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The Quality of Frozen Friesian Holstein Semen after Long-Term Storage

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ABSTRACT

Semen cryopreservation is the long-term storage at very low temperatures in liquid nitrogen for future use. This study investigates the quality of frozen Friesian Holstein (FH) semen after long-term storage. Samples of FH semen stored for 25, 20, 15, 10, 5, and 1 years were collected from one of the national centers for artificial inseminations. Frozen semen was stored in containers with liquid nitrogen at -196 °C in a room with a temperature of 20 °C The variables used after thawing were sperm motility, viability, and abnormalities, as well as plasma membrane integrity (IPM) using computer-assisted sperm analysis (CASA), eosin-nigrosine staining, and hypoosmotic swelling (HOS) test, respectively. Data were analyzed by one-way ANOVA and expressed as mean ± SEM. The result showed no significant differences in sperm viability, abnormalities, and IPM. Furthermore, sperm motility was >40%, consistent with the Indonesian standard for frozen bovine semen. CASA analysis showed that all variables of the motility pattern have no significant difference, except linearity (LIN). The Lin of sperm was lower in frozen semen after one and five years than after 20 and 25 years of storage. The overall quality of semen after 25, 20, 15, 10, 5, and 1 years of storage met the standard and was suitable for artificial insemination.

Keywords: FH bull; frozen semen; semen quality; storage periods

INTRODUCTION

Cryopreservation is the cooling and storing of cells, tissues, or organs at freezing temperatures for future use (Hinting & Agustinus, 2020). Furthermore, semen cryopreservation is a process used to preserve sperm cells. This technique offers great potential for preserving animal cells, conserving germplasm threatened with extinction, and supporting the artificial insemination (AI) program for wild and farm animals (Santiago-Moreno & Blesbois, 2022). According to Haugan *et al.* (2007), the technique allows long-term storage of sperm in liquid nitrogen (-196 °C). It also reduces the metabolic activity of cells.

The Artificial Insemination Centers (AIC) in Lembang, West Java, and Singosari AIC, East Java, established in 1976 and 1982, are important producers of frozen semen for Indonesian livestock. AIC has a straw for frozen semen production over 20 years old. In a previous study, Leibo *et al.* (1994) found that frozen bovine semen had normal sperm motility after 37 years of storage. According to Malik *et al.* (2015), the viability and motility of thawed semen were lower after 6 years of storage in liquid nitrogen than after one to two years. Furthermore, no statistical deterioration in sperm quality was observed in humans after 5 years of storage (Rofeim & Gilbert, 2005). Ramírez-Reveco *et al.* (2016) found that long-term storage at -196 °C has no significant effect on the basic sperm quality parameters of bovine semen. According to Malik *et al.* (2015), future use of frozen semen is possible, but the cryopreservation process affects sperm quality and fertility (Malik *et al.*, 2015). Nagata *et al.* (2019) reported that frozen semen of good quality and suitable for AI is stored in liquid nitrogen containers. Their lifetime in the form of frozen semen must be maintained until being used. Therefore, the quality must be maintained by properly handling the frozen semen in its containers. Improper handling leads to quality deterioration (Nagata *et al.*, 2019).

The study on sperm quality during long storage was conducted in Indonesia by Malik *et al.* (2015), which investigated and evaluated frozen semen from Friesian Holstein (FH) bulls after 6 years of storage. The frozen semen was stored longer and sperm motility was evaluated using the Computer Assisted Sperm Analysis (CASA) system. CASA offers the potential for a more accurate fertility prediction than the traditional microscopic technique of semen evaluation (Broekhuijse *et al.*, 2012; Murphy *et al.*, 2018; Tanga *et al.*, 2021). The national standard of Indonesia (SNI 4869.1-2017) for frozen semen quality evaluation has three requirements, namely sperm motility, movement score, and concentra-

tion in a straw. According to Halvaei *et al.* (2016), sperm must be alive, progressively motile, and have normal morphology with intact chromatin to fertilize an egg. The basis of this study is the importance of demonstrating the frozen semen quality that has been stored for a long time. Furthermore, this study aims to investigate the quality of frozen FH semen after a storage period of 1 to 25 years.

