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Effect of Thawing Technique on the Quality and Status of Acrosome Reactions

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ABSTRACT

The success of Artificial Insemination is affected by the thawing technique used by the inseminator. The difference in thawing technique can affect the quality of the spermatozoa produced. This study aims to determine the effect of temperature and duration of thawing on the frozen semen quality of Bali and Madura cattle. The material used was 80 frozen semen straws for Bali cattle and Madura cattle. The method used is an experiment with four treatments and 10 replications, that is thawing in water at 37°C for 30 seconds duration, 28°C for 30 seconds, 28°C for 45 seconds and 28°C for 60 seconds. The variables observed in this study were capacitation, intact acrosome, intact plasma membrane and chromatin damage. The data were analyzed using a Randomized Block Design with replication as a block. The results of the analysis of variance showed that thawing technique had significant effect ($p<0.05$) on not yet capacitated, intact acrosome, intact plasma membrane. Thawing technique had no significant effect ($p>0.05$) on the acrosome reacted and chromatin damage.

Keywords: thawing technique, capacitation, intact acrosome, intact plasma membrane, chromatin damage

INTRODUCTION

Bali cattle and Madura cattle are plasma nutfah cattle in Indonesia that have the potential to be empowered as supporters of domestic meat needs. Bali cattle and Madura cattle have advantages, including being able to adapt to hot temperatures, maintenance patterns as they are with low feed quality, fast growth rates, and good reproductive performance (Susilawati, 2017). According to the Ministry of Agriculture, Directorate General of Livestock and Animal Health (2021), the solution provided by the government is the Sikomandan program or the State Mainstay of Commodity Buffalo Cattle which was launched in early 2020. The program is to increase the production of cattle and buffalo in a sustainable manner by utilizing livestock reproduction technology through artificial insemination (AI) technique using frozen semen in bulk. However, this technique is not always successful, pregnancy failure often occurs due to many factors that affect the success rate, one of which is the factor of the inseminator in thawing frozen semen. Thawing in question is the re-thawing of frozen semen using a certain medium and duration so that it can be used properly for evaluation and for artificial insemination (Adnyani et al., 2018). The cooling, freezing and processes thawing cause cold shock and heat shock effects that affect membrane stability, reduce viability and ability to fertilize spermatozoa (Khalil et al., 2018). Frozen semen thawing can damage spermatozoa caused by changes in temperature and stress due to differences in osmotic pressure (Hezavehei et al., 2018).

The National Standardization Agency, namely SNI 4869-1:2017 special requirements for thawing frozen semen using water temperature 37°C - 38°C for 30 seconds, however inseminators often use water with a temperature of 28°C because it is easier to obtain than having to carry warm water. The results of the research of Indriastuti et al (2020) stated that the temperature of thawing 37°C for 30 seconds showed a motility value of 69.37± 0.41%. Salim et al., (2012) stated that the technique thawing has an effect on the motility and viability of frozen semen of Bali cattle, Madura cattle and PO cattle, with a temperature of thawing 37°C and a duration of 15 seconds which has the best quality in each breed. The opinion of Kurniawan et al. (2021) with a temperature of 26°C for 5, 15, 30, 60, 90, 120 seconds gave the best results in the 60 second thawing treatment, namely individual motility 68.27% and viability 93.28%. The variation in the quality of frozen post-thawing semen indicates the absence of a technique thawing that provides optimal results for achieving fertilization using frozen semen. The ability to formulate the technique thawing through variations in temperature and duration greatly determines the quality of frozen post-thawing semen, especially the technique used by the inseminator in the field.

MATERIAL AND METHOD

Location and Research

This research was conducted on August 3th - September 4th, 2021 at the animal reproduction laboratory, Faculty of Animal Science, Universitas Brawijaya.

