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Spermatozoa Quality of Pasundan Cattle Frozen Semen at Various Thawing Temperatures and Durations

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ABSTRACT

Pasundan cattle are West Java's indigenous Genetic Resources, and the proper frozen semen thawing technique is necessary for successful artificial insemination (AI) to increase the cattle population. This study aimed to evaluate the quality of spermatozoa of Pasundan cattle frozen semen post-thawing at various temperatures and thawing duration. A total of 24 frozen semen samples were thawed with two treatment factors: temperature (27 and 37°C) and thawing duration (15, 20, 25, and 30 seconds). The observed variables included motility, viability, and plasma membrane integrity. Data were analyzed using the General Linear Model (GLM) for these variables, followed by the Tukey test for multiple comparisons. The results showed no interaction between temperature and thawing time on the observed variables. There was no significant difference ($p>0.05$) in sperm motility between treatments, with values between 45.30±0.78 and 52.57±3.59%. Furthermore, the sperm viability rate was significantly different ($p<0.05$) due to temperature and thawing time. Sperm viability was higher in the 37°C thawing group for 15 seconds. In contrast to the integrity of the sperm plasma membrane, the 27°C temperature treatment group had a higher rate than the 37°C treatment group. It can be concluded that thawing frozen semen of Pasundan cattle at 27 or 37°C with a duration of 15-30 seconds produces normal spermatozoa quality, and a temperature of 37°C for 30 seconds is recommended for the thawing process. These findings contribute to optimizing thawing protocols in artificial insemination practices, enhancing the reproductive success of Pasundan cattle.

Keywords: Frozen semen, Pasundan cattle, Thawing temperature and duration

INTRODUCTION

Pasundan cattle are West Java's indigenous Genetic Resources, which must be protected. Pasundan cattle have been designated as an Indonesian local cattle breed based on the Decree of the Minister of Agriculture of the Republic of Indonesia with Number 1051/Kpts/SR.120/2014. The designation is a form of government effort to protect Pasundan cattle. However, until 2021, the Pasundan cattle population in West Java Province has declined since 2017, from 31,000 to 25,000 heads (Indra Kurniawan, 2021). Several factors are causing the population decline, including the fact that farmers prefer imported cattle, which are considered superior in terms of productivity and meat quality (Fitriani et al., 2020). In addition, government policies that are less supportive of local livestock development are also a contributing factor. Many farmers feel they lack support in terms of access to superior seeds and modern husbandry technology (Elsadina, 2021; Purwoko, 2016).

One of the efforts that can be made to increase the population of Pasundan cattle is by artificial insemination (AI). Artificial insemination can be done using fresh or frozen semen. The use of frozen semen often causes pregnancy failure. This is due to the mishandling of frozen semen, such as thawing, which can result in low motility and viability Salim et al. (2012). One of the primary issues is temperature management during thawing. Thawing frozen semen at inappropriate temperatures can lead to significant damage to sperm cells. Thawing at too low temperatures that are too low can result in incomplete thawing, while excessive temperatures can cause thermal shock, leading to cell death and reduced motility (Borah et al., 2015; Khalil et al., 2018).

Yekti et al. (2023) reported that thawing at 37°C for 30 seconds can increase motility and viability. Furthermore, Pesch dan Hoffmann (2007) suggested that for commercial AI in cattle, thawing should be done in water at 37°C for 20 seconds because it is more practical, and frozen semen should not be thawed below 15°C.

Rajbari et al. (2022) and Li et al. (2024) reported that prolonged thawing of frozen semen at extreme temperatures (below 30˚C or above 40˚C) could result in changes in pH and stop protein function, which ultimately leads to spermatozoa death. Meanwhile, Salim et al. (2012) Stated that the best thawing technique is to use water at a temperature of 37°C for 15 seconds. In the field, inseminators prefer thawing using tap water, which has a water temperature range of 27˚C because it is considered more accessible, easier and more practical (Zamrodah, 2016). The quality of frozen semen produced after the thawing process varies and is influenced by the temperature and length of thawing time.

Despite the various studies mentioned, there may still be uncertainties about the optimal thawing conditions specifically for Pasundan cattle. Based on these conditions, it is necessary to research to evaluate the effect of temperature and thawing duration on the quality of frozen semen spermatozoa of Pasundan cattle as a scientific information study, as well as to obtain data on the most appropriate thawing temperature and duration. This study aims to obtain information and practical guidance for inseminators in the frozen semen thawing process to increase the success rate of artificial insemination (AI).

