Effectiveness of Various Glycerol Concentrations as a Cryoprotectant in Frozen Semen of Pasundan Cattle

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ABSTRAK

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Penelitian dilakukan untuk mengetahui efektivitas gliserol sebagai bahan krioprotektan dengan berbagai konsentrasi terhadap kualitas semen Sapi Pasundan. Semen dikoleksi dari tujuh ekor pejantan Sapi Pasundan menggunakan vagina buatan setiap dua kali dalam satu minggu selama tiga bulan. Sampel semen selanjutnya ditambahkan bahan pengencer yakni TRIS-*Egg Yolk Extender* yang mengandung 20 % (v/v) kuning telur dan diberi perlakuan penambahan gliserol dengan lima konsentrasi berbeda (G5= 5%, G6= 6%, G7= 7%, G8= 8%, dan G9= 9%) kemudian dilakukan kriopreservasi. Rancangan Acak Lengkap (RAL) digunakan dalam penelitian ini untuk menguji pengaruh lima konsentrasi gliserol yang berbeda terhadap motilitas, Membran Plasma Utuh (MPU), Keutuhan Tudung akrosom (TAU), abnormalitas, dan laju pemulihan spermatozoa setelah proses kriopreservasi (post-thawing). Hasil evaluasi semen sapi Pasundan setelah proses pengenceran menunjukkan bahwa penambahan gliserol sebanyak 7% (G7) menghasilkan motilitas dan nilai TAU terbaik (83,68% dan 72,84%), penambahan gliserol sebanyak 7% dan 8% (G7 dan G8) menghasilkan nilai MPU terbaik (85,00% dan 84,50%), serta penambahan gliserol sebanyak 6%, 7%, 8%, dan 9% (G6, G7, G8, dan G9) menghasilkan nilai abnormalitas terendah (1%). Pada semen sapi Pasundan yang telah melalui kriopreservasi (*post-thawing*), penambahan gliserol sebanyak 7% (G7) menghasilkan nilai motilitas, TAU, MPU, dan laju pemulihan terbaik (54,49%, 38,57%, 54,29%, dan 72,28%). Sementara itu, penambahan berbagai konsentrasi gliserol tidak menunjukkan efek yang signifikan terhadap nilai abnormalitas spermatozoa setelah proses kriopreservasi. Secara umum, penambahan gliserol sebanyak 7% dalam pengencer semen menunjukkan hasil yang optimal sebagai bahan krioprotektan.

Kata Kunci: Krioprotektan, Gliserol, Sapi Pasundan, Kualitas Semen

ABSTRACT

Rasad SD, Solihati N, Winangun K. 2022 Effectiveness of various glycerol concentrations as a cryoprotectant in frozen semen of Pasundan cattle. JITV 27(3): 114-121. DOI:http://dx.di.org/10.14334/jitv.v27i3.3066.

This study was conducted to determine the effectiveness of Glycerol as a cryoprotectant with various concentrations on the quality of Pasundan cattle semen. Semen was collected from seven bulls of Pasundan Cow using an artificial vagina twice a week for three months. The semen sample was added with a TRIS-Egg Yolk Extender containing 20% (v/v) egg yolk and treated with the addition of Glycerol with five different concentrations (G5= 5%, G6= 6%, G7= 7%, G8= 8%, and G9= 9%) were then performed with cryopreservation. A Completely Randomized Design (CRD) was used to examine the effect of five different concentrations of Glycerol on motility, intact plasma membrane (IPM), the integrity of acrosome cap (IAC), abnormalities, and recovery rate (RR) of spermatozoa after cryopreservation (post-thawing). The results of diluted Pasundan cattle semen evaluation showed that the addition of 7% Glycerol (G7) resulted in the best motility and IAC values (83.68% and 72.84%), the addition of 7% and 8% Glycerol (G7 and G8) resulted in the best IPM values (85.00% and 84.50%). The addition of 6%, 7%, 8%, and 9% Glycerol (G6, G7, G8, and G9) resulted in the lowest abnormality values (1%). On the post-thawing Pasundan cattle semen evaluation, the addition of 7% Glycerol (G7) resulted in the best motility, IAC, IPM, and RR values (54.49%, 38.57%, 54.29%, and 72.28%). Meanwhile, adding various Glycerol concentrations did not significantly affect the abnormality value of post-thawing spermatozoa. Generally, the addition of 7% Glycerol in semen extenders shows optimal results as a cryoprotectant.