Research *Material*

This study used 80 frozen semen straws from Bali bull and Madura bull obtained from BBIB Singosari Malang. Materials and equipment used in this study include warm water at 37°C, water at 28°C, eosin-negrosin, light microscope, hand tally counter, container, tweezers, timer, water bath, thermometer, ose, object glass, cover glass, scissors, mini tube, micropipette (58µl-1000µl).

Research *Procedure*

The frozen semen of Bali bull and Madura bull was thawed in a water bath that had been temperature adjusted according to the treatment, then put the cement into an eppendorf tube. The semen quality test was carried out which included observing the integrity of the membrane, intact acrosome, chromatin damage and observing the reaction of the acrosome with Chlortetracycline (CTC).

Research Methods

The research used a laboratory experimental method with 4 treatments with 10 replications and a laboratory experiment using a Randomized Block Design. The treatments of this study were as follows: P0: thawing in water at 37°C for 30 seconds duration, P1: 28°C for 30 seconds, P2: 28°C for 45 seconds and P3: 28°C for 60 seconds.

Research Variables

The variables observed on this research were capacitation, plasma membrane, the acrosome intact and chromatin damage post thawing.

Data Analysis

The data were analyzed with a variety analysis (ANOVA) and using a randomized block design with replication as a block.

RESULT AND DISCUSSION

Percentage of Spermatozoa Capacitation and Acrosom Reacted after Thawing

Acrosome reacted is a continuation of physiological events in spermatozoa by eliminating decapacitation factors so that they can penetrate the zona pellucida. The averages spermatozoa and uncapacitated acrosome reacted after thawing in Bali cattle and Madura cattle are presented in a graph as shown in Figures 1 and 2.

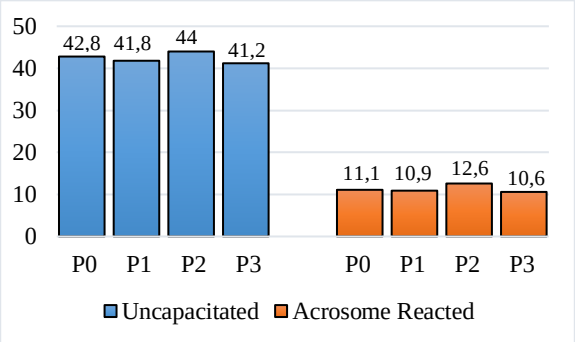

Figure 1. Averages spermatozoa capacitation and acrosom reacted post thawing in Bali Cattle

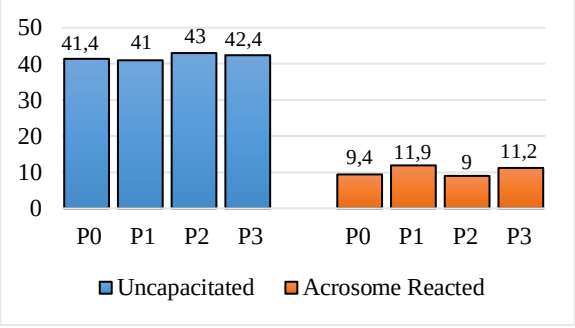

Figure 2. Averages spermatozoa capacitation and acrosom reacted post thawing in Madura Cattle

Figures 1 and 2 show that the percentages of spermatozoa uncapacitated and acrosome reacted after thawing give different results at all levels of treatment in all of cattle, but the percentage number are not much different. The results showed that in all treatments, more than 40% of spermatozoa had uncapacitated and less than 13% had acrosome reacted in the two of cattle. This condition is suspected because the spermatozoa have undergone early capacitation due to the cooling and freezing process, so that the reduced seminal plasma causes the spermatozoa membrane to change.