MATERIALS AND METHODS

Frozen semen, buffers, and location of study

A total of 24 Pasundan cattle frozen semen straws with 0.25 ml packaging were stored in a container filled with liquid N_2 (nitrogen) at -196°C for approximately one year. Pasundan cattle frozen semen straw was obtained from the Center for Breeding and Artificial Insemination Development (UPTD- BPPIB) of Ciamis Beef Cattle produced in May 2023 with bull code and batch 251507-T0530. It is known that the bull was 24 months old at the time of semen collection, and the quality of fresh semen before freezing has a motility rate of 70.4% with a concentration of 966 $x10^6$ $mm^{3\ -1}$ and several other characteristics, as shown in Table 1.

Table 1. Evaluation of pre-cryopreservation of Pasundan cattle semen

Bull and batch code	Volume	Color	pH	consistency	Motion Mass	Motility	Concentration ($mm^{3\ -1}$)
251507-T0530	7.5 ml	White milk	6.4	medium	++	70.4%	966.10^6

Source: UPTD-BPPIB beef cattle, Ciamis

All chemicals and places used to observe spermatozoa quality are laboratory facilities at UPTD BPPIB Beef Cattle Ciamis. The composition of Pasundan cattle liquid semen tris buffer before freezing consists of several chemicals, as shown in Table 2. The buffer was then added with 22% egg yolk of the total volume of diluent, 0.75 g penicillin antibiotics, 0.75 g streptomycin, and 6.5 ml glycerol.

Table 2. Egg yolk tris buffer composition

Additives	Composition
Citric acid (g)	0.965
Fruktosa (g)	2.795
Laktosa (g)	1.555
Aquabidest (ml)	88

Source: UPTD-BPPIB beef cattle, Ciamis

Research Method and Design

The research was conducted experimentally using two treatment factors: two levels of thawing temperature (27 and 37°C) and four levels of thawing duration (15, 20, 25, and 30 seconds). The selection of thawing temperature and duration was carried out with consideration to obtain practical guidance for inseminators in the field. Eight treatment combinations were obtained based on these two treatment factors, each repeated three times. The spermatozoa quality was examined in a randomised complete block design (RCBD) with a 2 x 4 factorial arrangement. The eight treatment combinations are:
Thawing process at 27°C for 15 seconds (T1D1),
Thawing process at 27°C for 20 seconds (T1D2),
Thawing process at 27°C for 25 seconds (T1D3),
Thawing process at 27°C for 30 seconds (T1D4),

Thawing process at 37°C for 15 seconds (T2D1), Thawing process at 37°C for 20 seconds (T2D2), Thawing process at 37°C for 25 seconds (T2D3), and Thawing process at 37°C for 30 seconds (T2D4).

Several variables were observed, including motility, viability, and plasma membrane integrity of spermatozoa.

Variable Measurement

Frozen semen samples of Pasundan cattle were thawed at 27 and 37°C with durations of 15, 20, 25, and 30 seconds. Furthermore, spermatozoa's motility, viability, and plasma membrane integrity were evaluated using the Darussalam method (Darussalam, 2019).

Spermatozoa motility was evaluated using Computer Assisted Sperm Analysis (CASA; Androvision Minitube Germany). A total of 10 µl of frozen semen that had been thawed was dripped on an object glass and then covered using a cover glass. The sample was observed in the Microscope-CASA for five fields of view with 200 x magnification. The variable motility value of CASA is progressive motility (PM) expressed in per cent (%).

The viability of spermatozoa was tested using eosin-nigrosin staining. Semen was mixed with eosin-nigrosin dye (1:3). The mixture of semen and eosin-nigrosin was made into review preparations and dried on a heating table at 37°C for 10 seconds. The dried preparations were observed under a microscope with a magnification of 40 × 10. Spermatozoa were observed in 10 fields of view or with a minimum number of spermatozoa of 200 cells. Live spermatozoa are not coloured (transparent); dead ones will be red on the head. The percentage of live spermatozoa is calculated by dividing the number of live spermatozoa by the total spermatozoa counted multiplied by 100%, with the formula:

$$\text{Spermatozoa viability} = \frac{\Sigma \text{ life spermatozoa}}{\Sigma \text{ total spermatozoa}} \times 100\%$$

Plasma membrane integrity was tested using the hypo-osmotic swelling (HOS) test. Semen as much as 10 µl was mixed in 1 ml of HOS solution and then homogenized. One drop of the HOS solution mixture with the semen was placed on a glass slide, covered with a covered glass, and evaluated under a 40 × 10 magnification microscope. A circular or bulging tail characterizes Spermatozoa with an intact plasma membrane, while those that are damaged are characterized by a straight tail. The percentage of spermatozoa with plasma membrane integrity was calculated by dividing the number of spermatozoa that reacted with HOS by the total spermatozoa multiplied by 100%, with the formula:

$$\text{Plasma membrane integrity} = \frac{\Sigma \text{ intact spermatozoa}}{\Sigma \text{ total spermatozoa}} \times 100\ \%$$

Statistical Analysis

The data obtained were analysed using the General Linear Model (GLM), and if there is an interaction between the two treatment factors, the test will be further tested with the Tukey test. The research data were processed using SPSS for Windows. Furthermore, the data are presented as mean ± standard error means (SEM).

