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Various Saccharides Addition of Extender Ram Efficiency to Improve Cryopreservation in Semen Etawah Crossbreed Bucks with Seminal Plasma Replacement

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ABSTRACT

Phospholipase in buck seminal plasma will trigger a coagulation incident resulting in the demise of the spermatozoa during liquid semen processing using milk or egg yolk substrates diluent. A plasma replacement process is performed to avoid this possibility. The cryopreservation process leads to sperm cell damage due to the freezing process, therefore cryoprotectant agents such as saccharides are required as protective agents. This study aimed to investigate the effect of various types of saccharides on the quality of frozen semen of etawah crossbreed (PE) bucks during cryopreservation and thawing with plasma replacement using Priangan ram semen plasma. Semen was collected using an artificial vagina once a week. Fresh PE bucks semen centrifuged at 3,000 RPM for 30 min. The supernatant (seminal plasma) was removed and replaced in equal volume with Priangan ram seminal plasma. Semen was divided into four treatments: goat semen with sheep semen plasma in Tris diluent (control); control with 0.6% dextrose added (monosaccharides); control with 0.6% sucrose added (disaccharides); control with 0.6% raffinose added (trisaccharides). Semen quality including percentages of motile spermatozoa, live spermatozoa, and intact plasma membrane (IPM) were evaluated after diluted, equilibrated, and thawing, respectively. Results of this study showed that viability and IPM of monosaccharides, disaccharides, and trisaccharides were significantly ($P<0.05$) higher than control (54.40 and 51.40; 55.00 and 53.60; 55.60 and 52.20 vs 48.40 and 52.20, respectively). The motility of disaccharides was significantly ($P<0.05$) higher compared to control and other diluent (47.00 vs 41.00, respectively). In conclusion, different types of saccharides were effective in maintaining the quality of etawah crossbreed buck frozen semen. The addition of disaccharides seems more effective compared to monosaccharides and trisaccharides in tris extender on the quality of etawah crossbreed buck frozen semen.

Keywords: Dextrose, Etawah Crossbreed, Priangan ram, Raffinose, Sucrose

Introduction

Etawah crossbreed goats (PE) are the result of a cross between Etawah goats and local Indonesian goats is considered to be dual purpose (meat and milk). Farmer interest in raising PE goats for milk production without compromising their ability to produce offspring for meat has increased in several regions (Sutama, 2009). PE goats can produce milk that is high enough for tropical areas. Thus, these goats have great potential to be developed as one of the milk-producing livestock in Indonesia. While the PE goat's milk production was not as great as that of some other dairy goats, it had the advantage of being able to adapt to the challenging local environment, particularly the weather and feed conditions (Susilorini *et al*., 2017). These goats can also increase the potential of other local goats through crossbreeding with reproductive technology, such as artificial insemination (AI).

Until now, the success of the AI program using frozen semen for goats has not been as expected (Faigl *et al*., 2012). Many factors cause the failure of AI program pregnancy in goats. One of the factors causing the low pregnancy rate is the poor quality of the frozen semen used. In frozen semen processing, several treatments are not beneficial for maintaining the quality of goat spermatozoa (Dorado *et al.*, 2010).

In the semen freezing process, due to very low-temperature treatment (-196°C), ice crystals

will form and changes in electrolyte concentration will cause damage to spermatozoa cells (Jang *et al.*, 2017). All those factors could affect the semen quality, decrease sperm integrity, and further lead to Deoxyribonucleic acid (DNA) fragmentation then resulting in lowered sperm viabilities (Pini *et al.*, 2018). To reduce this effect, a cryoprotectant compound must be added to the diluent. A good type of cryoprotectant that is very commonly used in the cement freezing (cryopreservation) process is various kinds of sugars. In addition to maintaining osmotic balance and offering cryoprotection in the diluent, sugars are crucial for sperm respiration. Given that seminal plasma already includes sugars, the inclusion of sugars in the diluent is logical (Gangwar *et al*., 2016). Several types of sugar added to the diluent have succeeded in improving the quality of frozen semen, such as trehalose in Pampinta sheep (Aisen *et al*., 2002), lactose (Rizal *et al*., 2003), and maltose in Garut sheep (Herdis and Darmawan, 2012). Moreover, different types of sugars have different effects on bull and rooster semen (Mohammadzadeh *et al*., 2019; Stanishevskaya *et al*., 2021). However, the effect of various sugars on PE buck is still unknown clearly.