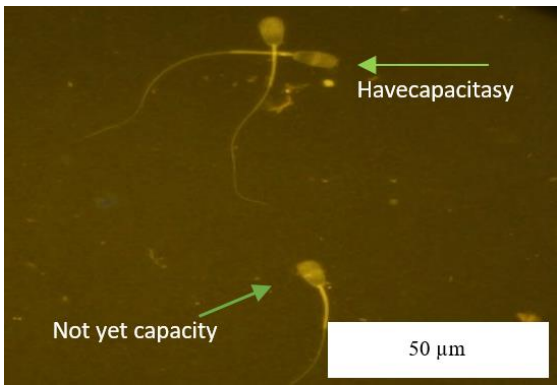

Figure3. Spermatozoa uncapacitated

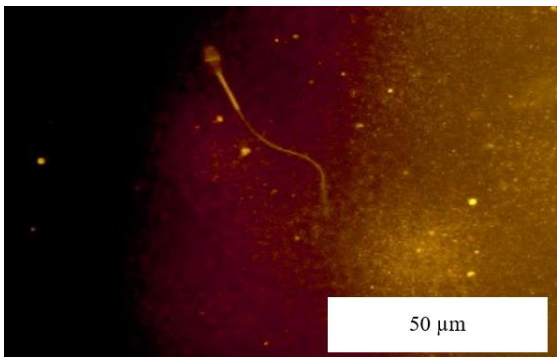

Figure 4. Spermatozoa acrosom reacted

The results of statistical analysis of the influence of temperature and length of *thawing* time on the frozen semen spermatozoa of Bali cattle and Madura cattle are presented in Tables 1 and 2. The results of the analysis of variance showed that temperature and duration thawing had a significant effect ($p<0.05$) on uncapacitated frozen semen in Bali cattle and Madura cattle. The results of descriptive statistics based on the average value ± standard deviation showed that frozen semen had the highest capacitation, in treatment P2 with a temperature of thawing 28ºC and a duration of 45 seconds in Bali cattle and Madura cattle, while frozen semen had the lowest capacitation in P3 treatment with temperature of thawing 28ºC and duration of 60 seconds in Bali cattle and P1 treatment with temperature of thawing 28ºC and duration of 30 seconds in Madura cattle. This is because the spermatozoa in all frozen semen in the two breeds of cattle had early capacitation due to the freezing and processes thawing which caused membrane dysfunction due to reduced seminal plasma.

The results of the analysis of variance showed that temperature and duration thawing no significant effect ($p>0.05$) on frozen semen for the acrosome reaction in Bali cattle and Madura cattle. Descriptive statistical results based on the mean ± standard deviation showed the highest acrosomal reaction frozen semen, in treatment P2 with a temperature of thawing 28ºC and a duration of 45 seconds in Bali cattle and P1 treatment with a temperature of thawing 28ºC and a duration of 30 seconds in Madura cattle, while the lowest acrosomal reaction frozen semen was in treatment P3 with a temperature of thawing 28ºC and a duration of 60 seconds in Bali cattle and P2 treatment with a temperature of thawing 28ºC and a duration of 45 seconds in Madura cattle. This means that treatment and breed do not affect the acrosome reacted on spermatozoa in thawing. Radomi et al., (2011) stated that cryopreservation processes including dilution, cooling, packaging and freezing significantly affect viability, motility and capacitation. It was added (Pamungkas et al., 2005; Salim et al., 2012) that cooling, freezing and thawing greatly affect the stability and function of the membrane.

Spermatozoa that have capacitation and hyperactivation will be an acrosomal reaction. The acrosome contains several enzymes, including hyaluronidase which functions to destroy cumulus osphorus, corona penetrating enzyme which functions to destroy the corona radiata (the layer of follicular cells above the zona pellucida layer), and acrosin which functions to destroy the zona pellucida (Sumarmin, 2018). Acrosomal reaction occurs before the spermatozoa reach the site of fertilization, the spermatozoa will lose the ability to fertilize the oocyte.