RESULTS AND DISCUSSION

This study focused on the role of thawing temperature and duration in producing the best quality spermatozoa before artificial insemination (AI). Two levels of temperature (27 and 37°C) and four levels of thawing duration (15, 20, 25, and 30 seconds) were combined as treatments to obtain the best sperm quality. More details on sperm quality (motility, viability, and plasma membrane integrity) post-thawing with these treatments are presented in Table 3.

Spermatozoa motility at various temperatures and duration of thawing

Post-thawing spermatozoa motility was not significantly affected ($p>0.05$) by both treatment factors, either temperature or thawing duration. The results of this study also showed no interaction ($p>0.05$) between temperature and thawing duration on spermatozoa motility rate (Table 3). It means that using a thawing temperature of 27 or 37°C and a thawing time of 15-30 seconds did not significantly affect spermatozoa motility. It showed that even though the temperature and thawing time varied, spermatozoa motility remained relatively the same and did not experience significant changes.

These findings are consistent with those of Utomo and Baquifai (2010), who also reported that thawing temperatures between 26 and 37°C did not significantly affect spermatozoa motility rates. It is likely due to the thawing process that has begun since the frozen semen was removed from the depot container. The release of mini straw from this container will automatically

increase the temperature (natural thawing) from freezing to room temperature. Meanwhile, 15-30 seconds of thawing does not significantly affect motility. The short size of the thawing time compared to the metabolic reactions of spermatozoa means that the thawing time limit of frozen semen before insemination should not be more than 5 minutes (Pugliesi et al., 2013). This statement shows that there is still a long time interval compared to the thawing duration in this study.

The motility rate of spermatozoa obtained in this study was in the range of 45.30-52.57%. This value is by the Indonesian National Standard, which is a minimum post-thawing sperm motility of 40% (SNI 4869-1: 2021). Data analysis also showed that at least one-third of the thawed spermatozoa could still move well. As a reference for the success of egg fertilization, motility variables have a higher role than sperm viability. As stated by Hong et al. (1985), good sperm motility is very important for the success of the artificial insemination process. Sperm with high motility are better able to reach the egg and increase the likelihood of fertilization.

These results suggest that to achieve optimum post-thawing sperm motility, thawing temperature, and duration can be selected within a flexible range as they do not significantly affect the results. However, it is still important to consider other factors and a more comprehensive thawing protocol in artificial insemination practice.

Table 3. Motility, viability, and plasma membrane integrity of post-thawing spermatozoa at different levels of thawing temperature and durations

Variable	Combination of thawing temperature and duration treatment					Probability (p-value)		
	Temp.	Duration						
		15”	20”	25”	30”	Temperature	Duration	Interaction
Motility	27°C	48.70±1.70	46.07±3.20	51.90±0.26	45.30±0.78	0.11	0.71	0.34
	37°C	50.90±3.50	50.40±2.81	50.07±3.18	52.57±3.59			
Viability	27°C	60.85±3.47^{ab}	57.63±3.61^{a}	55.03±0.51^{a}	64.68±2.78^{ab}	0.00	0.01	0.22
	37°C	71.37±0.70^{b}	61.09±2.14^{ab}	62.70±2.53^{ab}	65.37±1.29^{ab}			
Plasma Membrane integrity	27°C	60.52±5.20	57.30±0.34	65.72±1.03	66.50±1.93	0.03	0.34	0.05
	37°C	62.87±2.50	57.51±1.24	57.37±2.52	55.01±2.75			

Notes: Different superscripts on the same column significantly differed ($p<0.05$).

Spermatozoa viability at various temperatures and duration of thawing

Sperm viability refers to the ability of sperm cells to survive and maintain their reproductive function after collection, storage, and transfer into the female reproductive tract (Samplaski et al., 2015; Santiani et al., 2023). This parameter is often measured by various methods, including testing motility (movement), morphology (shape), and the ability of sperm to cope with osmotic and thermal stress (Momeni et al., 2020; Rowe & Pruett-Jones, 2011). High viability indicates that the spermatozoa are healthy and able to fertilise the egg, while low viability indicates a potential failure in the fertilization process. (Park et al., 2021)

This study found that both thawing temperature and thawing time significantly impact the viability of frozen semen from Pasundan cattle ($p<0.05$). In other words, the temperature and the time used for thawing are important for keeping sperm cells alive and healthy. This highlights the need to carefully control both factors to preserve the quality of the frozen semen. However, the study also found no interaction between the two factors ($p>0.05$). This means that the effect of thawing temperature on sperm viability doesn't depend on the length of thawing, and the impact of thawing time doesn't depend on the temperature. Each factor works independently to affect sperm viability.