MATERIALS AND METHODS

This study was performed in the Reproductive Rehabilitation Unit (URR) laboratory, Division of Reproduction and Obstetrics, Department of Clinics, Reproduction, and Pathology, Faculty of Veterinary Medicine, IPB University and Ciamis Regional AIC, West Java, Indonesia. Furthermore, frozen semen of FH bull was taken based on the storage time of 25, 20, 15, 10, 5, and 1 years from Singosari AIC. Since its establishment, Singosari AIC has consistently used Tris-egg yolk extender and the same freezing process. Data on the quality of the fresh semen is no longer available. However, the production of frozen semen refers to the Regulation of the Director General of Livestock, Department of Agriculture No. 12207/Hk.060/F/12/2007 on the technical guidelines for the production and distribution of frozen semen. In this case, all frozen semen was derived from fresh semen with sperm motility >70%.

In this study, eight straws were used per year of storage. Five straws were used each to test sperm viability, abnormalities, and integrity of the plasma membrane (IPM). Three straws were used to test sperm motility and movement with CASA (AndroVision, Minitube Germany). Furthermore, frozen semen was thawed one by one at 37 °C for 30 seconds and stored in a microtube at 37 °C during the examination (Santoso *et al.*, 2021).

The Evaluation of Sperm Motility

A total of 4 μL of thawed semen was placed on the slide and covered with an 18x18 mm coverslip. Assessment of sperm motility with CASA is based on analysis of digitized images from a computer connected to a microscope with 200x magnification. The test was repeated for all five fields of view. After CASA analysis, the following descriptors of sperm were determined: velocity curvilinear (VCL), velocity straight-line (VSL), velocity average pathway (VAP), linearity (LIN)=VSL/VCL×100%, straightness (STR)=VSL/VAP × 100%, wobble (WOB)=VAP/VCL) ×100%, lateral displacement amplitude (LDA), and beat cross frequency (BCF) (Oliveira *et al.*, 2013).

The Evaluation of Sperm Viability

Sperm viability was tested using the eosin-nigrosine dye according to Arifiantini (2012). Eosin-nigrosine was prepared by mixing 20 g nigrosine, and 1.5 g sodium citrate in 300 mL distilled water and dissolving with a stirrer. Furthermore, 3.3 g of yellow eosin was added to the nigrosine solution, and the pH was adjusted to 6.8-7. The solution was then left at room temperature and filtered to obtain the staining medium. Afterwards, 10 μL of semen was placed on the sliding glass, and 20 μL of eosin-nigrosine dye was added (Pardede *et al.*, 2020). The mixture of semen and eosin-nigrosine dye was spread on the slide and dried on a warm plate for 10 seconds.

Sperms were counted under Olympus CX 23 microscope at 400x magnification from at least ten fields of view, with a minimum count of 200 cells. Live sperms do not absorb color (transparent), while dead sperms absorb red color on the head. The percentage of viable sperm count was calculated by dividing the number of live sperms by the total number of sperms counted, comprising live and dead sperms, and multiplying by 100%.

The Evaluation of Sperm Morphology

Sperm morphology is examined to determine their normality and abnormalities. The method used is similar to the viability examination but with a 1000x magnification (Olympus CX 23). Morphological examination of spermatozoa considers the overall shapes from head to tail. Damage or anomalies at the sperm's head, acrosome, mid-piece, and tail tip were considered indicators of abnormality. Furthermore, the percentage of abnormal sperms was determined by dividing the total number of sperms and multiplied by 100%.

