Biochem. Anal. Biochem., S5-001.

Direktorat Jenderal Produksi Peternakan (2007) Petunjuk Teknis Produksi dan Distribusi Semen Beku (Technical Guidelines for Production and Distribution of Frozen Semen). Jakarta: Agricultural Ministry.

Ersoy E, et al. (2015) Individual features and contraceptive attitudes of women who had unintended pregnancy. Journal of Clinical and Experimental Investigations, 6(3): 250-5. https://doi.org/10.5799/ahinjs.01.2015.03.0528

Evans G, Maxwell WMC (1987) Salamon's Artificial Insemination of Sheep and Goats. Sydney: Butterworths.

Kleinsmith LJ (2006) Principle of Cancer Biology. 1st Edn., University of Michigan, Pearson: Benjamin Cummings.

Kola M, Uzun Ş, Zeybek ND, Sarıcaoğlu F, Akıncı SB, Aypar Ü (2015) Effects of intra articular tramadol on articular cartilage and synovium of rats. J Clin Exp Invest., 6(4): 337-42. https://doi.org/10.5799/ahinjs.01.2015.04.0546

Lessard C, Parent S, Leclerc P, Bailey JL, Sullivan R (2001) Cryopreservation alters the levels of the bull sperm surface protein p25b. J. Androl., 21: 700-7.

Nawang SS (2005) Integritas Membran Spermatozoa Mencit Pada Pemberian Peroral Fasa Air daun Justicia Gendarusa Dengan Metode Hypoosmotic Swelling Test. [Thesis]. Pharmaceutical Faculty. Surabaya, Airlangga University.

Purdy PH, Graham JK (2004) Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosom reaction, and fertility. Biol. Reprod., 71: 522-7. https://doi.org/10.1095/biolreprod.103.025577

Riadi (2004) Perubahan karakter Ejakulat dan Aktifitas Antioksidan pada masa Reproduksi Kambing Kacang dan Kambing Peranakan Ettawa (Changes in the character of the ejaculate and Antioxidant Activity during the Reproduction Nuts and Goat goats Peranakan Ettawa) [Disertation]. Post-graduated Program. Airlangga University.

Salisbury GW, Van Demark NL (1985) Fisiologi Reproduksi dan Inseminasi Buatan pada Sapi (Physiology of Reproduction and Artificial Insemination in Cattle). [R. Djanuar, trans]. Yogyakarta, Gadjah Mada University Press.

Santoso S, Fandy T (2001) Riset Pemasaran, Konsep dan Aplikasi dengan SPSS (Research, Concept and Application using SPSS). Jakarta: PT Gramedia.

Solmaz SE, Houshang S (2013) Recognition and analysis of effective factors on investors' decision making in stock exchange of Tehran. UCT Journal of Management and Accounting Studies, (4): 14-21.

Susilowati S (2007) Insulin Like Growth Factor-I Complex plasma Seminalis Kambing Meningkatkan Kualitas Spermatozoa hasil Sentrifugasi (Insulin Like Growth Factor-I Complex plasma Ram seminal spermatozoa Improving Quality centrifugation). Media Kedokteran Hewan., 23: 155-60. https://doi.org/10.23869/bphjbr.12.2.200711