Another main problem in processing goat semen is the presence of enzymes contained in seminal plasma. This enzyme is synthesized by the bulbourethral glands (Cowper's glands) which, when interacting with egg yolk or milk, will cause clumping (coagulation) of semen (Leboeuf et al., 2000). In order to protect sperm from cold shock, lecithin from milk or egg yolk must be added to the dilution solution. Previous research has demonstrated the presence of egg yolk agglutinate and phospholipase in the seminal plasma of dairy goats. Lecithin in yolks can be hydrolyzed by phospholipase to create lysophosphatidic, which are poisonous and have negative effects on sperm. Yolk coagulase can cause the yolk to coagulate in the diluent and accelerate the release of fatty acids from yolk lipids, which lowers the diluent's pH and compromises the sperm's ability to survive (Zou *et al*., 2022). Semen washing to remove semen plasma is one method to overcome the above problem (Santiago-Moreno *et al*., 2017). However, semen plasma is needed by spermatozoa to support their vitality during the processing and storage (preservation) process because it contains various nutrients. Therefore, replacing semen plasma is one way that can be taken to continue to provide the supply of nutrients needed by spermatozoa. This is because there are several nutrients contained in seminal plasma whose functions cannot be completely replaced by the nutrients contained in the diluent. In order to overcome this, several studies have been carried out to replace semen plasma with semen plasma from other livestock (Salmani *et al*., 2014). Rizal *et al*. (2008) Replacing PE goat semen plasma with Priangan sheep semen plasma can maintain the quality of PE goat semen stored in the refrigerator at a temperature of 3–5°C for three days, and prevent semen coagulation. Other research shows that replacing goat semen plasma with bovine semen plasma does not cause problems during freezing (cryopreservation), in fact, this substitution is able to maintain the quality of frozen mud buffalo semen compared to without semen plasma replacement) (Zou *et al*., 2022).

Therefore, this study aimed to evaluate the administration of different types of saccharides (mono, di, polysaccharides) into the semen diluent in the process of freezing the semen of PE goats which semen plasma had been substituted with Priangan sheep semen plasma with the consideration that goats and sheep are more closely related.

Materials and Methods

The animal management protocol for this experiment was approved by the bioethics and animal welfare committee BRIN with an ethical clearance number. 082/KE.02/SK/10/2022.

Semen collection and plasma replacement. Semen is collected using an artificial vagina once a week from four adult PE goats aged approx. 4 years with body weight approx. 50kg. Semen collection was carried out five times as several repetitions. Sheep semen plasma was obtained from six adult Priangan sheep which were mixed. The sheep semen was centrifuged at 3000 RPM for 30 min and the supernatant was collected. The supernatant was centrifuged again at the same speed and time, and then the supernatant (semen plasma) was collected and stored in a freezer.

Fresh PE goat semen that has been stored is immediately evaluated to determine its quality. Semen that meets quality requirements (70% motile spermatozoa, mass movement ++ or +++, and abnormal spermatozoa <15%). The semen was centrifuged at 3,000 rpm for 30 min. The supernatant (semen plasma) and pellet (spermatozoa) are separated. The supernatant in the test tube was removed and replaced with Priangan sheep semen plasma as much as the supernatant (PE goat semen plasma) was removed. Before being added to the pellets (PE goat spermatozoa), the sheep semen plasma which was previously stored in the freezer is thawed again at room temperature, so that when mixed the temperature is the same as the pellet temperature.

