Evaluation of Motility, Viability, and Integrity Plasma Membranes of Frozen Semen in Friesian Holstein with Storage Periods of 33, 30, 27, and 24 Years

Ahmad Budi Purnawan[1], Rimayanti[2*], Suherni Susilowati[3], Imam Mustofa[4], Tatik Hernawati[3], Erma Safitri[5]

[1]Biology Reproduction Program, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, [2]Laboratory of Veterinary Infertility and Sterility, Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, [3]Laboratory of Veterinary Artificial Insemination, Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, [4]Laboratory of Veterinary Obstetrics, Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, [5]Laboratory of Veterinary Physiology, Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

*Corresponding author: rimayanti@fkh.unair.ac.id

Abstract

The quality of frozen semen is one of the components of the artificial insemination program. The freezing method, kind of diluent, handling of frozen semen, and thawing process all affect the quality of frozen semen. This study aimed to determine the motility, viability, and plasma membrane integrity of frozen semen of Friesian Holstein cattle at storage periods of 33, 30, 27, and 24 years. The samples used were stored for 33 years (production 1987), 30 years (production 1990), 27 (1993 production), and 24 years (1996 production) stored in Singosari Artificial Insemination Center, Malang. Data were statistically analyzed using One-Way ANOVA followed by Duncan's Multiple Range Test ($p < 0,05$). Based on the study's findings, it can be concluded that the motility, viability, and plasma membrane integrity of frozen semen that had been preserved for 33, 30, 27, and 24 years were still acceptable and in good condition in comparison to the SNI quality criteria for bull frozen semen in Indonesia.

Keywords: Friesian Holstein, motility, plasma membrane integrity, storage periods, viability

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INTRODUCTION

The government has carried out various programs to increase the cattle population and reduce import levels. One program that has been implemented is expanding the reach of the artificial insemination program (Masyitoh *et al*., 2018). Artificial insemination is a reproductive technology that has been proven to be effective and can be widely applied (Singh and Balhara, 2016). One factor in the success of artificial insemination is the quality of the frozen semen used (Bahari *et al*., 2023). The quality of frozen semen is determined by the freezing technique, type of diluent, type, and concentration of cryoprotectant (Ariantie *et al*., 2013), handling of frozen semen (Janett *et al*., 2008), and thawing procedures (Al-Badry, 2012).

The quality of spermatozoa is based on SNI 4869.1-2017 for frozen cow semen, there are three quality requirements, i.e., motility, movement, and concentration of spermatozoa in one straw (Prayogo *et al*., 2022). The ability of spermatozoa to fertilize an egg cell must be alive (viable), motile (moving forward progressively), and have normal morphology with intact and good chromatin or deoxyribonucleic acid (DNA) (Okabe, 2018).

Frozen semen has the advantage that it can be used in the future, but so far it has been widely known that the freezing and thawing process causes damage to spermatozoa. Spermatozoa damage during the freezing process is caused by several factors such as cold shock, ice crystal formation, and lipid peroxidation. The critical point during freezing is in the transition phase

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(5°C to -60°C) because it is in that phase that cold shock occurs and the formation of ice crystals (Bojic *et al*., 2021). Many studies have been carried out to minimize damage caused by the cryopreservation process to meet established standards, such as modifying semen plasma (Susilowati *et al*., 2019), modifying freezing techniques (Dwinofanto *et al*., 2018), and adding antioxidants (Azura *et al*., 2020; Susilowati *et al*., 2019; Susilowati *et al*., 2020; Wurlina *et al*., 2020). However, not much is known about the processes that occur in spermatozoa during storage in liquid nitrogen.

Study regarding the shelf life of frozen semen shows no different results. Several studies reported that there were no differences in DNA integrity and fertility in human spermatozoa with different shelf lives (Pradika *et al*., 2019). Other studies also reported that there were no differences in motility and viability in bovine spermatozoa whether stored for short or longer periods in liquid nitrogen (Ramírez *et al*., 2016).