The Evaluation of the Plasma Membrane Integrity

The integrity of the plasma membrane of sperm was assessed using the hypoosmotic swelling (HOS) test. The HOS solution was prepared from a mixture of 0.9 g of fructose and 0.49 g of sodium citrate in 100 mL of distilled water, with osmotic pressure ranging from 100 to 150 mOsm/kg, according to Nalley *et al.* (2019). A total of 50 μL of thawed semen was mixed with 1 mL of HOS solution, homogenized, and incubated at 37 °C for 30-45 min. The assay was performed by dropping a mixture of incubated HOS solution and semen onto a covered slide. The evaluation was performed at 400x magnification under Olympus CX 23 microscope. Sperms with intact plasma membranes were identified with a circular or curved tail, while the damaged sperm had a straight tail. Furthermore, the percentage of sperms with intact membranes is obtained by dividing the number of intact sperms by the total number of sperms counted multiplied by 100%.

Data Analysis

The one-way statistical analysis (ANOVA) was used to test the differences between each variable of the six treatments of storage times. The data obtained are presented as means ± SEM, and Duncan's test was used to determine the significant differences.

RESULTS

Sperm Motility of Frozen FH Semen at Different Storage Times

The total motility of frozen sperms with different storage times showed values ranging from 47% to 78%, as shown in Figure 1a. Sperms stored for 5 years had the highest value ($p<0.05$) of progressive motility (72.83 ± 3.3%), followed by sperms stored for 10 and 15 years, with 63% and 66%, respectively. Semen stored for 25 years had the lowest motility with values of only 40.7 ± 9.2%, as shown in Figure 1b. Furthermore, there was no significant difference in progressive motility between semen stored for 25 and 20 years and between semen stored for 20 and 1 years. The sperm motility of frozen semen at one year of storage is 56.03%.

Kinematic Sperm Properties of Frozen FH Semen with Different Storage Times

The VCL value ranges from 100-150 μm/s with no significant difference between storage times of frozen semen. In addition, VSL and VAP showed no difference, with values ranging from 39 to 65 μm/s and 53 to 75 μm/s, respectively. The LIN shows a value range of 31 to 55%, but it is lower than the optimal standard <35%, with values of 31.69 ± 4.6% and 34.24 ± 5.5%, respectively, for frozen semen with storage times of 5 and 1 years. The STR values ranged from 67 to 92%, and the LDA in this study showed no difference with a value of 1 to 2 μm. The semen stored for one year had the lowest BCF with a value of only 10.34 ± 1.4%, and the highest BCF was found in the semen stored for 20 years. Furthermore, there was no significant difference between the BCF values at 5, 10, 15, and 25 years of storage, as shown in Table 1.

Sperm Viability of Frozen Semen from FH Bulls at Different Storage Times

The lowest sperm viability ($p<0.05$) was found for frozen semen with a storage time of 25 years, with a value of 45.92 ± 8.1%. Sperm viability at storage times of 1, 5, 10, 15, and 20 years did not differ, with a value ranging from 65% to 78%, as shown in Figure 2.

Sperm Abnormalities of Frozen Semen of Bulls with Different Storage Times

Frozen semen with a storage time of 10 years had the highest sperm abnormalities with a value of 10.61 ± 2.3%, as shown in Figure 3. There were no significant differences between other storage times.

The integrity of Sperm Plasma Membrane in Frozen FH Semen at Different Storage Times

This study found that the storage time of frozen semen does not affect the IPM value, as shown in Figure 4. At different storage times, the sperm IPM of frozen semen from FH bulls showed values from 85% to 95%.

