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Research Article

Effect of Storage Periods on DNA Fragmentation of Post-Thawed Bali Bull Sperm

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Abstract | Deoxyribonucleic acid (DNA) is the genetic material carried by paternal and maternal lines that would be passed on to the offspring. The integrity of the spermatozoa cell DNA affects the success of embryo development. DNA damage carried by the paternal line has an impact on the syngamy process, in which a certain amount of male and female gamete chromosomes unite to continue the division process. This study aimed to determine the effect of storage periods in liquid nitrogen on DNA fragmentation of post-thawed semen of Bali bull. This research was conducted at the Laboratory of Animal Physiology and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada. Seventy-five semen straws produced from one bull were divided into 3 groups; each group consisted of 25 semen straws as long as 3, 6, and 8 months of storage in liquid nitrogen. The quality of thawed semen included motility, viability, abnormality, plasma membrane integrity, and DNA fragmentation were observed. The result showed that the sperm DNA fragmentation (%) which was stored for 3 months (6.33±0.38), 6 months (6.84±0.61), and 8 months (6.97±0.36) still in good conditions. Sperm motility, viability, and DNA fragmentation remained unaffected in different storage periods (p<0.05). Based on the result above, it could be concluded that storage period affects the abnormality and integrity of the plasma membrane, however, it does not affect the motility, viability, and DNA fragmentation of spermatozoa. The best quality of thawed frozen semen is obtained at 3 months of storage.

Keywords | Bali bull, DNA fragmentation, Liquid nitrogen, Spermatozoa, Storage periods

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INTRODUCTION

Artificial insemination (AI), is a reproductive technology that has been used in an attempt to improve genetic quality and increase animal productivity by using superior male semen (Widayati, 2023; Susilawati *et al.*, 2016). Based on research conducted by Isa *et al.* (2023) on the cattle in the Gorontalo Regency area, it is known that the success rate of artificial insemination (AI) which is conveyed through the service per conception (S/C), conception rate (CR), and non-return rate (NRR) values obtained sequentially, such as S/C 1.5, CR 54.78% and NRR 66% are still in the good category. The success rate of the Sapi Induk Wajib Bunting (SIWAB) program in Sangkub District, North Bolaang Mongondow Regency with the implementation of AI has different results, such as service per conception (S/C) 1.12%, and conception rate (CR) of 100% (Mahyun *et al.*, 2021). Differences in AI success rates are influenced

by several factors, such as the semen quality, acceptor cattle quality, accuracy of estrus detection, and the skills of the inseminator (Isa *et al*., 2023).

One of the successes of AI is supported by the quality of the inseminated semen (Prabowo *et al*., 2016; Mahfud *et al*., 2019). Fertility can be achieved if spermatozoa of frozen sperm have good motility, viability, morphology, and plasma membrane (Syauqy, 2014). According to Morell *et al*. (2017), sperm fertility depends on various factors and cannot be accurately predicted by examining only one aspect of sperm quality. Variables such as motility, viability, abnormality, plasma membrane integrity, and DNA fragmentation differ among bull breeds. Thus, assessing a single variable is insufficient to determine the fertility level of sperm cells for fertilizing egg cells. Frozen semen is stored at a temperature of -196˚C in a container filled with liquid nitrogen (Sukmawati *et al*., 2014). Cryopreservation with cryogenic temperatures can reduce or stop biological and chemical reactions in cells, making it possible to preserve cells in the long term without excessive cell damage (Jang *et al*., 2017). However, Malik *et al*. (2015) and Ardhani *et al*. (2020) reported that long-term storage causes decreased sperm quality in cattle.

Microscopic examination may remain the first stage of diagnosis which is insufficient to predict the overall ability of spermatozoa cells to attain embryo development (Nandre *et al*., 2011; Indrastuti *et al*., 2020). Molecular testing is needed to provide stronger predicting tools for sperm quality, namely by detecting the integrity of Deoxyribose-Nucleid acid (DNA) of spermatozoa cells (Erenpreisa *et al*., 2003; Prinosilova *et al*., 2012; Wright *et al*., 2014; Lone *et al*., 2017). Deoxyribonucleic acid (DNA) is the genetic material carried by paternal and maternal lines that would be passed on to the offspring (Chabiburrochman, 2022).