The decrease in spermatozoa quality during storage is thought to occur due to the aging process where plasma membrane destabilization occurs and activation of the apoptosis mechanism (Fraser *et al*., 2014). Meyers (2012) states that there is the possibility of free radicals being active at a temperature of -196°C. This allows the activity of Reactive Oxygen Species (ROS) to occur which causes oxidative stress. DNA damage will be more severe in spermatozoa that are not fully mature (Fortunato *et al*., 2012).

The risk of prolonged storage of spermatozoa is that oxidative stress can form and trigger the formation of malondialdehyde (MDA) in seminal plasma (Insani *et al*., 2014; Prastika *et al*., 2018). Oxidative stress can stimulate the formation of toxic lipid peroxide compounds which can reduce fertility by disrupting sperm membrane integrity and inducing DNA fragmentation, changes in mitochondrial structure, and even death (Longobardi *et al*., 2020; Wijayanti *et al*., 2023). Another cause is also thought to be due to changes in temperature during storage up to -80°C which causes crystal formation (recrystallization) in the cells (Setyawan *et al*., 2019; Bojic *et al*., 2021).

The importance of proving the quality of frozen semen that has been stored for a long time at the National Artificial Insemination Center in Indonesia is the basis for this study. This study was conducted to evaluate the quality of frozen semen stored at Singosari Artificial Insemination Center from 1987 to 1996 in Friesian Holstein cattle.

MATERIALS AND METHODS

Study Period and Location

This study was carried out at the Singosari Artificial Insemination Center Laboratory and the Animal Disease and Diagnostics Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang.

Samples

The method used in this study was an experimental laboratory with variations in treatment in the form of storage time. The sample size in this study was determined based on standard procedures for determining the number of samples using the Federer Formula which resulted in each group using 5 samples with a total sample of 20 samples. The sample used in this study was frozen semen from Friesian Holstein (ID: Kitanohana Bull, stud code 38619), imported from Japan on March 14, 1987, and produced from June 1987 until the end of 1996 (Hedah and Ma'sum, 1996).

The quality of fresh semen produced between 1987 and 1996 with a volume range of 5-6 ml, milky white color, pH 6,2-6,6, medium-thick consistency, mass movement 2+-3+, individual movement 70-80%, spermatozoa concentration 1,136–2,174 10^6/ml (Hedah and Ma'sum, 1995). The shelf life of the samples used was 33 years (1987 production), 30 years (1990 production), 27 (1993 production), and 24 years (1996 production) which were stored in the Sperm Bank of the Singosari Artificial Insemination Center, Malang.

Motility Evaluation

Motility examination includes IVOS II (Integrated Visual Optical System), 0,9% NaCl,

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micropipette, micropipette tip, object glass, cover glass, 1,5 ml microtube, water bath, thermometer, and straw scissors. The examination was carried out by thawing frozen semen in warm water at a temperature of 37°C for 30 seconds. The liquid semen is placed in a microtube and dripped onto 20 µl slides. The examination was carried out using IVOS II.

Viability Evaluation

Viability examination consists of a microscope, slide warmer plate, micropipette, micropipette tip, microtube, object glass, cover glass, hand counter, and eosin nigrosine dye. Examination of spermatozoa viability was carried out using eosin nigrosine smear preparations. The examination was carried out using a microscope with 400× magnification by looking at 10 different fields of view. Viability was calculated by counting live spermatozoa, i.e., spermatozoa that did not absorb color from the total number of spermatozoa observed (Susilawati, 2011).