Key Words: Cryoprotectant, Glycerol, Pasundan Cattle, Semen Quality

INTRODUCTION

One of the local breeds of cattle in Indonesia is Pasundan cattle, which originated from the West Java region and spread in the buffer zone and the southern coast of the area (Aisah et al., 2017). As a local breed, Pasundan cattle have several superior traits, such as disease resistance, high feed quality, and climate change adaptability. Cattle is one of the livestock currently being developed to meet the national beef

needs (Arifin et al. 2014). Based on those characteristics, therefore, some efforts are required to increase the population of Pasundan cattle through artificial insemination (AI) programs.

Artificial insemination (AI) is one of the reproductive technologies and the most important technique that has been applied to improve the genetics of animals. The technology is widely used because several selected males could produce enough sperm to inseminate thousands of females recipient per year (Ax et al. 2000; Aurich et al. 2020). The success of an AI program depends on the quality of frozen semen. As we know, the semen cryopreservation process aims to maintain the quality of semen during the freezing and storage process. However, the freezing process of semen causes a decrease in the viability of spermatozoa cells due to cold shock and ice crystal formation. Undertaking a dilution process and adding the best cryoprotectant to the semen diluent can overcome the problems (Najafi et al. 2013; Mahendra et al. 2018). As it is required to optimize the semen quality of superior bulls, a dilution process is needed. The purpose of the semen dilution process is to increase the volume of semen and to maintain the survival of the sperm. One of the extenders commonly used for diluting cattle semen is the TRIS-Egg yolk. This choice is because the TRIS-Egg yolk has a good buffer capacity with low toxicity (Feradis 2010). Adding cryoprotectant substances to the TRIS-egg yolk extender can prevent a decrease in the quality of frozen semen (El-Sheshtawy & El-Nattat 2018; Hermansson et al. 2021).

Glycerol is the most common cryoprotectant agent used in semen cryopreservation. Moreover, applying Glycerol could prevent cell dehydration of spermatozoa cells and a buildup of H_2O molecules in the cells and minimize ice crystal formation during the freezing process (Gamal et al. 2016; Ma et al. 2022). Some research on using Glycerol as a cryoprotectant in cattle semen has been carried out (Gamal et al. 2016), and using 7% Glycerol was conducted to compare the viability and fertility of bovine semen diluted in Botu-Bov (BB) commercial extender with and without Glycerol as a cryoprotectant then cooled at 5^{o}C. Other research added 3, 5, and 11% Glycerol in buffalo semen (Fabbrocini et al. 2000), and their results showed that optimizing the timing of the Glycerol addition and the presence of energy source in the extender rendered a higher efficiency in the thawed spermatozoa of Mediterranean buffalo.

Other research indicated that by using different levels of Glycerol in sheep semen, as much as 5% can optimally maintain the semen quality (Rehman et al. 2013; Yánez-Ortiz et al. 2021), while Baharun et al. (2017); and Setiono et al. (2015) explained that the use of Glycerol in cow semen in a range of 5-7% could maintain the semen quality during the cryopreservation process. Based on the above description, this research was conducted to test the effectiveness of Glycerol as a cryoprotectant with various concentrations on the quality of Pasundan cattle semen.

MATERIALS AND METHODS

This study was carried out from October 2018 to September 2019 in the Artificial Insemination Center of Beef cattle, Cijeungjing-West Java, Indonesia. Fresh semen used in this research was collected from 7 Pasundan bulls (2-5 years old) that have characteristics of good semen quality. Parameters observed in this study were motility, intact plasma membrane (IPM), the integrity of acrosome cap (IAC), and abnormalities. These parameters were tested before and after cryopreservation. Recovery rate of spermatozoa was tested after cryopreservation (post-thawing).