Table1. Average percentage of spermatozoa uncapacitated

No	Breed	Treatment			
		P0	P1	P2	P3
1	Bali cattle	42.80 ± 0.92 ^{bc}	41.80 ± 0.79 ^{ab}	44.00 ± 0.94 ^{c}	41.20 ± 0.92 ^{a}
2	Madura cattle	41.40 ± 0.97 ^{ab}	41.00 ± 0.94 ^{a}	43.00 ± 0.94 ^{c}	42.40 ± 0.97 ^{bc}

Note: P0: thawing in water at 37°C for 30 seconds duration, P1: 28°C for 30 seconds, P2: 28°C for 45 seconds and P3: 28°C for 60 seconds, Different notations on the same line show a noticeable difference ($p<0.05$)

Table2. Average percentage of spermatozoa acrosom reacted

No	Breed	Treatment			
		P0	P1	P2	P3
1	Bali cattle	11.10 ± 4.86 ^{a}	10.90 ± 2.18 ^{a}	12.60 ± 3.03 ^{a}	10.60 ± 3.10 ^{a}
2	Madura cattle	9.40 ± 3.24 ^{a}	11.90 ± 4.91 ^{a}	9.00 ± 3.02 ^{a}	11.20 ± 2.49 ^{a}

Note: P0: thawing in water at 37°C for 30 seconds duration, P1: 28°C for 30 seconds, P2: 28°C for 45 seconds and P3: 28°C for 60 seconds, Different notations on the same line show a noticeable difference ($p<0.05$)

Intact Akrosom

An acrosome hood is a sheath found on the head of the spermatozoa that serves to protect the release of genetic material and the enzyme hyaluronidase from the head of the spermatozoa. The average percentage of the wholeness of the spermatozoa acrosome hood after *thawing* on the four types of treatment in the two breeds of cattle as in Figure 5.

Figure 5 shows that there is an average difference in the percentage of the integrity of the spermatozoa intact acrosome in all four types of treatment. The highest average percentage of the wholeness of the spermatozoa acrosome hood is in the second treatment. The higher the percentage of intact spermatozoa acrosome hood, fertility in frozen semen will increase. This condition is caused if the temperature *thawing* is appropriate. Sitepu and Marisa research (2021) states that the acrosome head of spermatozoa can be damaged due to *cold shock* and the presence of bacteria in frozen semen that can reduce the quality of frozen semen. Spermatozoa whose acrosome head is damaged will also not be able to fertilize the egg because the spermatozoa ability to enter the egg is determined by the head of the spermatozoa acrosome.

The results of statistical analysis of the influence of temperature and length of thawing time on concentration on each frozen semen of Bali cattle and Madura cattle are presented in Table 3.

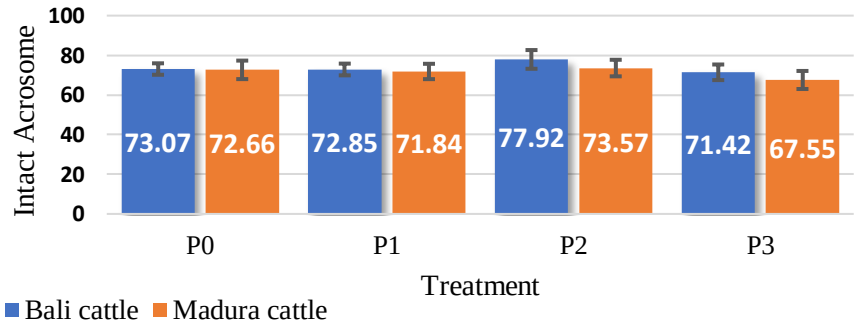

Figure 5. Average percentage of intact acrosome

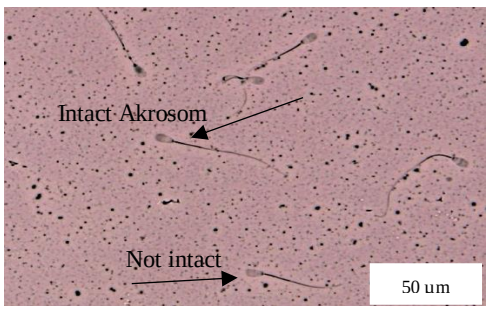

Figure 6. Intact akrosom

Table 3. Average percentage of acrosome integrity of spermatozoa at various levels of treatment in both types of cattle