DISCUSSION

Total motility is based on velocity classification and includes all sperms moving at a velocity greater than >5 μm/s. Furthermore, progressive motility includes all

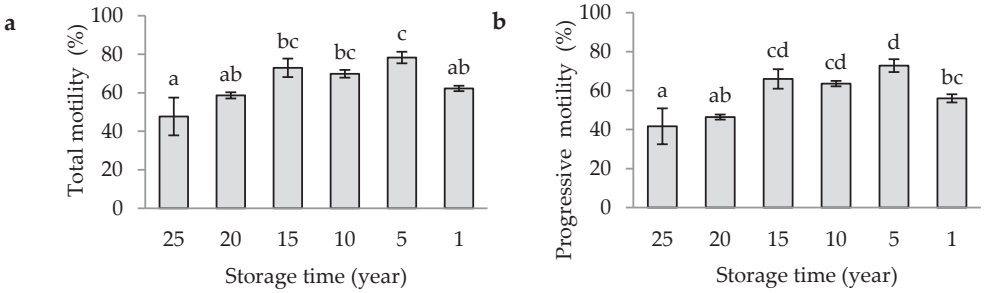

Figure 1. Total motility (a) and progressive sperm motility (b) of frozen FH semen with different storage times. Different letters on the bar show significant differences ($p<0.05$).

Table 1. Characteristics of sperm movement of frozen-thawed FH semen with different storage times

Variables	Storage periods (year)					
	25	20	15	10	5	1
VCL (μm/s)	142.29±17.6	119.76±7.7	120.25±10.5	108.28±18.3	140.44±20.0	127.27±12.5
VSL (μm/s)	64.07±7.3	63.85±8.0	61.89±9.0	39.95±6.4	49.43±9.5	39.96±6.1
VAP (μm/s)	74.73±8.3	71.54±6.1	70.05±6.4	53.27±9.0	63.64±9.5	57.77±6.0
LIN (%)	$46.37±2.7^{ab}$	$53.69±6.6^{b}$	$54.91±10.4^{a}$	$39.04±4.2^{ab}$	$34.24±5.5^{a}$	$31.69±4.6^{a}$
STR (%)	$86.27±3.5^{ab}$	$86.14±5.7^{ab}$	$92.43±21.0^{b}$	$77.91±6.2^{ab}$	$71.70±7.9^{ab}$	$67.15±6.3^{a}$
WOB (%)	$53.59±2.1^{ab}$	$60.76±4.9^{ab}$	$63.77±9.1^{b}$	$50.32±3.7^{ab}$	$45.59±4.2^{a}$	$45.98±3.5^{a}$
LDA (μm)	1.65±0.2	1.22±0.2	1.44±0.2	1.52±0.3	1.95±0.3	1.80± 0.2
BCF (Hz)	$18.15±1.4^{c}$	$23.12±2.6^{d}$	$16.73±1.2^{c}$	$11.59±1.8^{ab}$	$16.18±1.3^{bc}$	$10.34±1.4^{a}$

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). VCL= curvilinear velocity; VSL= straight-line velocity; VAP= average pathway velocity; LIN= linearity; STR= straightness; WOB= wobble; LDA= lateral displacement amplitude; BCF= beat cross frequency.

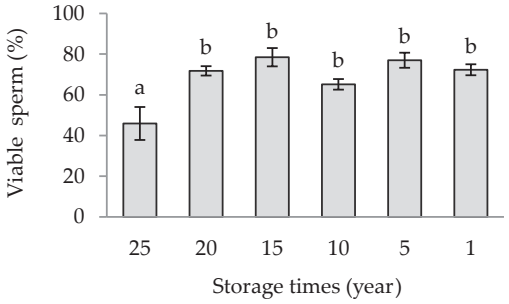

Figure 2. Sperm viabilities of frozen FH semen at different storage times. Different letters on the bar show significant differences in sperm viabilities (p<0.05).

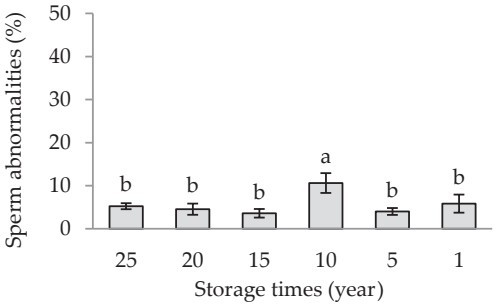

Figure 3. Sperm abnormalities of frozen FH semen at different storage times. Different letters on the bar show significant differences sperm abnormalities (p<0.05).

