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Evaluation of Friesian Holstein frozen semen storage time for 33, 30, 27, and 24 years based on sperm morphologic and related variables

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ABSTRACT

This study aimed to evaluate Friesian Holstein's frozen semen storage time for 33, 30, 27, and 24 years based on sperm morphologic and related variables. The sample used in this research was frozen semen from Frisian Holstein which were stored in the Sperm Bank of the Singosari Artificial Insemination Center, Malang. The level of MDA was measured using the Enzyme-linked immunosorbent assay method. Examination of the intact acrosomal cap using formol saline was then examined under a microscope. The chromatin maturity examination used a drop of semen immersed in a 10% solution of formaldehyde at room temperature for 30 minutes and rinsed twice with a PBS solution for five and then examined under the microscope. The chromatin integrity examination used a smear of one drop of semen prepared on a glass object, air-dried, and subsequently fixed in a solution of 96% ethanol and acetone at 4°C for 30 minutes, and then examined under the microscope. The morphology and motility examination used a drop of semen to the object glass and then examined under the microscope. The study found that storing sperm for longer periods resulted in higher levels of MDA and lower levels of chromatin maturity and integrity. The levels of MDA were significantly different among the groups stored for 33, 30, 27, and 24 years. The levels of chromatin maturity and integrity were significantly different among the groups stored for 24, 27, 30, and 33 years. However, sperm motility and morphology were not significantly different among the 24, 27, 30, and 33-year storage groups ($p>0.05$), but the 27-year storage group had the highest normal sperm morphology and motility percentage. It could be concluded that the impact of long-term storage on frozen semen causes changes in variables that have a direct or indirect influence on spermatozoa morphology.

Introduction

The quality of cryopreserved semen has a significant impact on the effectiveness of fertilization. It is influenced by a number of factors, such as the freezing method, the kind of diluent used, the type and concentration of cryoprotectant used (Ariantie *et al*., 2013; Iskandar *et al*., 2022), how the frozen semen is handled (Bahmid *et al*., 2023), and how long it takes to thaw (Biniová *et al*., 2018). The three quality requirements for frozen cow semen are spermatozoa motility, spermatozoa movement, and spermatozoa concentration in a single straw, as stated in SNI 4869.1-2017. Spermatozoa need to be living (viable), motile (moving forward gradually), have normal morphology, and show good chromatin/deoxyribonucleic acid (DNA) integrity in order to successfully fertilize an egg cell (Okabe, 2018).

The benefit of using frozen semen is that it can be stored for later use. It's important to remember, though, that the freezing and thawing process can harm spermatozoa. Many causes, including ice crystal formation, lipid peroxidation, and cold shock, are implicated in this damage. The freezing transition zone, which ranges in temperature from 5°C to -60°C, is the critical point. This stage is known to cause ice crystals to form and cold shock, both of which can harm spermatozoa (Bojic *et al*., 2021). Several investigations have been carried out to minimize the harm caused by cryopreservation and to adhere to established guidelines. These investigations include adding antioxidants (Azura *et al*., 2020; Susilowati *et al*., 2019; Wurlina *et al*., 2020), altering freezing methods (Dwinofanto *et al*., 2018), and modifying semen plasma (Susilowati *et al*., 2019). However, there is still limited knowledge about the processes that occur in spermatozoa during storage in liquid nitrogen.

The irreversible ageing of spermatozoa will eventually lead to death (Nagata *et al*., 2019). As spermatozoa age, they may experience mitochondrial damage and chromatin compactness alteration, which can trigger the apoptotic cascade and increase DNA fragmentation rates (Condorelli *et al*., 2020). Numerous research studies have investigated the impact of ageing on semen quality, with many of them focusing on the role of reactive oxygen species (ROS) (Nago *et al*., 2021). As age increases, oxidative stress increases, sperm motility decreases, and DNA fragmentation increases. Reactive oxygen species (ROS) have been the subject of most reports on the effects of aging on semen to date (Meyers, 2012). Significant DNA damage, such as incomplete protamination resulting in inadequately packed chromatin, is a common occurrence in immature spermatozoa. Because of this, they are more susceptible to oxidative damage brought on by reactive oxygen species (ROS) (De Martin *et al*., 2019). Thus, this study aimed to evaluate Friesian Holstein (FH) frozen semen storage time for 33, 30, 27, and 24 years based on sperm morphologic and related variables.