The integrity of spermatozoa cell DNA during fertilization influences the formation of pronuclei (Saili, 2006). The ability of DNA chromatin to form a pronucleus following the fusion of spermatozoa into the cytoplasm of the egg cell is the primary factor that influences fertilization success (Indrastuti *et al*., 2020). Spermatozoa cells release DNA chromatin, which causes modifications in the spermatozoa nucleus to form male pronuclei. In addition, spermatozoa cells that pass through the cytoplasm of the egg cell stimulate the second polar body to form female pronuclei. Male and female pronuclei will undergo a syngami phase in which the paternal and maternal chromosomes will unite to continue embryo development (Widayati, 2023). The level of DNA integrity of spermatozoa cells has a major impact on disorders of early embryonic development (Lone *et al*., 2017; Priyanto *et al*., 2019). The disruption of the syngamy process may affect how the female organism responds to the hormone release mechanism. Therefore, miscarriage and multiple mating cases in Indonesian cattle persist to be mentioned, one of which is theoretically assumed caused by sperm DNA damage. This study aimed to determine the effect of storage periods in liquid nitrogen on DNA fragmentation of post-thawed semen of Bali bull.

MATERIALS AND METHODS

Study period and location

The research was conducted from July to October 2023 at the Laboratory of Animal Physiology and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada.

Material

The Bali bull frozen semen utilized in this research was procured from commercial suppliers and has undergone quality assurance checks.

Thawing of spermatozoa

Straw-frozen semen was extracted from the container using tweezers and then thawed in a measuring cup filled with water at 37˚C for 30 seconds. Subsequently, the thawed frozen semen was transferred into a microtube (Kusumawati *et al*., 2016; Widayati and Pangestu, 2020).

Motility of spermatozoa

Dropped semen place on an object glass and covered with cover glass. Motility assessment was carried out based on the method Prabowo *et al*. (2023), sperm motility was subjectively assessed by examining it under a microscope at 40x magnification in several different object fields. Ratings are given in percentages on a scale of 0 to 100%.

Viability of spermatozoa

Sperm viability was assessed by the eosin-negrosin staining method. Live sperm would not absorb the stain of eosin-negrosin staining, but the dead sperm showed otherwise (Prihantoko *et al*., 2020). The procedure started by dropping 10 µL semen and mixing it with 50 µL eosin-nigrosin on an object glass, the smear was prepared using another object glass and followed to fixed with a bunsen burner. Sperm viability was assessed using a microscope at 40x magnification. The number of live and dead sperm from a total of 200 sperm cells was compared to determine sperm viability.

Abnormality of spermatozoa

Sperm abnormality was assessed by the eosin-negrosin staining method. The procedure started by dropping 10 µL semen and mixing it with 50 µL eosin-nigrosin on an object glass, smear was prepared using another object glass and followed to fixed with a bunsen burner. Sperm abnormality was assessed using a microscope at 40x magnification. The

number of sperm with abnormal morphology from a total of 200 sperm cells was compared to determine sperm abnormality (Cahyadi *et al*., 2016).

Plasma membrane integrity of spermatozoa

The sperm membrane integrity was assessed by modification method of hypo-osmotic swelling test (HOST) according to Prihantoko *et al*. (2022a) procedure started by 10 µL of semen diluted with 100 µL of HOST solution (a mixture of 0.9 fructose, 0.49 g of citrate sodium, and distilled water to a final volume of 100 ml) and incubated for 60 minutes at 37˚C. After incubation, the solution was smeared on the object glass with a cover slip followed by dried and fixed. Sperm with intact plasma membrane was assessed using a microscope at 40x magnification. The number of sperm with coiled tails (Figure 2) (positive to HOS solution) from a total of 200 sperm cells was compared to determine the sperm with an intact plasma membrane.