Plasma Membrane Integrity Evaluation

Plasma membrane integrity examination includes a microscope, water bath, micropipette, micropipette tip, microtube, object glass, cover glass, hand counter, and Hypo Osmotic Swelling (HOS) 150 mOsmol solution. The integrity of the spermatozoa cell membrane was checked using the HOS test. According to Susilawati (2011), the test was carried out by adding 0,1 ml of semen sample to 1 ml of 150 mOsmol hypoosmotic solution (solution made from 7,35 g of $2H_2O$ sodium citrate, 13,52 g of fructose dissolved in 1000 ml of distilled water), then incubated in temperature 37°C for 30 minutes. After incubation, take one drop and place it on an object glass, then cover it with a cover glass and observe using a microscope with a magnification of 400×. Observations were performed on the typical changes that occurred, i.e., the presence of a coiled tail tip which identified the integrity of the plasma membrane (Indriani *et al*., 2013). The calculation was carried out by counting the intact plasma membrane of the total number of spermatozoa observed.

Data Analysis

Data from the study results were analyzed statistically using the One-Way ANOVA followed by Duncan's Multiple Range Test test ($p < 0,05$).

RESULTS AND DISCUSSION

The results of the motility examination showed that the values for the 33, 30, 27, and 24-year storage groups were $43,05^a \pm 2,51$; $44,90^a \pm 6,62$; $47,00^a \pm 5,66$; $43,55^a \pm 2,28$. The results of the motility examination of the 33, 30, 27, and 24-year storage groups did not show any significant differences ($p > 0,05$). The 33-year storage group showed the lowest group motility level with a value of $43,05^a \pm 2,51$ while the 27-year storage group showed the highest motility level with a value of $47,00^a \pm 5,66$ (Table 1).

Viability was calculated by counting live spermatozoa, i.e., spermatozoa that did not absorb color from the total number of spermatozoa observed. Spermatozoa that were still alive had a transparent or white appearance while dead spermatozoa were colored and produced a crimson color (Figure 1).

The results of the viability examination showed that the average value for the 33, 30, 27, and 24-year storage groups was $54,00^b \pm 2,51$; $54,00^b \pm 6,62$; $51,16^a \pm 5,66$; $52,50^a$,b $\pm 2,28$. The results of the viability examination of the 24, 30, and 33-year storage groups did not show a significant difference ($p > 0,05$), on the other hand, the 27-year storage group and the 30 and 33-year storage groups had a significant difference ($p < 0,05$). The 27-year group showed the lowest mean Viability value with a value of $51,16^a \pm 1,47$ while the 30 and 33-year storage groups showed the highest viability level with a value of $54,00^b \pm 0,89$ (Table 1).

Examination of the integrity of the spermatozoa plasma membrane was carried out by calculating the typical changes that occur, i.e., the coiling of the tip of the spermatozoa tail. Calculation of the integrity of the plasma membrane was indicated by the curved tip of the spermatozoa tail shown in the arrow (A) and the

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Table 1. Motility, viability, and plasma membrane integrity of frozen semen in Friesian Holstein with storage periods of 33, 30, 27, and 24 years

Storage Time (years)	Motility (%)	Viability (%)	Plasma membrane integrity (%)
33	$43,05^{a} \pm 2,51$	$54,00^{b} \pm 0,89$	$58,00^{d} \pm 1,41$
30	$44,90^{a} \pm 6,62$	$54,00^{b} \pm 0,89$	$53,00^{c} \pm 0,89$
27	$47,00^{a} \pm 5,66$	$51,16^{a} \pm 1,47$	$44,50^{a} \pm 1,04$
24	$43,55^{a} \pm 2,28$	$52,50^{ab} \pm 1,87$	$49,00^{b} \pm 0,89$

[abcd]Different superscripts in the same column indicate significant differences ($p < 0,05$).