No	Breed	Treatment			
		P0	P1	P2	P3
1	Bali cattle	73.07 ± 2.88[a]	72.85 ± 2.96[a]	77.92 ± 4.73[b]	71.42 ± 3.94[a]
2	Madura cattle	72.66 ± 4.67[b]	71.84 ± 3.87[ab]	73.57 ± 4.18[b]	67.55 ± 4.55[a]

Note: P0: thawing in water at 37°C for 30 seconds duration, P1: 28°C for 30 seconds, P2: 28°C for 45 seconds and P3: 28°C for 60 seconds, Different notations on the same line show a noticeable difference ($p<0.05$)

The results of the analysis of variance showed that temperature and duration thawing had a significant effect ($p<0.05$) on the intact acrosome of frozen semen in Bali cattle and Madura cattle. Descriptive statistical results based on the average value ± standard deviation showed the highest frozen intact acrosome was in treatment P2 with a temperature of thawing 28º C and a duration of 45 seconds in Bali cattle and Madura cattle breeds, while the lowest frozen intact acrosome was in treatment P3 with a temperature of thawing 28ºC and a duration of 60 seconds in Bali cattle and Madura cattle. This shows that temperature and duration thawing the appropriateare neither too low nor too high. Frozen semen suitable for use in the AI program must have a motility percentage of at least 40% (Hafez and Hafez 2000), and a minimum TAU percentage of 30% (Evans & Maxwell, 1987; Rizal & Herdis, 2005). The average research result is 70 percent, which means that semen is suitable for Artificial Insemination.

The implementation of thawing from the frozen zone to the liquid zone was carried out by increasing the temperature gradually so that the thawing of the entire straw could be perfect so that the adhesion of the cell membrane freezing was not damaged. The plasma membrane is a part of spermatozoa which plays a very important role in the protection of cell organelles. Damage to the intact plasma membrane is usually accompanied by damage to the organelles of intact acrosomal cells, causing the release of enzymes needed during the fertilization process (Arvioges et al., 2021). The gradual decrease in temperature treatment can function to maintain the binding of the lipoprotein envelope on the spermatozoa membrane so that the plasma membrane remains stable when passing through the critical temperature zone.

Intact Plasma Membrane

The integrity of the spermatozoa membrane was observed by Swelling Test (HOS Test) based on the ability of the spermatozoa to swell after being put into a hypoosmotic solution (Susilawati, 2013). The functional integrity of a good spermatozoa membrane must be supported by a good structural integrity of the spermatozoa membrane. The membrane on the head of the spermatozoa functions during capacitation, acrosomal reactions and penetration of the zona pellucida during fertilization, while the membrane on the tail functions to obtain energy substrates used for movement (Rodiah et al., 2015). The average percentage of spermatozoa membrane integrity after thawing in the four types of treatment in the two breeds of cattle is shown in Figure 7.

Figure 7. Shows that the average number of intact spermatozoa membrane integrity from the four treatments is different. The highest average score was obtained in the second treatment for all breeds of cattle. This result is comparable to the mean value obtained in the acrosomal hood integrity test. The integrity of the plasma membrane is an absolute must for spermatozoa, the plasma membrane plays an important role in regulating all processes that occur in the cell (Rizal et al., 2003; Nofa et al., 2017). The percentage of intact plasma membrane of spermatozoa in both breeds of cattle was categorized as good. Damage to the intact plasma membrane is usually accompanied by damage to the organelles of intact acrosomal cap cells, causing the release of enzymes needed during the fertilization process (Arvioges et al., 2021).

The results of statistical analysis of the influence of temperature and length of *thawing* time on the whole plasma membrane of spermatozoa frozen semen of Bali cattle and Madura cattle are presented in Table 4.