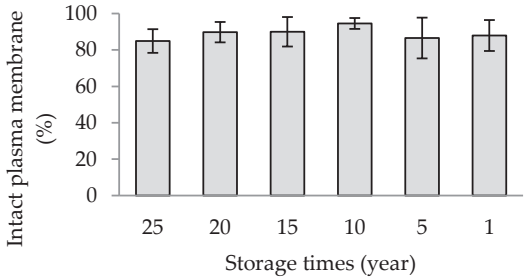

Figure 4. Intact plasma membrane of frozen FH semen at different storage times.

sperm moving at >20 μm/s (Massanyi *et al.,* 2008), and it is required to penetrate an egg (Ducha *et al.,* 2012). Sperm motility is an important parameter for bovine sperm fertility (Abavisani *et al.,* 2013). However, progressive motility of semen with different storage times is generally used for AI and follows SNI 4869.1-2017 for frozen bovine semen (BSN, 2017). The progressive motility shown in Figure 1b proves that the national AIC implements good container management and that semen still shows values >40% despite 25 years of storage.

Several factors influence the success of freezing, including the equipment used, the type of diluent, the freezing technique, and the technician's performance. Individual factors also influence the quality after thawing. The diluent used is the same, but the freezing process is becoming more efficient due to the improvements in equipment and technician expertise. This is probably the reason why the quality of frozen semen has improved in the last year. However, it is also determined by the individual bull (Indriastuti *et al.,* 2020).

The bull semen that has been frozen for 10-15 years may have a high freezing ability that produces a good PTM that the quality is maintained during the frozen storage until thawing. This also explains why frozen semen for 1 year is not better than frozen for 10-15 years. The most important information is that AIC only keeps frozen semen with a PTM of more than 40% according to the regulations for 25 years and maintains its quality. This result is consistent with the reports of Malik *et al.* (2015) that sperm motility was still 48% for frozen semen stored for 6 years. Furthermore, Ramírez-Reveco *et al.* (2016) reported that frozen semen still has 45% sperm motility after 45 years of storage, almost equivalent to sperm motility after one year of storage.

To successfully freeze cells, water must be removed and replaced with an antifreeze solution. Therefore, the cells are incubated in an antifreeze solution called cryoprotective solutes (Elliott *et al.,* 2017). Germann *et al.* (2013) state that water exchange with antifreeze solution occurs through a simple osmosis process. Cooling rates play an important role in successful cryopreservation, as they determine the sustained optimal cryo-dehydration that can be accomplished during transit through the high sub-zero temperature region to the range where true long-term cryo-stability can be assured.

Previous studies showed that the stability is below -100 °C (Liu *et al.,* 2014; Yao *et al.,* 2012). Cryobiological studies documented that for aqueous mixtures commonly used in cryopreservation, a physicochemical "glassy" state known as glass transition (Tg) exists at about -120 °C (Fuller, 2004). During freezing, sperm are cooled to a very low temperature below zero at -196 °C. At this temperature, all biological activity is effectively stopped (Getreu & Fuller, 2019). Sperm do not metabolize or use their energy reserves and are completely in a state of suspended animation, where all processes cease. Biologists believe that properly frozen cells in long-term storage can last forever, as long as the temperature is maintained correctly. Therefore, it can be ascertained that the artificial insemination center has carried out the maintenance of frozen semen properly.