Sperm DNA fragmentation

Detection of sperm DNA fragmentation was carried out using the sperm-bos-halomax in vitro diagnostic kit. The principle of the diagnostic kit halotech, USA is to examine the differential decondensation of spermatozoa cell DNA chromosomes which is characterized by halo-shaped luminescence due to the release of chromatin in the head area of the spermatozoa cell indicates sperm intact DNA (Priyanto *et al*., 2016; Prabowo *et al*., 2023). The procedure started by diluting semen with phosphate-buffered saline (PBS) (ratio 1;1.5) followed by melting the agarose for 5 minutes at 93˚C. The agarose was incubated at 37˚C for 5 minutes in a waterbath. After incubation, mixed 20 µL semen and 40 µL agarose in a microtube. Placed the semen suspension on the object glass, then covered it with cover glass. Incubate the slide in the refrigerator for 8 minutes. After the agarose forms into a solid, slowly lift the cover glass then continue by dripping Lysis Solution (LS) into the agarose submerged, and incubate at room temperature for 5 minutes. After that, it was incubated in aquadest for three minutes. Subsequently, they incubated in 70% and 100% ethanol for a minute each. The preparation was stained using fluogreen staining, and sperm DNA fragmentation was examined using a laser-scanning confocal microscope at a wavelength of 300 nm.

Statistical analysis

Analysis of data quality Bali bull frozen sperm post-thawed was carried out with one-way analysis of variance (ANOVA) using the SPSS analysis tool, Duncan's multiple range test was used for treatment comparisons. The test was used to compare the mean values resulting from the various treatments at a significance level α value used is $p<0.05$ (5%).

RESULT AND DISCUSSION

The effect of the storage period of frozen semen in liquid nitrogen on sperm motility, viability, abnormality, plasma membrane integrity, and DNA fragmentation are present in Table 1. Based on the research result that was listed in Table 1, semen quality would decrease with the length of time stored in liquid nitrogen. The ability of progressive movement (motility) of spermatozoa cells is required to achieve successful fertilization in the female reproductive tract. Sperm motility is needed to reach the meeting location with the egg cell and penetrate the zona pellucida of the egg cell so that fertilization occurs. The difference in storage period showed no significant effect on the percentage of frozen semen sperm motility of Bali bull ($p<0.05$) (Table 1). The higher value of sperm motility was 45.53±2.92% in 3-month storage, meaning that the frozen semen was still in good condition for Artificial Insemination (AI). The frozen semen motility standard that is suitable to be used for insemination is not less than 40 (Zelpina *et al*., 2012; Adrefani *et al*., 2019; Ardhani *et al*., 2020; Asni *et al*., 2022).

Table 1: The value of the quality sperm of Bali cattle.

Variable	Storage period		
	3 Month	6 Month	8 Month
Motility (%)	45.53±2.92	45.33±3.33	45.20±2.11
Viability (%)	74.68±3.93	75.70±5.68	74.24±6.99
Abnormality (%)	9.92±1.99^{a}	12.92±1.55^{b}	15.26±2.68^{c}
Plasma membrane integrity (%)	74.82±2.43^{b}	72.54±5.04^{a,b}	69.44±6.25^{a}
DNA fragmentation (%)	6.33±0.38	6.84±0.61	6.97±0.36

^{a,b,c} different superscript in the same row show the significant difference ($p<0.05$).

Sperm viability affects the ability of sperm to survive in the female reproductive system until fertilization is reached. The higher value of sperm viability on the statistical test of the research showed a value of 6-month storage that was 75.70±5.68%, which may indicate that it is still acceptable for use in insemination and falls within frozen semen guidelines (Table 1). The sperm viability value of frozen semen, as reported by Handhayani *et al*. (2021) and Prihantoko *et al*. (2020), is still comparatively good, with a percentage of life reaching a value of >40 until 50%. It is known from the statistical test findings that the sperm viability of Bali bull was not significantly affected ($p<0.05$) by the length of time frozen semen was stored.

The existence of different storage periods does not affect the sperm motility and viability of Bali bull frozen semen, possibly because the semen is stored at extremely low temperatures reaching -196˚C at the cryopreservation

temperature or below the freezing point of liquid nitrogen which could cause the metabolic process of spermatozoa cells to stop, so that the majority enzymatic reactions and cellular functions slow down or stop. Cessation of cell metabolic processes can reduce the negative impact of increased production of lactic acid, which is the end product of metabolism so that would be maintained the quality of sperm. Silvestre *et al.* (2021) stated that cell metabolism stop in frozen semen, according to Shannon and Curson (1984), it is known that cell metabolic activity could decrease until it stops as the temperature becomes lower during semen storage. The high production of lactic acid by the end product of the metabolism mechanism would be toxic to the life of sperm cells because of a decreasing pH level (Triana, 2006; Savitri *et al.*, 2014; Pasyah *et al.*, 2021).