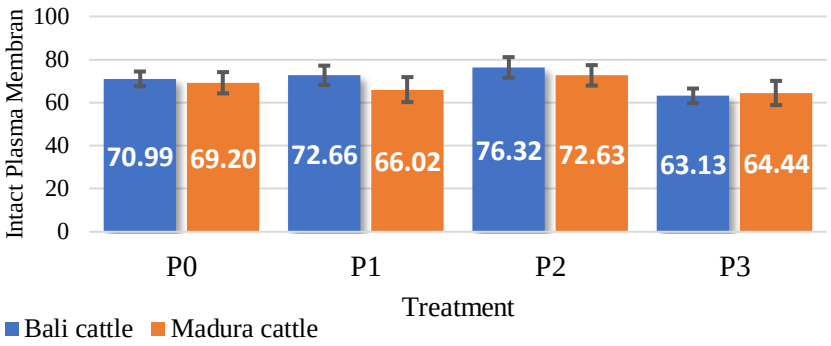

Figure 7. Membrane integrity

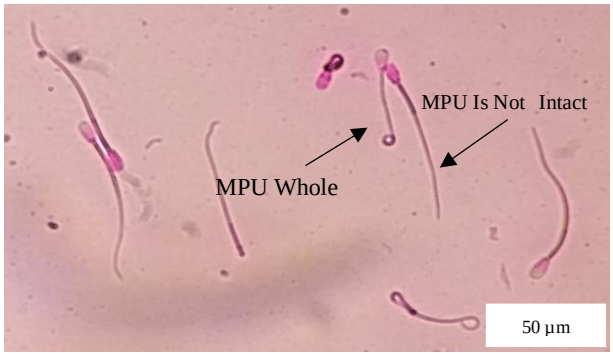

Figure 8. Integrity of Intact Membrane

Table 4. Percentage of Spermatozoa Plasma Membrane Wholeness at Various Levels of Treatment in both types of cattle (percent)

No	Breed	Treatment			
		P0	P1	P2	P3
1	Bali cattle	70.99 ± 3.44 [b]	72.66 ± 4.49 [bc]	76.32 ± 4.79 [c]	63.13 ± 3.39 [a]
2	Madura cattle	69.20 ± 4.95 [ab]	66.02 ± 5.81 [a]	72.63 ± 4.76 [b]	64.44 ± 5.62 [a]

Note: P0: thawing in water at 37°C for 30 seconds duration, P1: 28°C for 30 seconds, P2: 28°C for 45 seconds and P3: 28°C for 60 seconds, Different notations on the same line show a noticeable difference ($p<0.05$)

The results of analysis of variance showed that temperature duration thawing had a significant effect ($p<0.05$) on the integrity of the frozen semen membrane in Bali cattle and Madura cattle. Descriptive statistical results based on the average value ± standard deviation showed the highest frozen semen membrane integrity was in treatment P2 with a temperature of thawing 28ºC and a duration of 45 seconds in Bali cattle and Madura cattle breeds, while the lowest frozen semen membrane integrity was in P3 treatment with temperature of thawing 28ºC and duration of 60 seconds in Bali cattle and Madura cattle. This shows that a temperature of 28°C for 45 seconds is a suitable temperature for thawing.

The percentage of viable spermatozoa is determined by the intact plasma membrane. The plasma membrane of spermatozoa serves to protect spermatozoa organelles and transport electrolytes for spermatozoa metabolism (Salmah, 2014). Damaged plasma membranes can affect the physiological function and metabolism of spermatozoa, causing spermatozoa to die (Butarbutar, 2009). Rodriguez et al. (2005), reported that theprocess of thawing frozen bovine semen at 37°C for 60 seconds caused some damage to the spermatozoa membranes. Ansary et al., (2010) reported the highest motility, viability and membrane integrity, thawing in water at 37°C for 30 seconds. The difference in the quality of frozen post-thawing semen indicates that thawing at different temperatures and durations has a different effect on the quality of frozen semen. If the temperature is thawing too low, it will slow down the breakdown of straw snow, resulting in damage to the components that make up the spermatozoa membrane. Anwar et al. (2015) cell membranes function as active transport and have macromolecule

as nutrient metabolism which is used as the movement or life of progressively active spermatozoa.