Semen collection and initial evaluation

Semen was collected from each bull twice a week using an artificial vagina. Immediately after collection, the semen was evaluated using macroscopic and microscopic observations. Macroscopic evaluation is carried out by observing volume, color, smell, consistency, and pH. Microscopic qualities of fresh semen measured were sperm motility. Each ejaculate having less than 70% sperm motility was discarded.

Semen cryopreservation

Freshly collected semen was diluted in TRIS-Egg Yolk Extender. For every 100 mL, the extender contains 1.725 g Tris (hydroxymethyl) aminomethane crystal, 2.79 g Fructose crystal, 1.555 g Lactose, 0.95 g citrate monohydrate acid, 88 ml distilled water, 20% v/v egg yolk (Salamon & Maxwell 2017), and then treated with the addition of Glycerol with five different concentrations (G5= 5%, G6= 6%, G7= 7%, G8= 8%, and G9= 9%). Microscopic qualities of diluted semen measured were sperm motility, intact plasma membrane (IPM), intake of acrosome cap (IAC), and sperm abnormality. After dilution, the semen was packaged in a 0.25 ml straw with a motile sperm concentration of $25x10^6$, and the straw was equilibrated at 4^{o}C for 4 hours. After equilibration, straws were vaporized with liquid nitrogen at -80^{o}C for 9 minutes inside the Styrofoam box. Then, straws were plunged into a liquid nitrogen tank at -196^{o}C. Post-thawed semen evaluation is done by removing the straw from the liquid nitrogen tank and then stored at room temperature until the

semen melts completely. Then, the assessment is carried out by testing the same parameters as the diluted semen testing.

Evaluation of sperm motility

Sperm motility was calculated by calculating the total sperm concentration and the concentration of dead sperm. Semen concentration was measured by mixing 0.05 ml semen and 1 ml 3% Sodium Chloride and then calculated by using Hemocytometer (Neubauer chamber). Evaluation of semen concentration was done by counting the spermatozoa from five large squares. Semen concentration is the number of sperm cells that were calculated from five large squares cell $x10^7$ (Ax et al. 2000). Motile sperm are counted under a microscope with 400x magnification. The rate of motility was determined by percentage (Fabbrocini et al. 2000). The following formula calculated sperm motility:

$$Sperm\ Motility\ (Y) = \frac{\sum total\ sperm - \sum dead\ sperm}{Total\ Sperm} x\ 100$$

Evaluation of intact plasma membrane (IPM)

The intact plasma membrane (IPM) assessment was conducted using a Hypo Osmotic Swelling Test (HOST) solution prepared from 30 grams of NaCl dissolved in 100 ml of distilled water. Semen and HOS solution was homogenized with a ratio of 1:3 and then incubated at 37°C for 30 min. Furthermore, aliquots were smeared on the glass slide. IPM was evaluated using a 400x magnification microscope that observed at least 200 sperm cells. A circular-tail shape marked the intact membrane, while a straight-tail shape marked the damaged cell membrane. The calculation of the percentage of PMI used the following formula by Mitchell & Doak (2004):

$$\%IPM = \frac{\sum Intact\ plasma\ membrane\ of\ sperm}{200\ sperm\ cell} s\ x\ 100$$

Evaluation of intake of acrosome cap (IAC)

Evaluation of IAC sperm was conducted using 1% formalin fixation. The fresh or post-thawed semen was mixed with 1% formalin fixation with a ratio of 1:3. The observation was carried out on 200 sperm cells by using a microscope with 1000x magnification. The intake of the acrosome cap was characterized by a black line on the anterior portion of the head of sperm. The calculation of the percentage of IAC used the following formula (Mitchell & Doak 2004):

$$\%\ IAC = \frac{\Sigma Sperms\ with\ intact\ acrosome\ caps}{200\ sperm\ cells}\ x\ 100\%$$