In addition to sperm motility, viability is also very important. With storage times of 1, 5, 10, 15, and 20 years the sperm viability observed ranged from 65% to 78%, as shown in Figure 2. However, frozen semen at 25 years of storage has only 45.92 ± 8.1% viability. The sperm viability of frozen semen at 1-6 years of storage time ranged from 64% to 80% (Malik *et al.,* 2015). Ramírez-Reveco *et al.* (2016) reported that sperm viability of frozen semen has no significant difference when stored for 45, 40, 25, 10, and 1 years. Furthermore, live or viable sperm is required to fertilize an egg (Roca *et al.,* 2016). Non-viable sperm are destroyed by programmed death or apoptosis. Therefore, freezing, which induces extreme external cell stress, has a detrimental effect on the functional lifespan of currently viable sperm, resulting in an irreversible dysfunction that reduces their fertility potential and eventually leads to death (Roca *et al.,* 2016).

The values of sperm abnormalities at different storage times ranged from 3.5% to 6%. Frozen semen

at 10 years of storage times had the highest sperm abnormality with a value of 10.61 ± 2.3%. The high sperm abnormality at 10 years of storage time may not be due to the storage effect, but sperm abnormality may be higher in fresh sperm. Nonetheless, the amount of sperm abnormality is still below the requirements set by Permentan No. 10/Permentan/PK.210/3/2016. According to Purwantara *et al.* (2010), FH bull sperm abnormalities in fresh semen ranged from 1% to 8.4%. However, only sperm primary abnormalities were counted, whereas primary and secondary sperm abnormalities were counted in this study.

Freezing procedures affect sperm abnormalities by increasing the number of sperm cell abnormalities, mainly in bent and coiled tails, detached or damaged heads by about 10% (Khalil *et al.,* 2018), and a high number of abnormal sperm impairs fertility (Ferry, 2021). The maximum sperm abnormality in fresh bull semen to be processed into frozen semen is 20%. This is specified in Permentan No. 10/Permentan/PK.210/3/2016 on producing and distributing frozen semen for ruminants (Kementan, 2016). The bulls in the national AIC are selected and kept with good management, thereby having a relatively low sperm abnormality.

Freezing increased the number of sperm cell abnormalities, mainly in bent and coiled tails and detached or damaged heads, by about 10% (Khalil *et al.,* 2018). The IPM values are consistent with the reports of Ramirez-Reveco *et al.* (2016), which found high IPM values for frozen semen with storage times of up to 45 years. Furthermore, membrane integrity is important in frozen semen because it is a prerequisite for sperm survival (Sharma *et al.,* 2012). Sperm with intact membranes can bind to the zona pellucida (Ducha *et al.,* 2012). The membrane is the envelope found in all cells that separate their interior from the external environment. It regulates various sperm functions, and its integrity is directly related to fertility potential. During sperm cryopreservation, plasma membranes are particularly damaged by irreversible phospholipids changes during cold shock (Sieme *et al.,* 2015). The high IPM value in this study also proved that the National AIC has good freezing and container management techniques; hence, the quality of frozen sperm is maintained for 25 years.

In this study, eight kinematic parameters were determined, including three velocities, namely VCL, VSL, and VAP (μm/s), and three dimensionless motility indices, such as LIN, STR, WOB, and LDA (μm), as well as BCF (Hz). In this study, VCL values >100 μm/s reached the optimal value according to Oliveira *et al.* (2013). According to Agustinus & Pakpahan (2020), VCL is the velocity of sperm on their trajectory and indicates the movement's strength. Moreover, VCL only provides information about the strength of sperm movement but not the course and direction. The most commonly reported sperm movements are VAP, VSL, and VCL. Velocity and linearity contribute to important characteristics of sperm function. Fertilization potential is also related to velocity. VAP values can be used to predict the fertilization potential of thawed bull semen (Nagy *et al.,* 2015), while VSL illustrates important features of sperm function.

The results of the present study showed that the storage time of the frozen semen has no significant effect on the velocity values. Therefore, all frozen semen with different storage times should have good fertilization ability. The LIN refers to progressive motility, while STR is an indicator of the swimming pattern (Oliveira *et al.,* 2013). Oliveira *et al.* (2013) found that sperm move linearly in a straight line on average when LIN >35% and STR >50%.