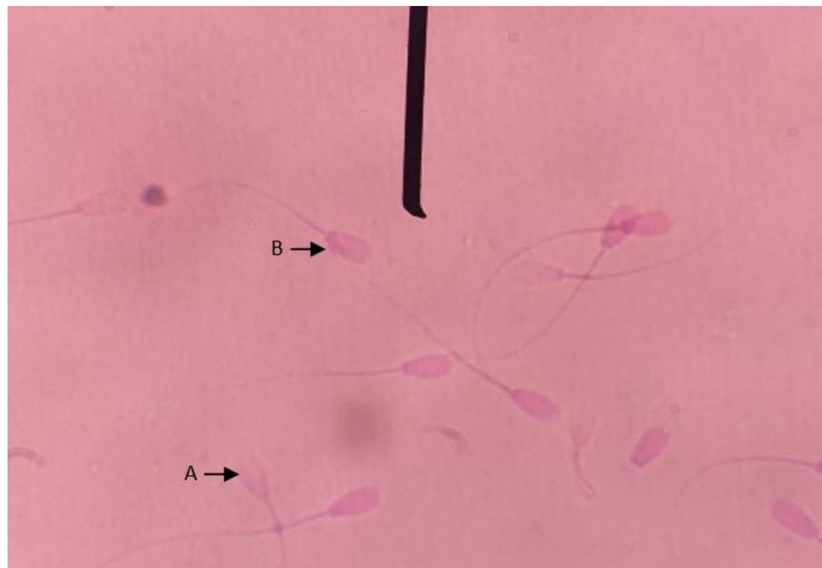

Figure 1. Eosin Negrosin staining is used to assess the viability of spermatozoa. Spermatozoa that (A) are alive are white, and those that (B) are dead are crimson.

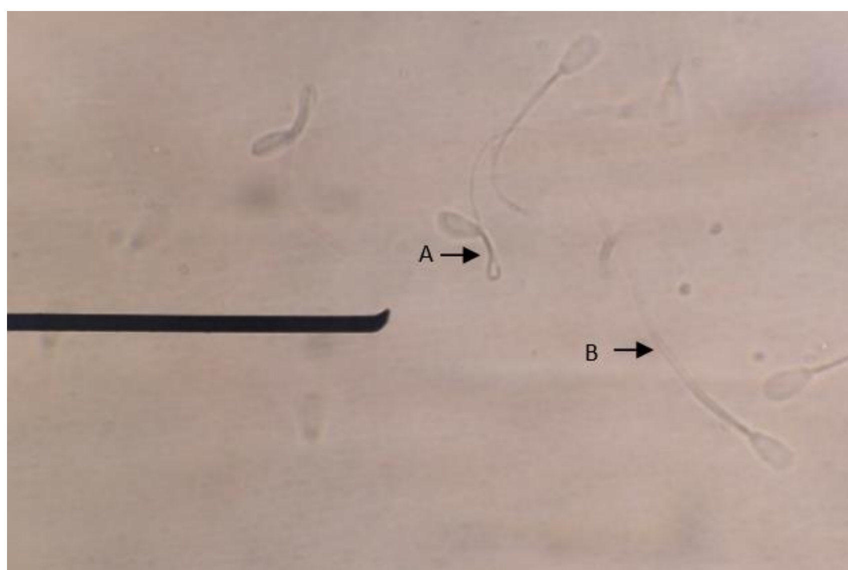

Figure 2. HOST solution is used to evaluate the integrity of the plasma membrane. The plasma membrane that (A) is intact is indicated by a circular tail in (B) while the damaged membrane is indicated by a straight tail in.

damaged plasma membrane was indicated by the straight tail in the arrow (B) (Figure 2).

The results of the plasma membrane integrity examination showed that the average value for the 33, 30, 27, and 24-year storage groups was $58,00^{d} \pm 1,41$; $53,00^{c} \pm 0,89$; $44,50^{a} \pm 1,04$; $49,00^{b} \pm 0,89$. The results of the plasma membrane integrity examination of the 24, 27, 30, and 33-year storage groups showed significant differences ($p < 0,05$) (Table 1). The 27-year

storage group showed the lowest level of plasma membrane integrity with a value of 44,50[a] ± 1,04, while the 33-year storage group showed the highest level of plasma membrane integrity with a value of 58,00[d] ± 1,41.

