Buletin Peternakan 45 (3): 155-158, August 2021

ISSTAP
Indonesian Society for Sustainable Tropical Animal Production

Bulletin of Animal Science

ISSN-0126-4400/E-ISSN-2407-876X Accredited: 36a/E/KPT/2016
http://buletinpeternakan.fapet.ugm.ac.id/

Doi: 10.21059/buletinpeternak.v45i3.65426

Effect of Different Pre-Freezing Time on Quality of Frozen Fat-Tailed Ram Semen

Sigit Bintara[1*], Andriyani Astuti[2], Panjono[3], and Tri Anggraeni Kusumastuti[4]

[1]Department of Animal Breeding and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada, 55281, Indonesia
[2]Department of Animal Nutrition and Feed Science, Faculty of Animal Science, Universitas Gadjah Mada, 55281, Indonesia
[3]Department of Animal Production, Faculty of Animal Science, Universitas Gadjah Mada, 55281, Indonesia
[4]Department of Livestock Sosio-Economics, Faculty of Animal Science, Universitas Gadjah Mada, 55281, Indonesia

Article history
Submitted: 23 April 2021
Accepted: 23 August 2021

* Corresponding author:
E-mail: sigitbintara@ugm.ac.id

ABSTRACT

This study aimed to determining the effect of temperature changes on the sperm quality of fat-tailed sheep during the freezing process using a microcontroller. This study was conducted from April to October 2018 at the Laboratory of Animal Physiology and Reproduction, Faculty of Animal Science UGM. The study was used twelve mature ram. The methods were conducted by collecting semen using artificial vagina, semen dilution, freezing semen, thawing and semen quality test. The pre-freezing temperature time were grouped for 6, 9, and 12 minutes. Data of frozen semen quality (motility, viability, and abnormality) the data were analyzed using one-way ANOVA and the study was arranged using Completely Randomized Design (CRD). The average of spermatozoa motility after semen freezing with a 5 to -140°C decrease in pre freezing temperature for 12 minutes (50±5.3%) was significantly different (P<0.05) compared to 9 minutes (48±4.8%) and 6 minutes (43±4.8%). The average of spermatozoa viability after semen freezing with a decrease in pre freezing temperature of 5 to -140°C for 12 minutes (55±4.7%) was significantly different (P<0.05) compared to that for 9 minutes (52±3.5%) and 6 minutes (49±5.7%). The average of spermatozoa abnormality after freezing with a decrease in pre freezing temperature of 5 to -140°C for 6, 9, and 12 minutes was not significantly different (10±2.4%, 9±0.8%, and 10±0.9%, respectively). Based on the findings, it is possible to conclude that semen freezing at a lower pre-freezing temperature of 0 to -140°C for 12 minutes can improve the quality of freezing results.

Keywords: Fat-tailed sheep sperm, Pre freezing, Semen Diluter, Semen freezing, Time Freezing

Introduction

Over decades, the rate of artificial insemination (AI) in sheep has increased in Indonesia. The most widely discussed advantages of AI it was capable to increase the numbers of offspring born from superior ram. AI with frozen semen is importance in breeding contributing to increased domestic species. These scheme were well developed in cattle but in sheep still rare. Evaluation of male semen quality during the entire course of processing as well as its subsequent fertilization ability are based on basic indicators such as spermatozoa concentration, morphology, motility, and viability (Dolezalova *et al.,* 2015). Further, it was important to make sure optimal semen processing including semen collection, dilution (Mostafapor and Ardebili, 2014), equilibration and pre-freezing (Camara *et al*., 2011), also evaluated (Rovegno *et al*., 2013) in ram semen.