This result proved that long-term storage of frozen semen has no significant effect on STR. The LIN of sperm was lower in frozen semen after 1 and 5 years than after 20 and 25 years of storage. A high value of the LIN and STR values indicated a progressive swimming pattern of the sperm, while low LIN showed hyperactivity. Several factors influence the occurrence of hyperactivity, including the origin of the sperm, individual variations, and temperature (Nagata *et al*., 2019). The reason for frozen semen stored for 1 and 5 years is more potent is yet unknown, but it is possibly due to individual influences.

Wobble is the maximum amount of sperm per second (Oliveira *et al.,* 2013). The results showed the highest WOB was found in frozen semen with a storage time of 15 years, and the lowest WOB was found in frozen semen with a storage time of 5 years. LDA and BCF values are variables of sperm wave patterns (Ratnawati *et al.,* 2017). Generally, larger VCL values are not desirable in LDA (LDA ≥ 7μm, VCL ≥ 70μm) as this indicates hyperactivated sperm (Raafi *et al.,* 2021). LDA values for frozen semen are <5 μm showing that sperms are not hyperactive at all storage times (Oliveira *et al.,* 2013; Raafi *et al*., 2021). These results were consistent with the result reported by Ramírez-Reveco *et al*. (2016) that an LDA value of 2-3 μm. Agustinus & Pakpahan (2020) found that the LDA value between trials depended on many factors, including CASA type, standard sperm trajectory, and device setting.

BCF indicates sperm strength and predicts fertilization *in vivo* (Oliveira *et al*., 2013). The BCF value in this study ranged from 10.34-23.12 Hz. This result was consistent with Maulana *et al.* (2021), which reported a BCF value ranging from 11.64.34 to 17.6±3.75 in the same breed.

In Indonesia, the minimum standard for sperm motility after freezing is >40%, and those below 40% are discarded. Sperm motility is an important factor indicating the progressive movement of sperm in the female reproductive tract to reach the egg and initiate fertilization. Therefore, frozen semen should be maintained during storage. The liquid nitrogen level should always be checked, and keep the straw submerged in nitrogen. This study showed that the total and progression motility of frozen semen stored for 1, 5, 10, 15, 20, and 25 years were different, but all values were >40%, which is consistent with SNI 4869.1-2017. Furthermore, Ramírez-Reveco *et al.* (2016) previously reported the evaluation of sperm with CASA in frozen semen stored for a long period. The result showed that frozen bull semen stored for 45, 40, 25, 10, and 1 years had the same movement patterns. Broekhuijse *et al*. (2012) state that VCL is related to the ability of sperm to penetrate the

cervical mucus, while VSL is related to the fertility of bulls (Gillan *et al.,* 2008). Therefore, this study's equal values of VCL and VSL can contribute to maintaining semen quality after long-term storage.

This study's limitation is that there is no data on motility after thawing immediately after freezing; hence, it is unknown whether motility decreases during storage. According to the regulations, AIC only stores frozen semen with more than 40% motility. All sperm motility within the storage time was above the standard, and this study also showed satisfactory values for sperm viability, abnormalities, and IPM. These results showed that the national AIC uses selected and well-managed bulls, a good freezing method, and management of the container system. This was evident by the frozen semen stored for 25 years and still suitable for artificial insemination. However, *in vitro* and *in vivo* studies are needed to ensure that the frozen semen stored for 25 years can still fertilize eggs.

CONCLUSION

Frozen semen stored for 25 years can still be used for artificial insemination. *In vitro* and *in vivo* fertility tests are required for frozen semen that has been stored for an extended period.

CONFLICT OF INTEREST

The authors certified that there is no conflict of interest with any financial, personal, and relationships with other people or organizations related to the material discussed in the manuscript.

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