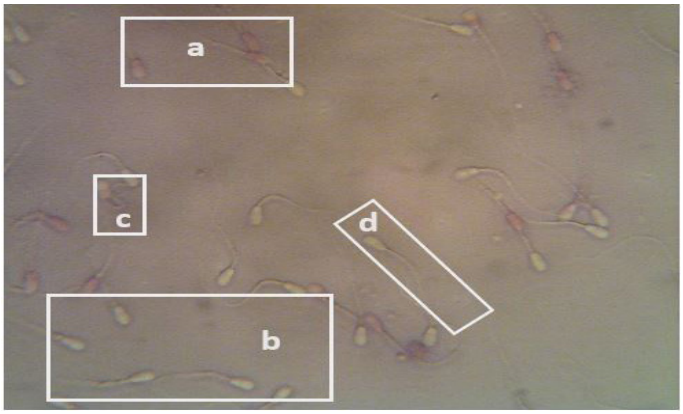

Figure 1: Viability and abnormality result using eosin-negrosin staining, spermatozoa in a red eosin showed dead sperm (a), while spermatozoa in a bright color showed viabil (b), sperm with morphology abnormal (c), sperm with normal morphology (d).

The difference in storage period in liquid nitrogen has a significant effect on the percentage of sperm abnormalities (Figure 1) of the frozen Bali bull semen (p<0.05) (Table 1). The lower percentage of sperm abnormalities showed a value of 9.92±1.99a%, which is still within the standard and suitable for insemination because it has a value of <20% following the standards for sperm abnormalities. A high number of sperm abnormal in the frozen semen to be inseminated could provide an opportunity for fertilization failure. Abnormal conditions that occur in the midpiece and endpiece may disturb the cell's metabolic process in producing the energy necessary to survive and sperm motility toward the egg cell. Furthermore, abnormal conditions in the sperm heads may interfere with the function of spermatozoa cells when they fuse with egg cells. Putri *et al.* (2015) stated that a high percentage of spermatozoa cell abnormalities can cause the cells to be unable to reach the fertilization site, fertilize the egg, or maintain the development of the early stages of the embryo. Ardhani *et al.* (2020) stated that the percentage of sperm abnormalities exceeding 30 to 35% indicates infertility.

The plasma membrane has a physiological function as a control for ion transport and prevents the entry of fluid outside the cells so that their integrity needs to be maintained (Prihantoko *et al.*, 2020). The result of the statistical test showed that differences in the storage period of frozen semen had a significant effect on the percentage of sperm plasma membrane integrity (p<0.05) (Table 1). The higher value of sperm membrane integrity was 74.82±2.43.%. The high percentage of plasma membrane spermatozoa of the Bali bull frozen semen found in this study was due to the reduced risk of oxidative stress in the frozen condition. Cellular respiration, which involves oxygen consumption and carbon dioxide production, practically stops at low temperatures. Sperm cells do not actively use oxygen or produce carbon dioxide, which helps prevent oxidative stress and cell damage. The extremely low temperature in liquid nitrogen can decrease and stop metabolic activity, which could inhibit the formation of Reactive Oxygen Species (ROS), which cause oxidative damage. Indriani *et al.* (2013) stated that the metabolic processes that continue to occur during storage are thought to be able to cause a decrease in the percentage of plasma membrane integrity due to an increase in Reactive Oxygen Species (ROS) originating from the remaining metabolic products of spermatozoa. Increasing ROS production would decrease the sperm membrane integrity therefore, cessation of metabolic activity has a large role in maintaining ROS production (Prihantoko *et al.*, 2022b).