Materials and methods

Location

This research was conducted at the Singosari Artificial Insemination Center Laboratory and the Animal Disease and Diagnostic Laboratory (ADD-Lab) of the Faculty of Veterinary Medicine, Brawijaya University, Malang.

Samples

The sample used in this research was frozen semen from Frisian Holstein cattle with the name Kitanohana stud, stud code 38619. Kitanohana cattle were imported from Japan on March 14, 1987. Frozen semen from

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Kitanohana stud began to be produced in June 1987 until the end of 1996. Diluent composition: Skim milk powder 10 g, Fructose 1 ml, Citric acid 1.70 g, Streptomycin sulfate 0.1 g/ml, Penicillin 1000 UI, Glycerol 8 ml, Aquabides add 100 ml (Hedah and Ma'sum, 1996). Straw semen is stored in a liquid nitrogen container at a temperature of –196°C.

According to Hedah and Ma'sum (1995), the quality of fresh semen from Kitanohana cattle produced between 1987 and 1996 ranged from 5 to 6 milliliters, had a milky white color, pH between 6.2 and 6.6, a medium to thick consistency, mass movement between 2 and 3 plus, individual movement between 70% and 80%, and had a spermatozoa concentration between 1,136 and 2,174x10^6 per milliliter. The sperm bank of the Singosari Artificial Insemination Center, Malang, was the storage location for the samples used for 33 years (1987 production), 30 years (1990 production), 27 years (1993 production), and 24 years (1996 production). The number of samples for this study was five straws from each storage period.

Determination of MDA Levels

The level of MDA was measured using the Enzyme-linked immunosorbent assay (ELISA) method (Malondialdehyde Competitive ELISA Kit Invitrogen, Thermo Fisher Scientific) (Wang *et al*., 2021).

The Intact Acrosomal Cap Examination

Using formol saline, which contains 2.54 g potassium dihydrogen phosphate, 5.41 g sodium chloride, 6.19 g disodium hydrogen phosphate dihydrate, 125 ml 37% formaldehyde, and 875 ml distilled water, the intact acrosomal cap was examined. Combine 1000 µl of formol saline solution with 10 µl of semen. After leaving it for an hour, take one drop of the mixture, put it on the glass object, and cover it with a glass cover. After that, the preparations were examined under a 400-times magnification microscope with ten distinct fields of view (Syafi'i and Rosadi, 2022; Priyanto *et al*., 2015). Spermatozoa that have an intact acrosomal cap is characterized by the presence of a black acrosomal cap, while the damaged one does not show the same signs (Syafi'i and Rosadi, 2022).

Chromatin Maturation Examination

a drop of semen was smeared onto a glass object and allowed to dry. Subsequently, the sample was immersed in a 10% solution of formaldehyde at room temperature for 30 minutes and rinsed twice with a PBS solution for five minutes each. The sample was then stained with a 2.5% solution of Aniline Blue by soaking it for seven minutes, after which it was rinsed again with PBS solution and air-dried. A light microscope with a magnification of 400 times was utilized for examining the sample. Spermatozoa with mature chromatin would appear colourless, while those with immature chromatin would tend to show a blue hue. Erenpreiss *et al*. (2001), the examination was carried out on 100 spermatozoa for each sample.

Chromatin Integrity Examination

One drop of semen was smeared on a glass surface, allowed to air dry, and then fixed for thirty minutes at 4°C in a solution of 96% ethanol and acetone (1:1). Following fixation, the preparations were allowed to air dry once more before being hydrolyzed for five minutes at 4°C in a solution of 0.1 N hydrochloric acid. Following three distilled water washes, the hydrolyzed preparations were soaked for ten minutes in 0.05% TB stain. Following another wash with distilled water, the stained preparations were twice dehydrated with t-butanol and twice cleaned with xylol. After that, the stained preparations were examined at 400x magnification using an Olympus BX-53 microscope. The heads of spermatozoa with good chromatin integrity appeared bright blue, while those with diminished chromatin integrity appeared dark blue. The examination was conducted on 100 spermatozoa for each sample (Erenpreisa *et al*., 2003).

Data Analysis

The statistical analysis of collected research data utilized a Completely Randomized Design (CRD) in conjunction with the Analysis of Variance (ANOVA) test. Should discrepancies arise, a further Duncan's Multiple Range Test (DMRT) was performed to determine the significance of the differences.