Semen dilution and cryopreservation. The semen is then divided into four treatments. The semen in each test tube was diluted with Tris diluent until it reached a concentration of 50 million motile spermatozoa per 0.5 mL according to the treatment given. Treatment was: Control = contained goat semen with sheep semen plasma in Tris diluent; Monosaccharides = contained goat semen with sheep semen plasma in Tris diluent with 0.6% dextrose added; Disaccharides = contained goat semen with sheep semen plasma in Tris diluent with 0.6% sucrose added; Trisaccharides = contained goat semen with sheep semen plasma in Tris diluent with 0.6% raffinose added. Tris basic diluent consists of: 2.42 g Tris

(hydoxymethyl) amino methane, 1.28 g citric acid, and 2.16 g fructose which is dissolved in sterile aquabidestylate to reach a volume of 100 mL, then 1,000 IU/mL penicillin and 1,000 streptomycin are added. g/mL (Herdis and Darmawan, 2012). The composition of Tris diluent is 80% Tris base diluent plus 20% egg yolk.

The diluted semen is packaged in mini straws and then equilibrated in the refrigerator at 5°C for three hours. Semen freezing begins by placing the equilibrated straw 10 cm above the surface of liquid nitrogen in a liquid nitrogen container (approximately –130°C) for 15 min. Then the straw is put into liquid nitrogen (approx. –196°C) and stored in a container. After being stored for seven days, frozen semen samples from each treatment were thawed again to evaluate their quality. Thawing is done by placing the straw in water at 37°C (in a water bath) for 30 sec.

Semen quality measurement. Semen quality is evaluated at the post-storage stage (fresh semen), dilution stage, equilibration stage, and thawing stage. The quality of semen evaluated at the fresh semen stage is volume, color, viscosity (consistency), pH (degree of acidity), spermatozoa concentration, mass movement of spermatozoa, motile spermatozoa, live spermatozoa, abnormal spermatozoa, and intact plasma membrane (IPM). Meanwhile, the evaluation of semen during the freezing process is the percentage of motile spermatozoa, percentage of live spermatozoa, and percentage of IPM after the dilution, equilibration, and thawing stages, respectively.

Percentage of motile spermatozoa: the percentage of spermatozoa that move progressively (move forward). It is subjectively evaluated in eight different fields of view with a light microscope at 400x magnification (Rizal *et al*., 2003). The numbers given range between 0 and 100% on a 5% scale.

Percentage of live spermatozoa: The percentage of live spermatozoa was evaluated with 2% eosin staining (Toelihere, 1993). A white head marks live spermatozoa, while dead ones are marked by a red head. A minimum of 200 spermatozoa were evaluated with a light microscope at 400x magnification.

Spermatozoa IPM percentage: the percentage of spermatozoa that have intact plasma membranes. Evaluated using the osmotic resistance test (ORT) or hypoosmotic swelling (HOS) test (Revell and Mrode, 1994). The composition of the hypoosmotic solution consists of: 0.9 g fructose + 0.49 g sodium citrate dissolved with aquabidestylate to reach a volume of 100 mL. A total of 200 μL of hypoosmotic solution was added to 20 μL of semen and mixed until homogeneous then incubated at 37°C for 45 minutes. Make a thin smear on a glass slide th, then evaluate with a light microscope at 400x magnification, withminimum of 200 spermatozoa. Spermatozoa that have intact plasma membranes are characterized by coiled or bulging tails, while those that are damaged are characterized by straight tails.

Experimental design and statistical analysis. The study was conducted using randomized block design (RBD) and analyzed using analysis of variance. Duncan's test was used to compare means when the interaction was significant. The statistical models were evaluated using the general linear model (GLM) procedure of SPSS ver. 22. A significant effect was considered at $p \leq 0.05$.