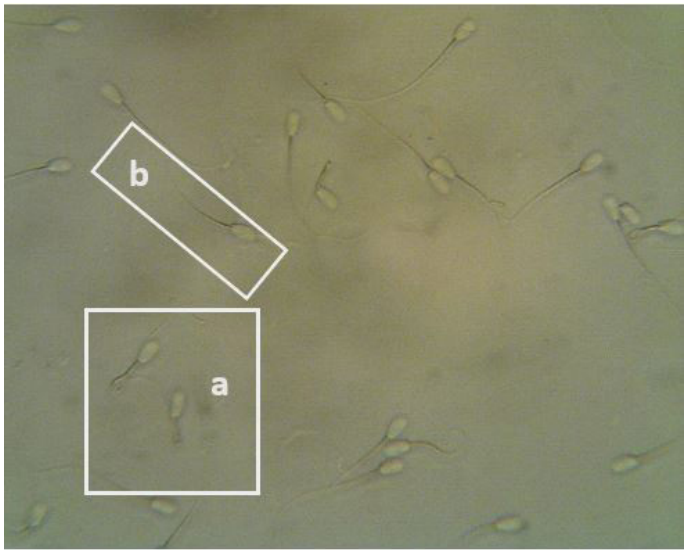

Figure 2: Sperm with coiled tail (positive to HOS solution) showed intact plasma membrane (a), sperm which has negative reaction to HOS solution showed damaged membrane (b).

Different storage times affected sperm abnormality and plasma membrane integrity (Table 1) of frozen Bali bull semen, possibly because the plasma membrane of spermatozoa cells is composed of lipids, carbohydrates,

and proteins. Spermatozoa have different membrane characteristics that are influenced by genetic factors. Esmaeili *et al.* (2015) stated that differences in lipid content in the plasma membrane of spermatozoa cells are one of the factors that can affect the cryotolerance and sensitivity of spermatozoa cells during storage at low temperatures, this is related to differences in the ratio of omega-3 and omega-6 concentrations in ruminant spermatozoa. The lipid content of different spermatozoa cell heads is controlled by hormones during spermatogenesis. Luteinizing Hormone (LH) and Adrenocorticotropin Hormone (ACTH) can change enzyme activity in the testes, resulting in changes in fatty acid composition to maintain fluidity and maturation of spermatozoa cells (de Catalfo and de Gomez Dumm, 2002; Esmaeili *et al.*, 2015). The process of cell cryopreservation can change the structure and function of membranes with low water content and high membrane fluidity (Hammerstedt *et al.*, 1990; Ugur *et al.*, 2019). Maintaining cell fluidity of spermatozoa during freeze storage in liquid nitrogen is necessary therefore plasma membrane integrity could protect intracellular spermatozoa.

The carbohydrate composition of the plasma membrane is necessary for maintaining the stability of the plasma membrane from the effects of intracellular and extracellular damage. Changes that occur in the carbohydrate concentration of spermatozoa cells during the cryopreservation process can reduce the integrity of the cell plasma membrane during storage (Di Santo *et al.* (2012); Hezavehei *et al.*, 2018). Changes in lipid, carbohydrate, and protein composition due to changes in osmotic pressure and ice crystal formation can disrupt the structure of the spermatozoa cell membrane in post-thawing frozen semen.

The presence of binders of sperm (BSP) such as BSP1, BSP3, and BSP5 protein profiles in bovine sperm cells can influence the stability and ability to maintain plasma membrane integrity during storage. Manjunath *et al.* (2007) stated that the BSP proteins are a family of phospholipid-binding proteins that make up the majority of the protein fraction (50-70) in bovine seminal plasma. During ejaculation, the BSP protein binds to the phospholipid choline in sperm membranes, causing alterations in the sperm plasma membrane through the stimulation of phospholipid and cholesterol efflux. Excessive exposure to BSP protein can damage the sperm membrane, disrupting its capacity to store sperm in frozen conditions. Binder of sperm BSP protein specifically binds to low-density lipoproteins found in egg yolk, compounds commonly used in semen thinners. The interaction between BSP and lipoproteins found in egg yolk can eliminate the detrimental effects of BSP protein on sperm membranes. Aslam *et al.* (2014) stated that a change in the polarity of the diluent used in spermatozoa cryopreservation can affect the stability of the spermatozoa membrane.