Evaluation of sperm abnormalities

Spermatozoa abnormalities were evaluated using eosin-nigrosine dye. One drop of fresh semen was put on the end of the object glass using an ossicle, then one drop of 2% eosin-nigrosine solution was added near the fresh semen. Both were mixed and covered with an object glass. Fixation of the smear preparation used Bunsen. Observe using a microscope with a magnification of 400x (Arifiantini 2012). Spermatozoa that absorb color were declared dead. The number of sperm observed was at least 200 spermatozoa by the formula (Susilawati 2017):

$$Sperm\ Abnormalities = \frac{abnormal\ sperm\ count}{sperm\ count\ observed} x100\%$$

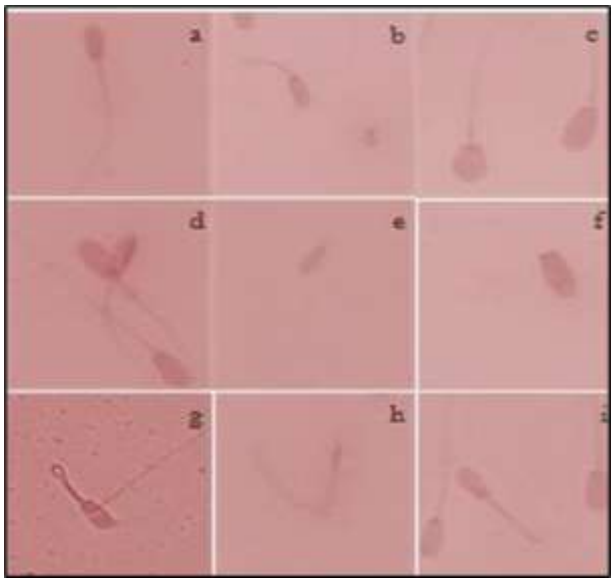

Figure 1. Normal and Abnormal Spermatozoa Morphology 1000x Magnification with Eosin-Nigrosine Solution. a) Normal Spermatozoa, b) Pear-shaped, c) Macrocephalus, d) Microcephalus, e) Detached Head, f) Head only, g) Circular Tail, h) Tail, and i) Stump Tail

Evaluation of recovery rate (RR)

Assessment of recovery rate was performed by comparing the data of post-thawing motility with fresh semen motility. The calculation of the percentage of recovery rate used the following formula (Arifiantini et al. 2005; Mahendra et al. 2018):

$$\%\ RR = \frac{\%post\ thawing\ motility}{\%fresh\ semen\ motility}\ x\ 100\%$$

Statistical analysis

This experimental study was analyzed using a completely randomized design (CRD). The study was conducted using five different Glycerol content

treatments (G5, G6, G7, G8, and G9) on extender cement and their effect on post-liquid semen quality. Each treatment is repeated six times. The data were analyzed using ANOVA followed by Duncan's multi-range test.

RESULTS AND DISCUSSION

Evaluation of semen before cryopreservation

Fresh semen was initially evaluated macroscopic and microscopically for its feasibility before treatment. The results of initial semen evaluations can be seen in Table 1. After the first assessment, semen that met the criteria was diluted with an extender and treated with various Glycerol levels. Based on table 1, the quality of fresh semen from Pasundan cattle macroscopically indicates normal conditions. The diluted semen was tested for microscopic quality. The results of diluted semen evaluations can be seen in Table 2.

Table 2 shows that the sperm motility of Pasundan cattle ranges between 83.68% for the highest value (G7) and 75.40% for the lowest value (G9). The results were higher than the other local cattle sperm motility. According to Aisah et al. (2017), Bali cattle semen had average motility of 64.65%. According to Romadhoni et al. (2014), Madura cattle had average motility of 70%. However, according to the results of Baharun et al. (2017), the average cow semen has a motility of 89.37%. Differences in the quality of semen can be caused by factors such as feed quality, weather conditions, livestock health, genetics, and livestock management programs (Ahirwar et al. 2018). However, based on the evaluation of the motility of semen of post-mortal cattle in this study, it was shown that fresh semen was feasible for the cryopreservation process.