Results

MDA levels varied significantly ($p<0.05$) among the storage groups of 33, 30, 27, and 24 years. However, a significant difference did not exist ($p>0.05$) between the storage groups of 27 and 24 years. The 30-year storage group displayed an average MDA level value of 7.57c±0.89 U/L, while the 24-year group displayed the lowest average MDA level value of 3.92a±1.26 U/L (Table 1). The group aged 27 exhibited the lowest average value of Intact Acrosome Cap, measuring 40.67a±0.81. In contrast, the storage group, aged 33, displayed an average value of Intact Acrosomal Cap, measuring 52.00c±0.89 (Table 1). The intact acrosomal cap is black, the damaged acrosomal cap is characterized by an unstained/shiny acrosomal cap (Figure 1).

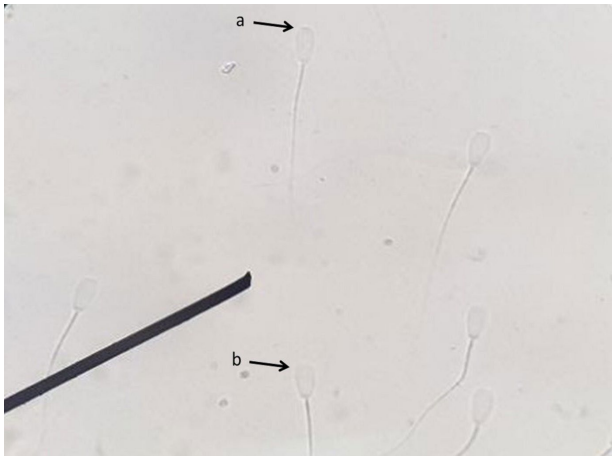

Fig. 1. Sperm Intact Acrosomal Cap Examination Results: (A) Intact acrosomal cap with black stained tip. (B) Damaged acrosomal cap with unstained and shiny white head.

Chromatin maturity is indicated by a clear colour in spermatozoa cells, while spermatozoa with immature chromatin show a blue colour (Figure 2). The chromatin maturity of the 24, 27, 30, and 33-year storage groups varied significantly ($p<0.05$). With an average Chromatin Maturity value of 79.50a±1.04, the 33-year-old group displayed the lowest value, and the 24-year-old storage group displayed the highest value, at 85.50d±1.04 (Table 1).

Table 1. Malondialdehyde (nmol/mL), intact acrosomal cap, chromatin maturity, and chromatin integrity (%) of Friesian Holstein frozen semen storage for 24, 27, 30, and 33-years.

Parameters	Storage time (years)			
	24	27	30	33
Malondialdehyde	3.92a±1.26	5.06^{a,b}±0.81	7.57c±0.89	6.01b±0.89
Intact acrosomal cap	49.00b±1.26	40.67^{a}±0.81	51.00c±0.89	52.00c±0.89
Chromatin maturity	85.50d±1.04	83.50^{c}±1.04	82.00b±0.63	79.50a±1.04

Spermatozoa with reduced chromatin integrity display a dark blue color on their heads, while those with good chromatin integrity have dark blue heads (Figure 3). The chromatin integrity of the 33, 30, 27, and 24-year storage groups varied significantly ($p<0.05$). With an average Chro-

matin Integrity value of 65.13a±0.59, the 33-year-old group displayed the lowest value, and the 24-year-old storage group displayed the highest value, at 75.63d±0.44 (Table 1).

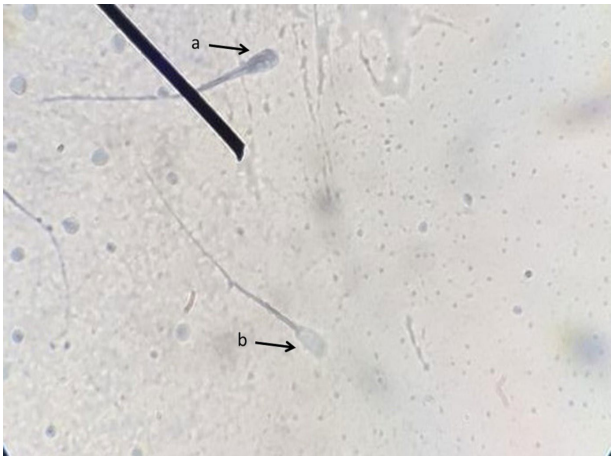

Fig. 2. Examination of Chromatin Maturity Using Aniline Blue Staining. (A) clear spermatozoa indicates mature chromatin, (B) Blue indicates immature chromatin.