Semen processing have many factors which need to evaluated which are extender, freezing rate, dilution concentration, time and method freezing condition (Foote and Kaporth, 2002). While the processing, spermatozoa were significantly affected by the materials and procedures to make frozen semen (Watson, 2000). The extent of spermatozoa damage caused by cold shock depends on temperature and temperature rate also. Mainly, the higher the cooling rate, make more damage in spermatozoa (Lemma, 2011) resolving the subsequent post thaw spermatozoa motility (Andrabi *et al*., 2008). The length of freezing curve type were responsible for many physiological changes leading to many spermatozoa damages (Forero-Gonzalez *et al*., 2012).

Reducing the temperature is related with a lower proportion unfrozen fraction as well as an increase of osmotic pressures. This fact indicates that the freezing speed should be maximized, but

fast freezing also causes intracellular ice crystal formation. Therefore, freezing has to be slow enough for sufficient spermatozoa dehydration (Watson, 2000). Based on those study, hypothesis could be drawn that the freezing rate or time would significantly affected the spermatozoa quality after thawing. Therefore, the aims of this study was to identify the effect of selected freezing time on spermatozoa quality after thawing determined by motility, viability and abnormality of spermatozoa.

Materials and Methods

Semen collection

This study was carried out at the Laboratory of Animal Physiology and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada. The animals were maintained under standard field, laboratory conditions and performed following the University guidelines. Semen was collected from 12 matures Fat-tailed rams using an artificial vagina at an artificial vaginal temperature between 42-45°C. The semen was then macroscopically and microscopically examined, comprising of concentration, motility, viability and abnormalities of spermatozoa.

Semen dilution and processing

In this study, semen was diluted with Andromed at 25°C by pouring diluent slowly through the wall of erlenmeyer glass containing semen until the diluent was homogeneously mixed. The mixing was carried out at room temperature. After the dilution, the semen was then equilibrated for 2 hours in a refrigerator at temperature of 5°C. Semen was loaded into 0.25 mL straws at a $30x10^6$ spermatozoa/straw concentration. Diluted sperm pre-freezing was attained using styrofoam that was filled with liquid nitrogen to a depth of 4 cm, and the method was achieved using an automatic system controlled by a microcontroller. To reach temperatures ranging from 5°C to -140°C, three different pre-freezing times were used: Freezing time I (6 minutes), Freezing time II (9 minutes), and Freezing time III (12 minutes). Thereafter, doses were handled into a liquid nitrogen container and stored at -196°C. Following that, doses were transferred to a liquid nitrogen container and stored at -196°C.

Evaluate the quality of semen and analyze statistical data

The straws cryopreserved were subsequently thawed in a water bath at 38 ± 1°C for 30 s. The data observed were concentration, motility, viability and abnormalities of spermatozoa before and after freezing. The sperm motility was observed by putting and homogenizing 10 µL of diluent mixed with NaCl (1:4) and then placed on the microscope (Olympus CH 20). The view was taken from ten fields at a magnification of 100x400, scores were given in the range 0-100% with a 5% scale. Eosin staining procedure was used for sperm viability. A total of 200 spermatozoa were counted per sample using light microscope (Olympus CH 20) to differentiate the reacted and non-reacted spermatozoa. The death sperm with damaged acrosome emitted the strong red color whereas non-reacted with were live sperm emitted with light pink or no-color. The all data were analyzed using one-way Completely Randomized Design (CRD).

Results and Discussion

The present study showed that the qualities and quantities such as motility and viability by macroscopically or microscopically of fresh ram semen semen were normal also reported by other investigator (Camara *et al.,* 2011). The assessment results of fresh ram semen quality was shown in Table 1. The present study showed significant effect of freezing times affecting semen motility and viability of spermatozoa which similar with previous research (Defoin *et al.,* 2008), the data revealed that the better quality was observed in the pre-freezing time 12 min (Table 2).