Other results of the Fresh semen evaluation of this study showed that the fresh semen of Pasundan cattle had Intact Plasma Membrane (IPM) ranging between 85.00% and 84.50% for the highest value (G7 and G8) and 63.30% for the lowest value (G5). This result is not much different from other local species of cattle living in the tropics. Hapsari et al. (2018) stated that the fresh semen of Bali cattle at four years and seven years of age had an average percentage of IPM of 60.85% and 54.84%, respectively. The results of the other studies stated that the average IPM in the fresh semen of Madura cattle was around 78.83% (Romadhoni et al. 2014). Moreover, the results of this research showed that the Intake of Acrosome Cap (IAC) of fresh semen of Pasundan cattle ranges between 72.84% for the highest value (G7) and 68.29% for the lowest value (G5). The quality of IAC in this study is not much different from Bali cattle, which was 68.25% (Anwar et al. 2015). The quality of IAC in local cattle (*Bos sondaicus*) is still shallow compared to those in *Bos indicus* and *Bos taurus*. According to the results of Nofa et al. (2017), it was stated that fresh semen of Brahman and Limousine cattle had IAC of 90.85% and 90.40%, respectively.

Abnormality in this research's fresh semen of Pasundan cattle was very low, i.e., at 1-1.5% (Table 2). Research results were better than other local cattle sperm motility. Romadhoni et al. (2014) reported that fresh semen from Madura cattle had an abnormality of 4.5%. Research reported by Prastowo et al. (2018) showed a high percentage of abnormalities in the semen of Bali cattle that, reached 3.89%. Overall, the G7 treatment showed the best results on the motility and IAC of Pasundan cattle cement after dilution. The G7 and G8 indicate the best value in IPM while the abnormality value in each treatment was the same except for G5, which had a higher abnormality value.

Evaluation of Pasundan cattle post-thawing sperm motility

The quality of Pasundan Cattle post-thawing semen at a different Glycerol level could be seen in Table 3. Sperm motility of G7 had the highest sperm motility by 54.49% compared with other Glycerol levels.

Table 1. Initial Evaluation of Pasundan Cattle Fresh Semen

Bull	Volume (mL)	pH	Smell	Consistency	Color	Motility (%)
1	6.0	6.5	Specific	Viscous	White-Cream	84.34
2	5.5	6.5	Specific	Moderate	White-Cream	82.47
3	5.3	6.8	Specific	Viscous	White-Cream	81.78
4	4.5	6.5	Specific	Viscous	White-Cream	83.07
5	4.2	6.5	Specific	Viscous	White-Cream	81.25
6	4.5	6.5	Specific	Viscous	White-Cream	83.22
7	4.2	6.8	Specific	Moderate	White-Cream	81.69

Table 2. Microscopic quality of Pasundan cattle diluted semen

Level of Glycerol	Motility (%)	IPM (%)	IAC (%)	Abnormalities (%)
G5 (5%)	81.18[b]	63.30[a]	68.29[a]	1.5[b]
G6 (6%)	82.19[c]	70.00[b]	69.73[c]	1[a]
G7 (7%)	83.68[d]	85.00[d]	72.84[e]	1[a]
G8 (8%)	82.72[c]	84.50[d]	71.29[d]	1[a]
G9 (9%)	75.40[a]	72.00[c]	69.27[b]	1[a]

IPM= intact plasma membrane, IAC= intake of acrosome cap. G5= 5%, G6= 6%, G7= 7%, G8= 8%, G9= 9%