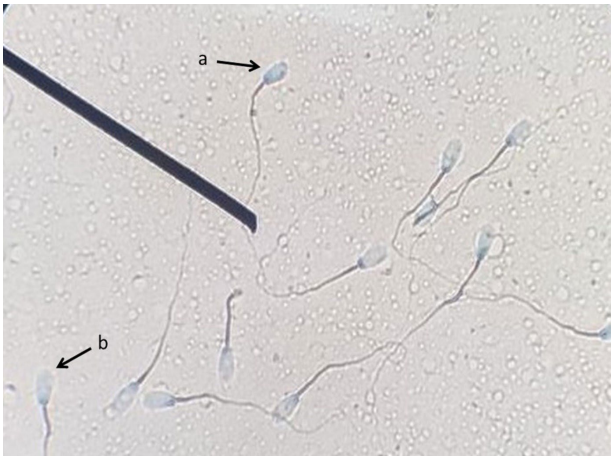

Fig. 3. Examination of Chromatin Integrity Using Toluidine Blue Staining. (A) Spermatozoa with damaged DNA are stained dark blue, (B) intact DNA are stained light blue.

The analysis of the sperm morphology revealed no appreciable variations in the morphology between the storage groups for 33, 30, 27, and 24 years (p>0.05). Among the three groups, the 27-year-old group exhibited the highest level of normal sperm morphology (96.32±2.20), while the 30-year-old group showed the lowest level of normal sperm morphology (95.06±1.84) (Table 2).

Table 2. Sperm morphologic (%) of Friesian Holstein frozen semen storage for 24, 27, 30, and 33-years.

Parameters	Storage time (years)			
	24	27	30	33
Normal Morphology	95.62±2.64	96.32±2.20	95.06±1.84	95.66±2.65
Abnormal Morphology	4.64±2.78	3.92±2.27	5.28±1.86	4.58±2.79
- Bent Tail	1.64±0.77	1.40±0.43	1.82±0.58	1.60±0.76
- Coiled Tail	0.22±0.39	0.06±0.09	0.24±0.38	0.24±0.38
- DMR	2.18±1.71	1.82±1.60	2.48±1.32	2.12±1.73
- Proximal Droplet	0.10±0.17	0.12±0.16	0.12±0.16	0.12±0.16
- Distal Droplet	0.50±0.29	0.52±0.29	0.62±0.08	0.50±0.29

Note: DMR: distal midpiece reflex. No significant different (p > 0.05) among storage years

There were no appreciable variations in the morphology between the 33, 30, 27, and 24-year storage groups, according to the sperm motility test (p>0.05). In comparison to the other three groups, the 27-year-old group exhibited the highest motility percentage (55.38±3.13), while the 24-year-old group displayed the lowest motility percentage (53.50±3.44) (Table 3).

Table 3. Sperm motility (%) of Friesian Holstein frozen semen storage for 24, 27, 30, and 33-years.

Parameters	Storage time (years)			
	24	27	30	33
Motile	53.50±3.44	55.38±3.13	53.68±3.73	54.94±3.87
- Progressive	43.50±2.97	45.16±3.12	44.66± 3.71	43.80±3.47
- Slow	9.26±3.16	9.32±3.15	8.52±2.44	10.24±1.62
Static	46.50±3.44	44.62±3.13	46.32±3.73	45.06±3.87

Discussion

Assessment of Malondialdehyde (MDA) levels has been widely used to measure the occurrence of lipid peroxidation. According to Arif *et al.* (2020), heightened MDA levels are indicative of a more significant degree of lipid peroxidation, particularly during the freezing and thawing phases. Lipid peroxidation can result in damage to the cell membrane of spermatozoa, resulting in a decrease in the quality of frozen semen. The extent of damage to the spermatozoa cell membrane is directly proportional to the decline in the quality of frozen semen.

According to the statistical analysis, the examination of malondialdehyde levels showed that the average value of the 33-year storage group was 6.06 U/L, for 30 years it was 7.57 U/L, for 27 years it was 5.06 U/L, and for 24 years it was 3.92 U/L. The findings show that the levels of malondialdehyde in the 33-year storage group, as well as the 30, 27, and 24-year storage groups, differed significantly. But there was no discernible difference between the groups that had been stored for 30 and 27 years, or between the groups that had been stored for 27 and 24 years.