Motility examination using Computer Assisted Sperm Analysis (CASA) IVOS-II. Frozen Friesian Holstein semen which had been thawed again in warm water at a temperature of 37°C for 30 seconds was then taken as much as 6 μL and dropped into the Leja counting chamber which had been previously warmed to a temperature of 37°C. A total of 1000 spermatozoa were analyzed from 30 images in 8 fields of view taken randomly.

The results of statistical analysis of the average Motility examination values show that the storage group for 33 years produced a percentage of 43,05%, 30 years of 44,90%, 27 years of 47,00%, and 24 years of 43,55%. This study revealed that the motility examination for the 33, 30, 27, and 24-year storage groups did not show any significant differences.

The results of the motility examination showed that the percentage was > 40%, this is in accordance with the quality standards set by the SNI regarding frozen cow semen. According to SNI (2021), the special requirements for frozen semen post-thawing at a temperature of 37-38°C for 30 seconds must show a minimum progressive spermatozoa motility of 40%.

Semen motility is an important parameter to evaluate the fertility potential of bulls. One of the most frequently observed parameters is progressive motility. According to Amann and Waberski (2014), spermatozoa must be given special treatment to achieve maximum potential for fertilization. Motility is also closely related to DNA damage, motility is crucial in fertilization (Puglisi *et al*., 2017). Immotile spermatozoa or spermatozoa that do not move and abnormalities in motility can be used as indicators of infertility in bull cattle (Longobardi *et al*., 2017). Tests and calculations that produce the percentage of spermatozoa that have forward movement are standard tests for measuring male fertility (Berg *et al*., 2018).

Spermatozoa have a tail that is used to mobilize movement within the female reproductive tract and fertilize the oocyte. Spermatozoa move quickly and strongly shortly after being expelled, capacitation is needed to activate the acrosome reaction and fertilization (Moghbeli *et al*., 2016). In general, ATP plays an important role in spermatozoa tail movement. When spermatozoa move quickly and strongly, it results in faster intracellular energy consumption. On the other hand, ATP is a potential indicator of fertilization (Blanco *et al*., 2011).

Spermatozoa cell viability is an important aspect in assessing ejaculate quality which determines the success of fertilization because it is related to the number of viable spermatozoa (Kumaresan *et al*., 2017). The percentage of viable spermatozoa cells can be measured in the laboratory by identifying the number of spermatozoa cells that are not stained with eosin-negrosin (Baiee *et al*., 2017).

Based on the results of statistical analysis, the average value of spermatozoa viability examination showed that the storage group for 33 years produced a percentage of 54,00%, 30 years of 54,00%, 27 years of 51,16%, and 24 years of 52,50%. This study showed that the results of the spermatozoa viability examination for the 33, 30, 27, and 24-year storage groups, there is a significant difference between the 33 and 30 storage groups and the 27-year storage group, but there is no significant difference between the 33, 30, and 24-year storage groups.

In this study, the percentage of spermatozoa viability in the 33, 30, 27, and 24-year storage groups was above 50%. According to Khalil *et al*., (2019), the gradual decrease in the percentage of spermatozoa viability in the frozen semen production process is caused by the cryopreservation process. The percentage of spermatozoa viability in fresh semen is in the range of 94%, after undergoing the freezing process it will decrease by 20-35%, and after the thawing process the percentage of spermatozoa viability will be > 50%.

The significant difference between the 33 and 30-year storage group and the 27-year storage group is thought to be caused by the percentage of

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spermatozoa viability in fresh semen in the 33 and 30-year storage group being higher than in the 27-year storage group, thus affecting the percentage of spermatozoa viability in frozen semen post-thawing. Dead spermatozoa have high membrane permeability so that the dye can easily enter along with Ca^{2+} transport and color the cytoplasm of the stained spermatozoa. Meanwhile, live spermatozoa still have acceptable membrane permeability so it is difficult for dyes to infiltrate spermatozoa (Purnama *et al*., 2019; Prabowo *et al*., 2021).