Table 3. Post-thawing Pasundan cattle microscopic semen quality

Level of Glycerol	Microscopic Quality			
	Motility (%)	IPM (%)	IAC (%)	Abnormality (%)
G5 (5%)	44.89[a]	36.86[ab]	46.86[a]	1.36[a]
G6 (6%)	53.41[d]	36.71[ab]	47.14[a]	1.14[a]
G7 (7%)	54.49[e]	38.57[b]	54.29[b]	1.07[a]
G8 (8%)	48.64[c]	35.86[ab]	51.86[ab]	1.64[a]
G9 (9%)	45.80[b]	33.29[a]	47.00[a]	1.07[a]

Differences in the superscription (a, b) in the same row show a significant difference (P<0.05). G5= 5%, G6= 6%, G7= 7%, G8= 8%, G9= 9%

According to Baharun et al. (2017), the frozen semen of Pasundan cattle added with 6% Glycerol produced lower post-thawed motility of 49.45%. Another study of Bali cattle frozen semen added with 8% Glycerol stimulated lower average post-thawed sperm motility of 51.88% (Nalley et al. 2016). Use of Glycerol more than 7% caused a decrease in post-thawed motility (Kulaksiz et al. 2013; Villaverde et al. 2013).

In each treatment, motility was lower than that of fresh semen (before treatment). A decrease in sperm motility occurs gradually, starting from dilution to thawing. Baharun et al. (2017) stated that a reduction in the quality of sperm of frozen semen could be caused by plasma membrane damage of sperm during freezing processes affecting sperm motility. This membrane is rolled to facilitate substances and ion exchanges needed for sperm metabolism to produce energy for sperm movement (Storey 2008).

Evaluation of integrity plasma membrane (IPM) of Pasundan cattle post-thawing sperm

The integrity of the plasma membrane is one of the semen quality determinants. Its fluid properties and flexibility are needed to help sperm flagella movement. Table 3 shows that the integrity plasma membrane (IPM) of Pasundan cattle decreased dramatically after freezing and thawing. The decrease in the plasma membrane and drastic temperature changes affect sperm membrane structures and characteristics (Rehman et al. 2013).

Table 3 shows that the differences in Glycerol levels in the dilution of semen of Pasundan cattle did not significantly affect the sperm IPM on G5, G6, and G8. However, G7 resulted in a higher percentage of IPM (38.57%); the lowest was G9 (33.29%). Hapsari et al. (2018) stated that the average IPM of post-thawed semen of Bali cattle (4 and 7 years old) was frozen with 7% Glycerol equal to 44.6% and 33.8%, respectively. The plasma membranes of sperm have unsaturated fatty acids, which are very susceptible to cryopreservation damage.

Research results showed a decrease in IPM of sperm of Pasundan cattle frozen semen between 36.86-42.14% after thawing. Villaverde et al. (2013) stated that 40 to 50% of fresh semen that has been frozen would be damaged in their plasma membrane. Using Glycerol in semen dilution could help to protect against plasma membrane damage due to osmotic pressure changes and mechanical damage because of ice crystals formation in the plasma membrane during freezing (Mahendra et al. 2018).

Evaluation of intake of acrosome cap (IAC) of Pasundan cattle post-thawing sperm

The acrosome of sperm is a part that plays a vital role in the fertilization process as a carrier of enzymes and genetic materials. This part of the acrosome equator is an important part of the spermatozoa, this is because the anterior part of this post acrosome initiates the merger with the oocyte membrane in the fertilization process (Susilawati 2017). The quality of IAC of Pasundan cattle sperm in this study decreased after the freezing and thawing. Table 3 shows that IAC dropped from the highest of 54.29% (G7) to the lowest of 47.00% (G9). Zekariya et al. (2011) stated that freezing and thawing processes negatively affected the integrity of sperm acrosomes because this process could change the structure of chromatin in the sperm DNA. The Duncan test showed that Pasundan cattle frozen semen with 7% Glycerol produced a higher percentage of IAC (54.29%) compared to other treatments (Table 3). Shah et al. (2016) stated that sperm acrosomes integrity in semen diluted with 7% Glycerol was higher than those diluted with DMSO without Glycerol.