According to the study, the MDA levels increased as the frozen semen storage time increased, however, in the case of the 33-year storage group, there appears to be a lower MDA value than the 30-year storage group. This could be due to several factors, such as changes in regulations over time or human error during post-thawing treatment, as per Galián *et al.* (2023), numerous factors influence post-thawing sperm quality, such as the extender used, the dilution and freezing protocol, or the evaluation procedure. It is crucial to thoroughly analyze and understand these discrepancies to interpret the results of the study accurately.

According to Aitken (2020), Sperm cells exhibit a natural mechanism of defence that relies on the presence of antioxidants within semen to repair any damage. However, a failure of this defence system may result in an imbalance between reactive oxygen species (ROS) and antioxidants in semen. This imbalance is considered the primary cause of the suboptimal quality of frozen semen. During the process of capacitation, the maintenance of low ROS values is crucial for optimal outcomes. Thus, careful management of ROS and antioxidant levels in semen is essential to ensure consistent quality in semen samples used for artificial insemination or other reproductive technologies. According to Alyedthodi *et al.* (2021), the primary reasons for low cryopreservability are attributed to an elevation in lipid peroxidation and reactive oxygen species (ROS) levels and a subsequent decline in antioxidant capacity.

To ensure the safety and quality of frozen semen, it is crucial to incorporate antioxidants in the diluter during the freezing and thawing processes. This prevents lipid peroxidation, which can harm the semen. Vitamin C, among other antioxidants, is recommended to be added to the diluter. Recent studies have found that the addition of 0.9 mg/ml of vitamin C significantly improves the quality of frozen semen. Vitamin C can reduce the negative effects of vitrification on sperm parameters, chromatin quality, and apoptosis in semen (Mangoli *et al.*, 2018). Additionally, Vitamin C accumulates in the aqueous phase of the cell. The hydroxyl groups located at the double bond in the lactone ring act as donors of protons and electrons, which then convert into the diketone

moiety of DHA. This conversion gives ascorbic acid its strong reducing properties, which help protect other cellular components from oxidation (Kaźmierczak-Barańska *et al*., 2020). As science and technology progress, we have access to various antioxidants that can be utilized in diluters. According to Susilowati *et al*. (2019), including 4 mM L-arginine in the dilute can help maintain the quality of frozen semen post-thawing. This includes the viability, motility, and integrity of the spermatozoa's plasma membrane, while also decreasing the percentage of necrosis and apoptosis in the spermatozoa.

The observation of the acrosomal cap in spermatozoa is a crucial aspect to consider when conducting examinations. Successful fertilization requires intact acrosome and proper sperm morphology, particularly the acrosome region, which is associated with the ability of sperm to bind with zona pellucida (Sun *et al*., 2021). The deleterious effects of freezing and thawing processes on the acrosome cap have been documented, with the release of acrosomal enzymes such as hyaluronidase and acrosin being a primary characteristic of such damage. Spermatozoa with an abnormal Acrosome cap exhibit flattening of the apex, the anterior of the head is brighter than the posterior, the nucleus ring is not visible, and the surface of the spermatozoa head is not smooth (Mahendra *et al*., 2018). The colour change in the acrosomal cap is due to the oxidation reaction of the ions that bind to the formol saline compound. The Ca^{2+} ions present in spermatozoa cells react with the H_2O in the formol saline solution to form $Ca(OH)_2$.

Furthermore, the KH2PO4 in the formol saline solution reacts with $Ca(OH)_2$ to form KOH. This is an oxidation reaction that is caused by the reduction of hydrogen ions in KH_2PO_4 to KOH. The reaction equation can be expressed as $3Ca(OH)_2 + 2KH_2PO_4 \rightarrow Ca_3(PO4)_2\ 2KOH + 4H_2O$. The reaction results in the acrosome cap turning black (Cahyani *et al*., 2020).

Based on statistical analysis, there were differences in the average percentage of intact spermatozoa acrosomal caps amongst the storage groups. In particular, the group that stored materials for 33 years produced 52%, the group that did so for 30 years produced 51%, the group that did so for 27 years produced 40.67%, and the group that did so for 24 years produced 49%. These results indicate that there are notable variations between the 33, 30, 27, and 24-year storage groups when it comes to the intact acrosomal cap of spermatozoa. But there was no discernible difference between the groups that had been stored for 30 and 33 years; the group that had been stored for 27 years had the lowest value. All things considered, these findings offer crucial information about the preservation and storage of spermatozoa and may influence procedures involving assisted reproductive technologies.