The addition of diluents in the process of making frozen semen is very necessary to maintain the quality of spermatozoa cells in optimal conditions. Diluents added in the process of making frozen semen should consider some of the side effects of interaction with spermatozoa cells while the semen is stored frozen. Royere *et al.* (1996) cit. Hezavehei *et al.* (2018) stated that cryoprotectants added to the diluent necessary to protect spermatozoa cells by lowering the freezing point of intracellular and extracellular water. The presence of cryoprotectants in the diluent is necessary to protect the spermatozoa from damage due to cold shock and induction of ice crystal formation during storage (Abdelhafez *et al.*, 2009; Hezavehei *et al.*, 2018). Based on the information obtained, it is known that the diluent composition used in the frozen semen in this research was glycerol and egg yolk tris. Glycerol and egg yolk tris were added to maintain sperm structure. This follows the statement by Hezavehei *et al.* (2018)stated that glycerol works by passing through the plasma membrane and replacing water in sperm cells therefore it can maintain intercellular spermatozoa after thawing. Glycerol entering the plasma membrane of spermatozoa cells functions to reduce intracellular water levels, therefore the formation of intracellular ice crystals is reduced. The correct concentration of added glycerol in the diluent is necessary to provide effective protection against spermatozoa (Wetzels, 1996). Based on this, it is known that the changes that occurred in the plasma membrane integrity of post-thawed Bali bull frozen semen in the study were influenced by the composition of lipids, carbohydrates, and proteins contained in the plasma membrane of Bali bull spermatozoa cells which were related to the level of cryotolerance at freezing point temperatures and accuracy in the concentration of the addition of the diluent provided.

Evaluation of sperm DNA damage in this study was carried out with the commercial kit DNA fragmentation and fluoro-green stain. The examination was carried out using a laser-scanning confocal microscope, the result was easy to analyze and produced a clearly halo-shaped luminescence (Figure 3). Following the statement of Priyanto *et al.* (2018) which stated that the test using commercial kit DNA fragmentation is more sensitive for determining DNA damage. The statistical analysis showed that different storage periods had no significant effect on the sperm DNA of Bali bull (p<0.05) (Table 1). Damage to the sperm DNA of Bali bull had a lower mean of 6.33±0.38% in 3-month storage, which was good. According to this study, DNA is not significantly affected

during storage due to the DNA sperm cell has a highly stable, compact, and dense chromatin. Widayati (2023) stated that during the maturation process, spermatozoa cells undergo chromatin condensation; the exchange of histone proteins and protamines maintains DNA integrity with a more compact structure.

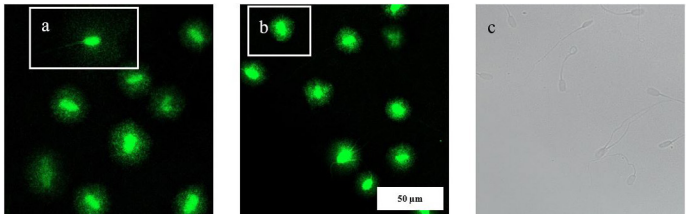

Figure 3: DNA Fragmentation results using the diagnostic commercial kit, spermatozoa without halo-shape indicated sperm DNA damage (a), while spermatozoa with halo-shaped luminescence due to the release of chromatin in the head area of the spermatozoa cell indicates integrated sperm DNA (b), spermatozoa on bright field microscope (c).

Damage to spermatozoa cell DNA can be caused by disruption of the spermatogenesis process, spermiogenesis, and exposure to oxidative stress. Disruption of the spermiogenesis process in the maturation process of spermatozoa in the epididymis can affect the ability of spermatozoa to form a compact chromatin structure. A compact chromatin structure is needed to maintain the integrity of spermatozoa cells against changes that occur during cryopreservation and storage in liquid nitrogen. Exposure to oxidative stress due to metabolic processes and exposure to oxygen can damage spermatozoa cell DNA. This is following the statement of Dutta *et al.* (2019) and Ismail *et al.* (2020) that oxidative stress conditions caused spermatozoa DNA damage. The high content of saturated fatty acids in the plasma membrane of spermatozoa cells makes spermatozoa cells susceptible to ROS. Excessive ROS levels could change sperm function, increase spermatozoa cell abnormalities in the midpiece, and DNA spermatozoa damage (Alhamar, 2019). Oxidation of DNA spermatozoa caused a high frequency of DNA single and double-strand breaks (Said *et al.*, 2010; Hezavehei *et al.*, 2018). Oxidative stress conditions could change the structure of nucleoproteins, disulfide bonds, and complexes protamine DNA (Johnston *et al.*, 2012; Hezavehei *et al.*, 2018). However, ROS production can be inhibited as long as the frozen semen is stored in liquid nitrogen at -196°C, the interaction with oxygen and cellular metabolic processes will be stopped. Therefore, different semen storage periods did not have a significant effect on the level of DNA damage in this study.