The pre-freezing rate time affected the spermatozoa motility and viability ($P<0.05$), the data showed the average of spermatozoa motility after thawing with freezing temperature 0 to -140°C time in 12 minutes (50±5.3%) was significantly different ($P<0.05$) compared to that

Table 1. Fresh ram semen quality before pre- freezing

Variable	Assessment
Volume (ml)	0.86±0.12
Colour	Cream
pH	6.83±0.06
Consistency	Thick
Odor	Specific
Motility (%)	82.69±2.54
Viability (%)	86.58±4.30
Abnormality (%)	13.77±2.20

Table 2. Diluted frozen semen quality in each pre-freezing method

	0 Minutes	6 Minutes	9 Minutes	12 Minutes
Motility (%)	73.65±5.40*	43±4.8[a]	48±4.8[b]	50±5.3[c]
Viability (%)	77.88±3.22*	49±5.7[a]	52±3.5[b]	55±4.7[c]
Abnormality (%)	14.38±1.60	10±2.4	9±0.8	10±0.9

*[abc] Means were significantly different in each columncolumn (*P<0.05)*

for 9 minutes (48±4.8%) and 6 minutes (43.48%). Also, the average of spermatozoa viability after thawing with freezing temperature 0 to -140°C time in 12 minutes (55±4.7%) was significantly different (P<0.05) compared to that for 9 minutes (52±3.5%) and 6 minutes (49±5.7%) which also similar with previous study in Bull (Chen *et al*., 1993) that obtained a successful protocol from freezing bull semen commercially cooling semen by the rate 15°C/min from +5 to –100°C which representative in Freezing time III in this present study.

The freezing rate time can adversely affect the nucleus, plasma, acrosome and mitochondrial membranes of spermatozoa and is associated with production of reactive oxygen species which can generally lead to increase permeability of membranes, release of soluble inter-membrane mitochondrial regulator proteins and apoptotic factors that might activate apoptogenic metabolic pathways (Mohan *et al.,* 2011). In general, the faster the rate of cooling, the more severe the damage. There is further evidence which suggests that the rate of temperature drop also determines the subsequent active life of spermatozoa (Andrabi *et al*., 2008). The type of extender used and the speed of temperature drop are known to affect susceptibility of spermatozoa to cold shock and the success rate of freezing semen (Shahverdi *et al.,* 2014). Interaction of equilibration length and freezing time documented positive effect of prolonged equilibration combined with less demanding slower temperature decline of the 2-phase curve. Mentioned results have to be taken into account within the adjustment of AI doses processing. Although, the average of spermatozoa abnormality after thawing with different freezing temperature 0 to -140°C time not significantly in each group, this result was similar with previous study (Dolezalova et al., 2015; Nur *et al.,* 2011). The speed of temperature is drop is known to have an effect on the susceptibility of spermatozoa to cold shock and on the quality of semen after freezing. Generally, cold shock damage manifests as a decline in cell metabolism, altered membrane permeability, loss of intracellular components, irreversible loss of spermatozoon motility, and increase in the number of dead spermatozoa (Watson, 2000).

The additional data also showed the decreased of ram semen quality after freezing, the freezing process significantly (*p<0.01*) lowered semen quality in terms of motility and viability. Lower motility after being frozen could be affected by several factors such as freezing method, the swelling in the area acrosome was a results of cold shock spermatozoa after pre-freezing (Ustuner *et al.,* 2015), pre-freezing also reduced semen membrane integrity which showed the plasma and membrane of acrosome were more vulnerable and thus affected spermatoza motility and viability (Apu *et al.,* 2015). Also the spermatozoa of goat did not have a high adaptability with temperature changes which contributed in spermatozoa sensitivity (Dorado *et al*., 2007; Bearden *et al.,* 2004).

Conclusions

The recent research findings led to the conclusion that, of the three freezing rates time, freezing the straw from 5°C to -140°C in 12 minutes was superior, as higher semen quality was obtained.

Acknowledgments

Thanks and high appreciations are due to the Directorate of Research and Community Service, Directorate General of Research Strengthening and Development, Ministry of Research, Technology and Higher Education which has funded this research.

References

Andrabi, S. M. H., M. S. Ansari, N. Ullah, M. Anwar, A. Mehmood, and S. Akhter. 2008. Duck egg yolk in extender improves the freezability of buffalo bull spermatozoa. Anim. Reprod. Sci. 104: 427-433.