According to the study, the percentage of intact acrosome caps and plasma membrane integrity showed a consistent trend, with the 33-year storage group showing the highest percentage and the 27-year storage group showing the lowest. The underlying reason for this trend is believed to be the damage caused by free radicals to the cell membrane of the spermatozoa. According to Ayala *et al*. (2014), these radicals react with unsaturated fatty acids, leading to lipid peroxidation. The outcome of this process may hinder oxidative metabolism, impair intracellular enzymes, and damage the structure of the plasma membrane, particularly the acrosomal cap.

The 33-year storage group had a higher percentage compared to the other storage groups, thought to be due to the use of egg yolk in the diluter so that it could maintain the membrane and integrity of the spermatozoa acrosome cap. According to Tarig *et al*. (2017), during the process of freezing sperm cells, one effective method to protect sperm cells is by breaking the protein bonds from low-density lipoproteins (LDL) in egg yolk. In addition, egg yolk is a good source of long-chain polyunsaturated fatty acids (PUFA). Studies have shown that egg yolk can be added to diluters with various concentrations to aid in the production process of frozen semen. This is in line with Anzar *et al*. (2019), that the active ingredient responsible for protecting spermatozoa cells in the cryopreservation process is LDL found in egg yolk. Orrego *et al*. (2019), also stated that soluble and LDL egg yolk fractions may prevent sperm protein tyrosine phosphorylation during cooling from 17°C to 5°C, maintaining sperm quality during cryopreservation.

Based on the statistical analysis results, the average percentage value of chromatin maturity indicates that the storage groups for 33 years produced 79.50%, 30 years produced 82%, 27 years produced 83.50% and 24 years produced 85.5%. This suggests that there are significant differences in the chromatin maturity examination results between the storage groups of 33, 30, 27, and 24 years. The study also found a similar pattern between the results of chromatin maturity and chromatin integrity, where the longer the storage time, the lower the chromatin maturity and chromatin integrity. This is because chromatin maturity and chromatin integrity are interconnected.

Spermatozoa with mature chromatin will be clear in colour or light blue, while spermatozoa with immature chromatin will show a dark blue colour (Agudo-Rios *et al*., 2023). Sperm chromatin condensation is crucial for spermatogenesis and fertility, with various events interacting both inside and outside the nucleus to support chromatin changes (Okada, 2022) Sperm chromatin structure plays a complex role in oocyte fertilization and embryo development, with abnormalities potentially affecting male infertility and reproductive outcomes (Marchiani *et al*., 2020). Mammalian spermatozoa carry a small amount of cytoplasm that harbours a nucleus with chromatin hypercondensation. Chromatin is a protamine-binding protein (PRM) containing arginine and cysteine of male-specific DNA (Gill-Sharma *et al*., 2011).

Protamine (PRM) is the most abundant protein in the spermatozoa nucleus and has an important role during the spermiogenesis process, especially during the protein turnover process. Histone proteins which initially dominate the spermatozoa nucleus are replaced by protamines in a very complex process such as methylation, phosphorylation and ubiquitination (Pardede *et al*., 2020). Protamine will attach to and surround spermatozoa DNA to increase chromatin condensation. This functions to protect the genetic integrity of the male genome from damage caused by nuclear enzymes, mutagens and factors that can damage DNA (Balhorn *et al*., 2018), damage can result from factors including reducing the volume, concentration, viability and membrane integrity of bull sperm (Fortes *et al*., 2014).

2 types of protamine play a role in normal spermatozoa, namely protamine 1 and protamine 2. Protamine 1 is widely reported to be very dominant, Protamine 1 is composed of 50 amino acids with 3 main domains: a central arginine-rich domain; a high affinity for DNA, flanked by serine and cysteine residues domain; and the last containing threonine segments and several phosphorylation sites (Nagaki *et al*., 2022), PRM1 potentially crucial for sperm motility and PRM2 for DNA compaction (Arévalo *et al*., 2022). Protamine deficiency in sperm leads to oxidative stress-mediated DNA damage, impairing fertilization, and potentially requiring new treatment options for infertile men with impaired protamination (Schneider *et al*., 2020). DNA damage will inhibit embryo development, and reduce the ability to implant, resulting in pregnancy failure. Furthermore, it can cause damage to the membrane which will damage the acrosome and chromatin DNA (Dutta *et al*., 2021).