Results and Discussion

Fresh semen quality

Data on the characteristics of fresh PE goat semen obtained (Table 1) shows that the semen is of good quality and meets the requirements for the freezing process. This is because fresh semen has a slightly thick to viscous consistency, spermatozoa mass movement is 2.83, motile spermatozoa are 70%, and abnormal spermatozoa are less than 15%. According to Holt (2000), the motility of normal spermatozoa for further development ranges from 70-90%.

Table 1. The characteristics of fresh semen of etawah crossbreed (PE) bucks

Semen characteristic	Value	Normal value (Souhoka *et al*., 2009)
Macroscopic		
Volume	1 ± 0.0	05-1.00
Colors	Cream	Cream
Consistency	Thick	Thick
pH	7.00 ± 0.0	7
Microscopic		
Mass motility	3.00 ± 0.00	2-3
Concentration (10^6 /ml)	4.310 ± 0.0	3,900 4,500
Motility (%)	70.00 ± 0.0	70
Viability (%)	86.00 ± 0.0	81-86
Abnormality (%)	8.00 ± 0.0	6-9
Intact plasma membrane (%)	88.00 ± 0.0	83-86

The research results showed that the average volume of fresh semen was 0.65 ml (Table 1). The average volume of fresh semen obtained in this study was lower than that reported and the average volume of PE goat semen is 1 ml (Hafizuddin *et al*., 2021). other report 1.6 – 2 ml on 1-6-year-old PE (Heriyanta *et al*., 2014) However, 0.68 ml of semen volume was also reported (Souhoka *et al*., 2009). This difference is thought to be due to differences in livestock and experimental conditions. The mass movement of spermatozoa obtained was 2.83 and the average pH was 7. Tambing *et al.* (2001) reported that the mass movement of PE goat spermatozoa was an average of 3 and an average pH of 7.13. in agreement with the report in PE bucks mass motility ranges from 2-3 with a pH of 7 (Souhoka *et al*., 2009). Motile spermatozoa obtained in this study was an average of 70% and live spermatozoa had an average of 86.00%. The results obtained were lower compared to Dorado *et al*. (2010) who reported that the motility of goat spermatozoa was 94.06%, but the results obtained were more or less

the same as those reported by Tambing et al. (2001) that the percentage of motile spermatozoa and live spermatozoa in PE goats averaged 72.79 and 82.54%, respectively. Souhoka *et al.* (2009) reported the same value in PE bucks motility was 70% and viability was 83%.

The research results showed that the average spermatozoa concentration was 4,124 million cells/ml. Dorado *et al*. (2010) reported a goat spermatozoa concentration of 3,690 million cells/ml. The results obtained in this study were higher than those reported by Tambing *et al.* (2001), namely an average of 2,940 million cells/ml. Another concentration report in PE buck's was 4,144 million cells/ml (Souhoka *et al*., 2009). Heriyanta *et al*. (2014) report PE buck from 1-6 y old had sperm concentrations ranging from 3,600 – 5,025 million cells/mL.

The abnormal spermatozoa obtained was an average of 8.0%. The results obtained were lower than (Dorado *et al*., 2010) who reported goat spermatozoa abnormalities of 13.3%. Liu and Baker (1992) stated that spermatozoa abnormalities should not be more than 10%. This is important because spermatozoa abnormalities are related to male fertility in general. According to Delgadillo (1992), the percentage of abnormal spermatozoa in normal goats is around 6 to 10%. The research results showed that the average MPU percentage was 84.40%.

Semen quality after diluting, equilibrating, and thawing

After dilution (Table 2), there was no significant effect (P>0.05) of different types of saccharides on motility, viability, and intact plasma membrane. Motility after dilution was 70%. PE bucks ' viability and intact plasma membrane after dilution ranged from 81.60-82.20 and 81.40-82.00 respectively.