Apu, A. S., Yahia, M. A. M. Kandhoker, S. S. Husain, M. Fakruzzaman, and D. R. Notter. 2015. A com- parative study of fresh and frozen-thawed semen quality in relation to fertility of Black Bengal Goats. Irania J. Appl. Anim. Sci. 2: 157–161.

Bearden, H. J., J. W. Fuquay, and S. T. Willard. 2004. Applied Animal Reproduction, 6th edn. Pearson Prentice Hall, Upper Saddle River.

Camara, D. R., S. V. Silva, F. C. Almeida, J. F. Nunes, and M. M. P. Guerra. 2011. Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen. Theriogenology, 76: 432-350.

Chen, Y., R. H. Foote, C. Tobback, L. Zhang, and S. Hough. 1993. Survival of bull spermatozoa seeded and frozen at different rates in egg yolk tris and whole milk extenders. J. Dairy Sci. 76: 1028-1034.

Defoin, L. A., A. Granados, and I. Donnay. 2008. Analysing motility parameters on fresh bull semen could help to predict resistance to freezing: A preliminary study. Reprod. Dom Anim. 43: 606-611.

Dolezalova, M., L. Standnik, Z. Biniova, J. Duchacek, and J. Beran. 2015. The effect of the freezing curve type on bull spermatozoa motility after thawing. Acta Vet. Brno. 84: 383-391.

Dorado, J., I. Rodríguez, and M. Hidalgo. 2007. Cryopreservation of goat spermatozoa: Comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology. 68: 168–177.

Foote, R. H. and M. T. Kaporth. 2002. Large batch freezing of bull semen: effect of time of freezing and fructose on fertility. J. Dairy Sci. 85: 453-456.

Forero-Gonzalez, R. A., E. C. Celeghini, C. F. Raphael, A. F. Andrade, F. F. Bressan, and R. P. Arruda. 2012. Effects of bovine sperm cryopreservation using different freezing techniques and cryoprotective agents on plasma, acrosomal and mitochondrial membranes. Andrologia 44: 154-159.

Lemma, A. 2011. Effect of cryopreservation on sperma quality and fertility. In: M. Manafi (editor). Artificial Insemination in Farm Animals. pp: 191-216

Mostafapor, S. and F. F. Ardebili. 2014. Effects of diluting medium and holding time on sperm motility analysis by CASA in ram. Veterinary Research Forum 5: 101-105.

Mohan, J., R. P. Singh, K. V. H. Sastry, R. P. Moudgal, A. Biswas, and N. Shit. 2011. Influence of chicken native breeds on some physical and biochemical characteristics and short-term storage of semen. Br. Poult. Sci. 52: 395-400.

Nur, Z., B. Zik, B. Ustuner, S. Tutuncu, H. Sagirkaya, C. G. Ozguden, U. Gunay, and I. Dogan. 2011. Effect of freezing rate on acrosome and chromatin integrity in ram semen. Ankara Üniv Vet Fak Derg. 58: 267–272.

Rovegno, M., W. B. Feitosa, A. M. Rocha, C. M. Mendes, J. A. Visintin, and M. E. O. D. Assumpcao. 2013. Assessment of post-thawed ram sperm viability after incubation with seminal plasma. Cell Tissue Bank. 14: 333-339.

Shahverdi, A., A. Rastegarnia, and T. R. Topragaleh. 2014. Effect of extender and equilibration time on post thaw motility and chromatin structure of buffalo bull (bubalus bubalis) spermatozoa. Cell J. 16: 279-288.

Ustuner, B., N. Zekariya, A. Selim, B. T. Mehmet, S. Hakan, and K. S. Mustafa. 2015. Effect of freez- ing rate on goat sperm morphology and DNA integrity. Turk. J. Vet. Anim. Sci. 39: 110–114.

Watson, P. F. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60: 481-492.