In addition, the addition of antioxidants to the semen diluent is also one of the factors that cause spermatozoa cells to remain in good condition despite being stored for a long time in liquid nitrogen. Antioxidants have an important role in inhibiting oxidation reactions in stored frozen semen. Antioxidants can bind free radicals that can cause damage to spermatozoa by filling the space on unpaired ROS electrons. This is following the statement of Ismail *et al.* (2020) which states that the addition of antioxidants to cement diluents can maintain the stability of cell DNA chromatin. Morell *et al.* (2018) added that ROS production will be inhibited in post-thawing frozen semen with the addition of antioxidants, and stop metabolic activity in frozen semen stored in liquid nitrogen. Therefore, the different diluents applied to semen affect cell survival during storage, especially in DNA and mitochondrial fragmentation.

The resilience of spermatozoa cells in maintaining DNA integrity is also related to differences in the expression of spermatozoa cell protein profiles. Heat-Shock Protein 90 (HSP90) is a protein profile found in spermatozoa cell DNA. Heat-Shock Protein 90 (HSP90) has a function in the DNA repair system. DNA repair mechanisms are indispensable during the cryopreservation process to maintain its integrity. Therefore, the expression of different HSP90 protein profiles may affect the level of DNA integrity of spermatozoa cells during storage. This is following the meta-analysis conducted by Ugur *et al.* (2019) which states that DNA damage in spermatozoa cells can be influenced by a decrease in the number of DNA repair enzymes in spermatozoa cells. The durability of DNA integrity during cryopreservation at low temperatures are influenced by genetic factors (Morell *et al.*, 2018). This is related to cell plasma membrane integrity and spermatozoa cell protein expression (Memili *et al.*, 2020). Differences in the composition and level of protein expression in spermatozoa cells are related to differences in the ability of spermatozoa cells to survive at low temperatures. Heat-Shock Protein 90 expression in semen is associated with spermatozoa cell cryotolerance, high HSP90 expression indicates a positive reaction to cell survival during storage (Wang *et al.*, 2014). Therefore, this could explain that genetic differences may result in differential expression of protein profiles on cryotolerance during semen storage in liquid nitrogen.

Evaluation of the detection of DNA damage to spermatozoa cells is very important therefore the success of artificial insemination in embryo development could be achieved. According to a study by Sedó *et al.* (2017), DNA fragmentation should be considered when evaluating semen quality. The extent of DNA damage may indicate the degree of blastulation during embryonic development. According to a meta-analysis review by Kopeika *et al.* (2015), fragmentation in the chromatin structure of spermatozoa is associated with miscarriage rates but does not affect fertilization success. Spermatozoa DNA damage

at the 10% level is known to be repaired by the oocyte repair system after fertilization.

CONCLUSIONS AND RECOMMENDATIONS

Storage period affects the abnormality and integrity of the plasma membrane, however, it does not affect the motility, viability, and DNA fragmentation of spermatozoa. The best quality of thawed frozen semen is obtained at 3 months of storage. To determine the cryotolerance of spermatozoa, it is recommended to conduct a specific protein analysis. Heat-Shock Protein 90 is one of the candidates that can be used for cryotolerance research.

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NOVELTY STATEMENT

The current study shows the effect of storage period on the level of DNA damage of Bali bull spermatozoa stored in liquid nitrogen. This finding is important for semen quality control in AI activities in the society to reduce pregnancy failure due to DNA damage.

AUTHOR'S CONTRIBUTION

ANB contributed to the original ideas of the research, research design, data collection, analysis, interpretation, and manuscript writing. NAM and SBF contributed to the research design and data collection. KDP contributed to the research design, data collection, and analysis, manuscript preparation, and reviewed the manuscript. DM contributed to the statistical analysis, supervised the study, research preparation, and manuscript preparation. DTW contributed to the research ideas, supervised the study, coordinated the research funding, data collection, and manuscript preparation, and reviewed the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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