After the equilibration stage (Table 2), treatment significantly (P<0.05) affects the intact plasma membrane (IPM). IPM was higher (P<0.05) in PE bucks semen diluted with sugar monosaccharides, disaccharides, and trisaccharides compared to control (73.60, 73.40, 72.80 vs 71.80). Different types of saccharides did not affect (P>0.05) the percentage of motility and viability after equilibration on PE bucks. The administration of saccharides could ensure the stability of protein-lipid structures in sperm cell membranes (Saleh *et al*., 2017) or maintenance of osmotic balance (Pieper *et al*., 2023). Motility decreases by about 2-4% after equilibration compared to the dilution stage (Figure 1). Viability decreases by about 5-7% after equilibration (Figure 1). The decrease in quality in this stage is reasonable due to the presence of intact oxygen in the diluent which causes oxidation reactions during the process (Pieper *et al*., 2023).

After thawing (Table 2), viability and IPM of monosaccharides, disaccharides, and trisaccharides were significantly (P<0.05) higher than control (54.40 and 51.40; 55.00 and 53.60; 55.60 and 52.20 vs 48.40 and 52.20, respectively). Percentage of motile spermatozoa after thawing of monosaccharides (44.00%) and trisaccharides (44.00%) was higher than control (41.00%), but no significant difference (P>0.05). Motility of disaccharides was significantly (P<0.05) higher compared to control (47.00 vs 41.00, respectively).

The results of the research showed that replacing PE goat semen plasma with Priangan sheep semen plasma and adding different sugars was able to maintain spermatozoa motility in the semen freezing process and still had a quality that met the requirements for use in the AI program because it had a percentage of motile spermatozoa of 40%. Based on the Indonesian National Standards (SNI), semen that meets the quality requirements used in the AI program must have a minimum percentage of motile spermatozoa of 40%. The research results obtained are in line with what was stated by Zou *et al*. (2022) removing Guanzhong dairy goat plasma semen with bovine plasma semen at different levels shows that 100% replacement of goat plasma semen significantly improves sperm motility, plasma membrane, and acrosome integrity after thawing—furthermore resulting in improvement, in the fertilization ability of sperm. Semen cryopreservation in goats is unique compared to another domestic animal in terms of secretion-specific lipase enzyme that interacts with lipids from egg yolk or skim milk triglycerides, resulting in toxicity to the sperm (Roof *et al*., 2012). Goat pancreatic lipase-related protein

Table 2. Average percentage motility, viability, and intact plasma membrane frozen seme of etawah crossbreed (PE) bucks spermatozoa at various types of sugar

Parameter	Treatment	Stage of observation		
		Dilution stage	Equilibration stage	Thawing stage
Motility (%)	Control	70.00 ± 0.00	66.00 ± 2.24	41.00 ± 2.24 ^{a}
	Monosaccharides	70.00 ± 0.00	67.00 ± 2.74	44.00 ± 2.15 ^{ab}
	Disaccharides	70.00 ± 0.00	67.00 ± 2.74	47.00 ± 5.70^{b}
	Trisaccharides	70.00 ± 0.00	68.00 ± 2.74	44.00 ± 2.24 ^{ab}
Viability (%)	Control	81.60 ± 0.55	74.00 ± 2.92	48.40 ± 2.30^{a}
	Monosaccharides	81.80 ± 1.30	75.00 ± 2.24	54.40 ± 4.04 ^{b}
	Disaccharides	81.80 ± 1.24	74.00 ± 2.12	55.00 ± 2.55^{b}
	Trisaccharides	82.20 ± 0.84	75.00 ± 1.87	55.60 ± 2.07 ^{b}
Intact plasma membrane % (IPM)	Control	81.80 ± 0.84	71.80 ± 0.84^{a}	44.80 ± 0.84^{a}
	Monosaccharides	82.00 ± 0.71	73.60 ± 1.14^{ab}	51.40 ± 3.05 ^{b}
	Disaccharides	82.00 ± 0.71	73.40 ± 1.14^{ab}	53.60 ± 2.19 ^{b}
	Trisaccharides	81.40 ± 1.14	72.80 ± 1.00^{ab}	52.20 ± 1.79^{b}

Control = contained goat semen with sheep semen plasma in Tris diluent; Monosaccharides = control with 0.6% dextrose added; Disaccharides = control with 0.6% sucrose added; Trisaccharides = control with 0.6% raffinose added.