Different results were shown by Villaverde et al. (2013), who indicated that the use of Glycerol could not improve the integrity of sperm acrosomes of cats, but

3% Glycerol gave a higher percentage of IAC compared with 5% and 7% Glycerol. Sperm acrosomes damage during the freezing process is due to changes in the acrosome membrane connected with the capacitation process of sperm, so the presence of Glycerol does not affect the integrity of sperm acrosomes.

Evaluation of Pasundan cattle post-thawing sperm abnormality

Evaluation of sperm abnormalities is classified into primary and secondary abnormalities. Primary abnormalities in sperm are more influenced by genetic factors, while secondary abnormalities are influenced by environmental factors (Toelihere 1985). Table 3 shows freezing and thawing did not increase abnormalities of Pasundan cattle sperms. The dilution process with various levels of Glycerol also did not affect the level of sperm abnormalities. The increase in sperm abnormality could be caused by its morphological development and handling processes. Observations showed that the average abnormalities of sperms of Pasundan cattle at each treatment were not significantly different (1.07%-1.64%). Post-thawed semen of Pasundan cattle is suitable for artificial insemination because the standard level of abnormality is below 20% (BSN 2008; Manjunath 2012).

Evaluation of recovery rate (RR) of Pasundan cattle post-thawing sperm

The recovery rate is one of the indicators of the successfulness of the freezing process of semen, which describes the rate of recovery of sperms after freezing (Pileckas et al. 2013; Bhat et al. 2020). The results showed that the addition of Glycerol at levels of 7% before the freezing process of Pasundan cattle semen could produce a high recovery rate of 72.28% (G7), respectively, compared to the other treatments. The result found in this research is higher than those reported by the other studies. Baharun et al. (2017) stated that frozen semen of Pasasundan cattle added with 6% Glycerol produced a recovery rate of 59.62%. Hapsari et al. (2018) report that the recovery rate in Bali cattle semen (4 and 7 years old) frizzed with 7% Glycerol showed the percentage of RR of 65% and 61.3%, respectively. Moreover, Aisah et al. (2017) and Yendraliza et al. (2019) stated that the average recovery rate of Bali cattle semen was 56% to 60%, while the remained 40% was thought to be damaged to cell death caused by temperature stress. Cell damage due to freezing could occur due to dehydration, increased electrolyte concentration, and the formation of intracellular ice crystals that can affect cell wall permeability. In the end, the spermatozoa lose their motility (Zelpina et al. 2012).

Table 4. Post-thawing Pasundan cattle sperm recovery rate

Level of Glycerol	Fresh Semen Motility (%)	Thawed Semen Motility (%)	Recovery Rate (%)
G_5 (5%)	81.18^b	44.89^a	55.30^a
G_6 (6%)	82.19^c	53.41^d	64.99^c
G_7 (7%)	83.68^d	54.49^e	72.28^d
G_8 (8%)	82.72^c	48.64^c	58.12^b
G_9 (9%)	75.40^a	45.80^b	55.36^a

G5= 5%, G6= 6%, G7= 7%, G8= 8%, G9= 9%

CONCLUSION

Diluted Pasundan cattle semen evaluation showed that adding 7% Glycerol (G7) resulted in the best motility. IAC values (83.68% and 72.84%), the addition of 7% and 8% Glycerol (G7 and G8) resulted in the best IPM values (85.00% and 84.50%), and the addition of 6%, 7%, 8%, and 9% Glycerol (G6, G7, G8, and G9) resulted in the lowest abnormality values (1%). On the post-thawing Pasundan cattle semen evaluation, the addition of 7% Glycerol (G7) resulted in the best motility, IAC, IPM, and RR values (54.49%, 38.57%, 54.29%, and 72.28%). Meanwhile, adding various Glycerol concentrations did not significantly affect the abnormality value of post-thawing spermatozoa. Generally, the addition of 7% Glycerol in semen extenders shows optimal results as a cryoprotectant that can protect and maintain the quality of spermatozoa during the cryopreservation process and ultimately was expected to increase the success of artificial insemination.

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