According to Sukmawati et al. (Sukmawati et al., 2014), spermatozoa viability is influenced by differences in seminal plasma characteristics between individuals and between semen from the same animal collection and extreme changes in environmental conditions during freezing. Very low temperatures during freezing result in the leakage of vital substances in sperm so that intracellular enzymes, lipoproteins, adenosine tri Phosphate (ATP), and

intracellular potassium are reduced, which in turn can cause damage to the plasma membrane so that viability decreases. The temperature range susceptible to sperm damage during freezing is the cooling period (0°C to -5°C) and ice crystal freezing (-6°C to -15°C) and thawing between temperatures of -60 to -15°C.

The viability of Pasundan cattle spermatozoa after thawing in this study ranged from 55.03 - 71.37 % (Table 3). This figure shows that the viability of spermatozoa after dilution has met the standard. It is following the opinion of Zampini et al. that the minimum post-thawing spermatozoa viability rate is 40%.

Spermatozoa plasma membrane integrity at various temperatures and duration of thawing

The integrity of the spermatozoa plasma membrane is a cellular structure that encloses and protects spermatozoa, serving as a selective barrier that regulates the entry and exit of substances into and out of the cell. This membrane is a lipid bilayer rich in phospholipids, proteins, and cholesterol, providing cell stability and fluidity (Prastiwi et al., 2021). The integrity of the spermatozoa plasma membrane is an essential indicator of sperm cell health and viability. When the plasma membrane is intact, spermatozoa can perform their physiological functions properly, including motility and fusing with the ovum (Fannessia et al., 2015).

There is a close relationship between the three variables observed, namely motility, viability, and plasma membrane integrity, in determining the quality of spermatozoa after thawing frozen semen. Spermatozoa with an intact plasma membrane show a higher level of viability. Viability is important because healthy spermatozoa can survive longer in the female reproductive tract, thus increasing the likelihood of fertilization (Nurcholis et al., 2016). Furthermore, plasma membrane integrity contributes to spermatozoa motility, an important factor in reaching the ovum. Motile spermatozoa can move efficiently through the female reproductive fluid to find and fertilize the ovum. Decreased motility due to plasma membrane damage can reduce the chances of successful insemination. The condition of the plasma membrane affects the ability of spermatozoa to fuse with the ovum membrane. Damage to this membrane can disrupt the fertilization process, thus reducing the success rate of insemination (Puwantara et al., 2022)

The results of this study showed that the integrity of the plasma membrane of post-thawing spermatozoa at various temperature levels and thawing duration ranged from 55.01 ± 2.75 - 65.72 ± 1.03. The factor that plays a significant role ($p<0.05$) in the plasma membrane integrity (PMI) of spermatozoa is thawing temperature (Table 3). The total MPI value between the frozen semen group diluted at 27°C was higher ($p<0.05$) than the frozen semen group diluted at 37°C, namely 62.51 ± 5.75% vs. 58.19 ± 4.57%. This situation may be caused by the jump in thawing temperature to 37°C being too high, resulting in a higher level of spermatozoa stress compared to the thawing temperature of 27°C.

The results of research by Sukmawati et al. (Sukmawati et al., 2014) show that plasma membrane integrity is influenced by temperature during the cooling or thawing process, which is a trigger for sperm stress because it will change the configuration of plasma membrane phospholipids and disrupts membrane function and permeability. Furthermore, Gonzalez-Castro et al. (Gonzalez-Castro et al., 2024) explained that the freezing process causes the plasma membrane to be damaged due to the formation of lipid peroxidation, which results in changes in membrane structure and function. The next process, when the frozen semen is thawed, can cause changes in protein activity and permeability to water and solutes. The varying percentage of plasma membrane integrity occurs due to the biophysical and biochemical characteristics of the sperm membrane. The composition of the semen plasma can also affect plasma membrane stability.

CONCLUSION

The quality of spermatozoa after thawing at 27°C and 37°C with a thawing time ranging from 15 to 30 seconds met acceptable standards, and a temperature of 37°C for 30 seconds is recommended for thawing frozen semen of Pasundan cattle. The factors of temperature treatment and thawing time showed no interaction with the quality of spermatozoa and no significant effect on spermatozoa motility rate. However, temperature and thawing time factors influenced the viability rate individually. Meanwhile, the thawing temperature factor only influenced the plasma membrane integrity rate. These findings suggest that temperature and time during thawing should be carefully controlled to

optimize sperm viability and plasma membrane integrity in artificial insemination practices.

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