^{a,b,c} Different characters in the same column indicate significant differences at the 5% test level (DMRT multiple range test).

2 (PLRP2), a glycoprotein released by bulbourethral glands, is the enzyme responsible for this interaction that lead to egg yolk coagulating enzyme by catalyzation and hydrolysis of egg yolk phospholipids into toxic lysolecithin and free fatty acid (Chauhan and Anand 1990; Sias *et al.*, 2005) The PLRP2 enzyme, also known as BUSgp60, was found to cause spermatozoa motility to decrease, acrosome degradation, and epididymal spermatozoa mortality when skim milk was utilized as an extender for cryopreservation (Sias *et al*., 2005).

The result on improvement in semen dilution with different types of saccharides compared to control showed that monosaccharides, disaccharides, and trisaccharides result better to maintaining semen quality. Sugar can protect the plasma membrane of spermatozoa cells because on the outside of the cell plasma membrane, there are carbohydrates that are bound to lipids (glycolipids) or proteins (glycoproteins) which are called the cell sheath or glycocalyx (Cheon and Kim, 2015). It is assumed that the sugar added in the diluent will associate with the carbohydrate so that it is protected from mechanical damage during the cryopreservation process (Gangwar *et al*., 2016) even if the carbohydrates in the spermatozoa cell plasma membrane are damaged during the cryopreservation process (Talaei *et al*., 2010). sugar added to the diluent can be a replacement so that the cell envelope structure remains intact. Sugar also has an important role in reducing the salt content of the diluent solution, thereby reducing the solution effect (Fernández-Santos *et al*., 2007). This causes sugar to prevent damage to cells due to increased salt levels during the freezing process.

According to Aisen *et al*. (2002) the cryoprotective effect of sugar results from the formation of hydrogen bonds between the hydroxyl groups of sugar and the polar heads of phospholipids in the plasma membrane of spermatozoa cells, so that sugar replaces the position of water molecules during the dehydration process during freezing. Thus, sugar can regulate the fluidity of the plasma membrane of spermatozoa cells Ahmad and Aksoy (2012). According to Giraud *et al*. (2000), motility and viability of spermatozoa after thawing can be increased if the fluidity of the cell plasma membrane is high before freezing.

The positive effect of the sugar addition treatment which appears in the post-thawing stage is thought to be because, during freezing and thawing, there is heavy pressure on the spermatozoa cells due to the drastic decrease in temperature during freezing, and the also drastic increase in temperature during thawing (Kankofer *et al*., 2005). In circumstances like this, sugar will provide optimal protection for the integrity of the cell plasma membrane and spermatozoa cells. At the dilution and equilibration stage, spermatozoa are not under heavy pressure such as during freezing and thawing. This is an indication that in the semen cryopreservation process, the sugar added to the cement diluent acts more as an extracellular cryoprotectant than as an energy source substrate (Gangwar *et al*., 2016). Sugars from the disaccharide and polysaccharide groups can be used as energy source substrates by spermatozoa, only if there are enzymes in the semen plasma or diluent that break them down into several monosaccharide units (Misro and Ramya, 2012). The result also showed that all parameters di- and tri-saccharides were found more effective in preventing decreased sperm quality than monosaccharides even though the data is not significant, this could occur due to the higher