Toluidine Blue (TB) is used in spermatozoa DNA examination to observe changes in spermatozoa chromatin structure which is closely related to spermatozoa DNA stability (Erenpreisa *et al*., 2003). Toluidine Blue (TB) staining was used in this study, the same thing used by Rarani *et al*. (2019) to evaluate sperm nuclear chromatin condensation. The average percentage value of chromatin integrity, as determined by statistical analysis, indicates that the storage group generated percentages of 79.50%, 82.00%, 83.50%, and 85.50% over 33 years. This demonstrates that the percentage value of chromatin integrity for the 33, 30, 27, and 24-year storage groups differs significantly from one another.

From the 24 to 33-year storage group, there is an increase in the percentage of chromatin integrity. This is thought to be because the production process in the 24-year storage group is more modern and the

standard operating procedures applied are almost similar to the current production process. The same thing was expressed by Arif *et al.* (2020) who compared 3 types of stages of making diluters on frozen semen from Limousin bulls looking at the quality of the frozen semen that had been thawed. The stages in the mixing process and the division of equilibration time after the filling and sealing process affect the viability, plasma membrane integrity and AST levels in post-thawing frozen semen. Diluter mixing with 2 and 3 stages has the disadvantage of causing temperature changes when opening and closing the cooling cabinet during the second and third addition processes. Unstable temperatures damage semen (Khalil *et al*., 2018). Changes and osmotic pressure cause stress in semen which can result in molecular changes, DNA damage, changes in plasma membranes and increased ROS. Furthermore, it will reduce motility which will overall reduce semen quality (Ugur *et al*., 2019).

The nucleoprotein structure consists of protamine 1 (P1) protamine 2 (P2), and histones (5–15%) which cause damage to DNA during freezing and thawing. Protamines 1 and 2 disrupt disulfide bridges between cysteine residues. (Fortes *et al*., 2014). The size of this disorder depends on the type of protamine (P1 and P2) in the spermatozoa chromatin (Ribas-Maynou *et al*., 2021). The difference between protamine 1 and 2 is that protamine 1 is found in all species but protamine 2 is only in certain species, there are differences in the proportion (P1:P2), the content of the protamine, and the number of histones contained, an abnormality in protamine, frequently found in infertile males, is an increased P1/P2 ratio along with decreased levels of P2 and increased levels of P2 precursors (Rahiminia *et al*., 2020; Gosálvez *et al*., 2011). In addition, differences in temperature reduction, oxidative stress caused by the production of large amounts of ROS, and mechanical stress caused by cellular contractions that compact the chromatin in the genome can damage the plasma membrane during the freezing and thawing processes (Kopeika *et al*., 2015).

Using the Computer Assisted Sperm Analysis (CASA) IVOS-II to examine motility, the Leja counting chamber was preheated to 37°C before frozen Friesian Holstein (FH) semen was taken, up to 6 µL, and placed into it. The semen had been frozen and then thawed again in warm water for 30 seconds. 30 randomly selected fields of view containing a total of 1000 spermatozoa were examined. Taaffe *et al.* (2022) conducted an investigation on the semen of young FH bulls using the same methodology.

The 33-year storage group produced a percentage of 43.05%, 30 years of 44.90%, 27 years of 47%, and 24 years of 43.55%, according to the statistical analysis of the average Motility examination values. This indicates that no discernible differences were found in the motility examination results for the storage groups of 33, 30, 27, and 24 years. According to the National Standardization Agency's (BSN) quality standards for frozen cow semen, SNI 4869-1:2021, the percentage remains above 40% based on the results of the motility examination. SNI 4869-1:2021 states that frozen semen must meet specific requirements for post-thawing at a temperature of 37 to 38 degrees Celsius for 30 seconds. This includes a minimum progressive spermatozoa motility of 40%.

The number of progressively motile spermatozoa within an insemination straw positively correlates with the conception rate, and the progressive motility of bovine spermatozoa is linked to fertilization competence (Kogan *et al*., 2021). The motility of sperm is a critical factor in determining fertility outcomes. To improve the success rate of assisted reproductive technology, it is possible to enhance sperm motility using pharmacological agents and biomolecules (Dcunha *et al*., 2022). Spermatozoa with poor motility is considered one of the main factors contributing to male infertility. (Shahrokhi *et al*., 2020). The common methods for assessing male fertility involve computations and tests that yield the proportion of spermatozoa with forward motion (Berg *et al*., 2018).