Spermatozoa viability is influenced by the integrity of the plasma membrane by protecting the cytoplasm and DNA inside the cell (Almadaly *et al*., 2014). There are 40 miRNAs (small ncRNAs) in frozen sperm that have been thawed which influence spermatozoa viability which regulates metabolism and the occurrence of processes similar to apoptosis in spermatozoa cells (Capra *et al*., 2017). According to Munazaroh *et al*., (2013) a decrease in spermatozoa viability can be caused by friction between spermatozoa and each other, temperature, storage time for cell metabolism, and availability of nutrients. According to Mustofa *et al*., (2021) one solution to increase the percentage of spermatozoa viability after thawing is to mix 0,1 mg/100 green tea extract into the dilute.

Spermatozoa with an intact plasma membrane is characterized by a spermatozoa tail that looks circular, whereas spermatozoa with a damaged plasma membrane are characterized by a tail that looks straight (Mustofa *et al*., 2021). The addition of HOS solution to semen makes the pressure on the spermatozoa cells become hyperosmotic compared to the outside of the cells. Spermatozoa cells will absorb water linked to K^+ and Cl^- and are driven by Na^+ to reach an isoosmotic state (Safitri *et al*., 2022). The coiling of the tail occurs because the tail is more flexible than the other parts and is a sign that the plasma membrane is still intact (Larsen and Hoffmann, 2020).

Based on the results of statistical analysis, the average value of intact plasma membrane integrity examination of spermatozoa shows that the storage group for 33 years produced a percentage of 58,00%, 30 years of 53,00%, 27 years of 44,50% and 24 years of 49,00 %. This shows that the results of examining the integrity of the intact plasma membrane of spermatozoa in the 33, 30, 27 and 24-year storage groups contained significant differences.

The results were similar with a study conducted by Almadaly *et al*., (2014) which produced a percentage of plasma membrane integrity of frozen semen that had been thawed in several bulls ranging from 52–58% which was carried out using the HOS test method. The real differences between each storage group are thought to be caused by several things, namely the quality of fresh semen, the quality of semen before freezing, the vitamins mixed in the diluter and the type of diluter used during the production process.

Skim milk is used in the semen freezing process, most of which has gone through a reconstitution process combined with arabinose, fructose, or egg yolk (Raheja *et al*., 2018). Skim milk protein buffers semen pH (Perea *et al*., 2017). The lactose content in skim milk has hydrophilic properties and cannot diffuse into the cell walls of sperm cells, thereby protecting the cell walls and preventing freeze shock. Skim milk-based diluters are superior to Tris-based diluters when used in the frozen semen production process (Rahman *et al*., 2018).

The addition of egg yolk to the dilute can provide protection to the spermatozoa membrane thereby maintaining its integrity (Diliyana *et al*., 2014). Egg yolk is a non-penetrating substance used to thin semen protect sperm from freeze shock during the cooling process and prevent the loss of membrane phospholipids during the freezing process (Sun *et al*., 2019). Egg yolk low-density lipoprotein (LDL) maintains sperm membrane phospholipids during the cryopreservation process (Layek *et al*., 2016). Other study shows that sperm is protected during the freezing process by separating lipid-binding proteins from LDL in egg yolk (Raheja *et al*., 2018). Several studies have been carried out on the combination of several ingredients in diluters, which aims to improve the quality of frozen

semen. According to Susilowati *et al*., (2021), the combination of green tea extract 0,1 mg/100 ml dilute skim milk and egg yolk can improve plasma membrane integrity so that it can increase pregnancy rates in cows.

CONCLUSION

It can be concluded that frozen semen with storage periods of 33, 30, 27, and 24 years has no effect on the quality of frozen semen in Friesian Holstein based on motility, viability, and plasma membrane integrity evaluation. In particular, motility in all storage groups was ≥ 40%. The viability of the 33 and 30-year storage groups had the highest values. The plasma membrane integrity of the 33-year storage group had the highest value. It was found that SNI 4869-1:2017 is still in compliance with all storage period groups.

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