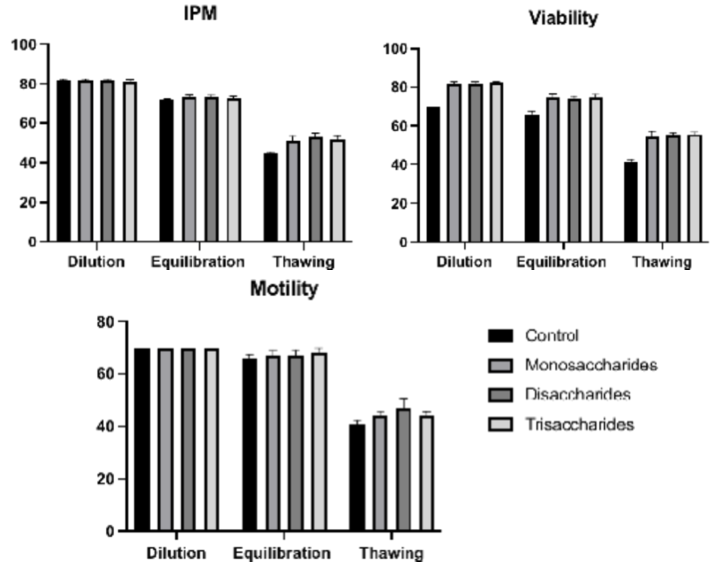

Figure 1. comparation of viability, motility and plasm integrity of different type of sacharides in every phase. Control = contained goat semen with sheep semen plasma in Tris diluent; Monosaccharides = control with 0.6% dextrose added; Disaccharides = control with 0.6% sucrose added; Trisaccharides = control with with 0.6% raffinose added.

osmotic dehydration in di-and tri-saccharides (Garde *et al*., 2008).

The result also showed that utilizing disaccharides could maintain motility better than other saccharides this finding is also similar to previous research which stated lactose/disaccharide is the best saccharide type to preserve frozen semen (Lapwood and Martin, 1966), our result is in agreement with findings in boar that comparing monosaccharides and disaccharides on sperm quality of boar sperm cryopreserved with an egg yolk based extender (Gómez-Fernández *et al*., 2012) this occurs due to disaccharides more being able to cross the cell membrane than the trisaccharides also less easily converted into energy than monosaccharides thereby better able to maintain the membrane integrity of cells than other saccharides (Kamal *et al*., 2023). Disaccharides could prevent the formation of ice crystals by forming hydrogen bonds between molecules in the lipid bilayer of cryopreserved semen (Uchida *et al*., 2007). This occurs because disaccharides restrict the availability of free water molecules from the solution to semen, causing dehydration in the cell and decreasing intracellular ice formation (Sussich *et al*., 2001). Disaccharides containing glucose (lactose, maltose, and trehalose) may have a larger cryoprotective impact than sugars that do not (lactulose) (Golshahi *et al*., 2018).

The results obtained in this research support the results of research on various types of animals and livestock. Yulnawati *et al*. (2009) reported that dextrose can maintain the quality of frozen semen from the epididymis of striped buffalo. Aisen *et al.* (2002) reported that the percentage of motile spermatozoa in the frozen semen of Pampinta sheep was 64% for semen diluted with Tris diluent with trehalose added and 52.10% for those with EDTA added. The same thing was also reported regarding the addition of trehalose sugar to frozen goat semen (Aboagla and Terada, 2004). The addition of sugar in the form of 60 mM lactose (Rizal *et al*., 2003).

Conclusion

Different types of saccharides were effective in maintaining the quality of etawah crossbreed buck frozen semen. The addition of disaccharides seems more effective compared to monosaccharides and trisaccharides in tris extender on the quality of etawah crossbreed buck frozen semen.

Conflict of interest

The authors have no conflict of interest to declare. All authors have seen and agree with the contents of the manuscript.

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Author's contribution

The authors confirm their contribution to the paper as follows: study conception and design. H., and M. R.,; data collection: A.H., D. A. M., F. B. I. L., R. I. A., S.; analysis and interpretation of results: A.N., H., M.R., and P.I.S.; draft manuscript preparation: A.N., H., and P.I.S. All authors reviewed the results and approved the final version of the manuscript.

Ethics approval

There are no human subjects in this article and informed consent is not applicable. This study was approved by the Administration Committee of Experimental Animals, BRIN

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