According to Vyklicka and Lishko (2020), spermatozoa use their tails to propel themselves around the female reproductive system and fertilize the ovum. After being expelled, spermatozoa move swiftly and powerfully; capacitation is required to initiate the acrosome reaction and fertilization. The present study reveals that sperm undergo substantial protein modifications during the capacitation and acrosome reaction processes. These findings suggest that protein modification may play a significant role in sperm maturation as well as male infertility (Moghbeli *et al*., 2016; Castillo *et al*., 2019;). Low glucose conditions activate mitochondrial activity for ATP generation, inducing high-speed linear motility in sperm. Male fertility is linked to the crucial role of sperm mitochondria in providing energy for motility (Zhu *et al*., 2019; Thomas *et al*., 2021).

The statistical analysis of the average normal morphological examination values revealed that the percentage of normal sperm was similar across different storage groups as it sounds in Table 2. This implies that there were no significant differences in the average normal morphological examination results for the different storage groups. Similarly, the results of the statistical analysis of the average abnormal morphological examination values for the different storage groups also did not show any significant differences.

The results of the research indicate a discernable variation in the average number of normal and abnormal morphology sperm across each storage group, although there was no statistically significant difference. The observed trend in the research results shows a decline in the average number of normal morphology sperm in the 33 and 30-year storage groups, followed by an increase in the 27-year storage group, and subsequently another decrease in the 24-year storage group. It is worth noting that various factors can influence the quality of post-thawing sperm, leading to variations in its performance, such as the extender used, the dilution and freezing protocol, or the evaluation procedure. Therefore, it is crucial to thoroughly analyse and understand these discrepancies to accurately interpret the study results (Galián *et al*., 2023).

Based on the findings of a study about the effects of MDA on sperm morphology, it has found a positive correlation between higher MDA values and a higher prevalence of spermatozoa exhibiting morphological abnormalities. This trend was particularly evident in the 30-year storage group, as reported by Ammar *et al.* (2019), According to the study, sperm morphology problems, specifically those involving aberrant head and tail forms, were strongly correlated with high seminal MDA concentrations. Furthermore, the study provides evidence that the cytoplasmic membrane may be the principal structure causing spermatozoa morphological abnormalities and the main structure targeted by ROS in sperm.

Certain stages of embryonic development appear to be significantly influenced by the shape and structure of sperm (Nikolova *et al*., 2020). Furthermore, in both dairy and beef herds, there is a strong correlation between the percentage of morphologically normal bull sperm and days to conception and calf output (Perry, 2021). Furthermore, research on sperm morphology has shown that viable abnormal sperm and defective tails are associated with poor sperm motility (Feyisa *et al*., 2018), this is per the results of sperm morphology research which shows that the highest abnormality value in the tail is owned by the 30-year storage group.

There is a strong correlation between sperm morphology and chromatin integrity, especially in the fertilization rate (Unchupaico-Payano *et al*., 2022; Boe-Hansen *et al*., 2018). The findings of Banaszewska and Andraszek (2021) corroborate the notion that a higher proportion of sperm with morphological defects is positively associated with a rise in the percentage of chromatin instability. This implies that morphological defects in sperm can have grave implications for the stability of chromatin, which plays a significant role in the overall health and viability of the sperm. Another variable examined also affected sperm morphology, namely acrosome. Widyastuti *et al.* (2022) stated that the loss of intact acrosome causes an increase in primary morphological abnormalities in the sperm head.

The study's findings collectively underscore the criticality of meticulous handling and preservation techniques in maintaining the quality of frozen semen over extended storage periods. Moreover, the study emphasizes the interconnections between different parameters, such as the association between MDA levels, sperm morphology, and chromatin

integrity. Such insights are pivotal for optimizing protocols in assisted reproductive technologies and ensuring successful outcomes in fertility treatments.

Conclusion

Tthe present study's results provide significant evidence supporting the need for monitoring and maintaining the quality of frozen semen over extended storage periods. The study's findings have far-reaching implications for developing effective assisted reproductive technologies and ensuring successful outcomes in fertility treatments.

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Conflict of interest

The authors have no conflict of interest to declare.

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