Effect of Temperature and Duration *Thawing* Frozen Semen Bali Cattle and Madura Cattle on Chromatin Damage

DNA testing using Toluidine Blue (TB) in principle evaluates the chromatin structure of DNA. Toluidine Blue is a DNA stain used to evaluate the chromatin integrity of spermatozoa by detecting the presence or absence of broken disulfide bonds in the chromatin, so that damaged spermatozoa DNA will be dark blue and intact will be bright blue (Kim et al., 2013). The average percentage of spermatozoa chromatin damage after thawing in the four types of treatment in the two breeds of cattle is shown in Figure 9.

Figure 9 shows that the average number of spermatozoa chromatin damage from the four treatments was different. The results of this study showed that the average DNA damage in the two breeds of cattle was less than 6%, this indicates that frozen *post-thawing* semen is still suitable for insemination. According to Karoui *et al.*, (2012), DNA damage that exceeds 7% to 10% in FH cattle, interferes with fertility. 10.34% DNA damage in frozen semen of cattle *White Holstein* was reported by Bochenek and Smorag (2010) meanwhile, Nandre et al. (2011) reported the presence of sperm DNA damage of 3.00% and 4.61% in buffalo before and after freezing in winter. DNA damage in summer was 7.61% and 13.61%, respectively.

The results of statistical analysis of the influence of temperature and length of *thawing* time against the damage to chromatin spermatozoa frozen semen of Bali cattle and Madura cattle are presented in Table 5.

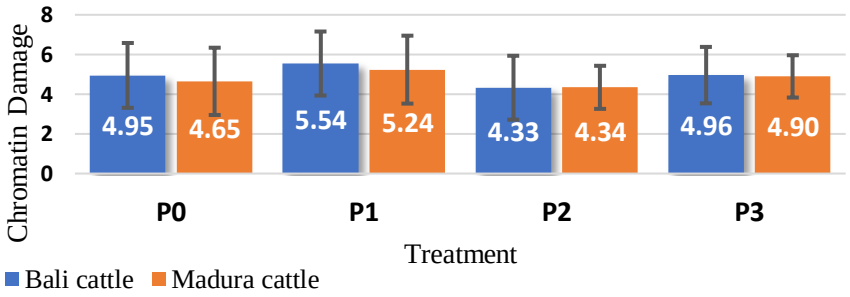

Figure9. Average Percentage of Spermatozoa Chromatin Damage

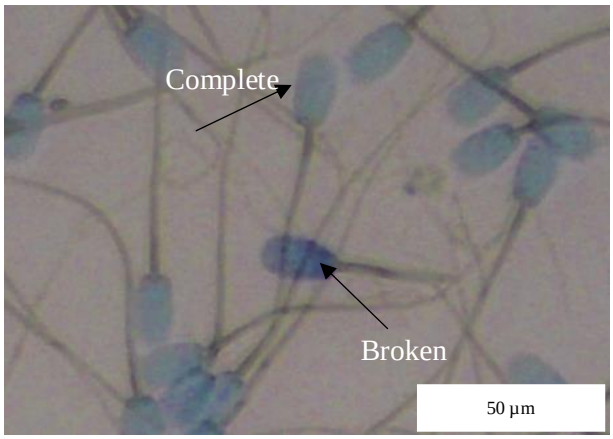

Figure 10. Damage to Chromatin Spermatozoa

Table5. Average percentage of damage chromatin spermatozoa at various levels of treatment in both types of cattle

No	Breed	Treatment			
		P0	P1	P2	P3
	Chromatin Damage (DNA) Frozen Semen				
1	Bali cattle	4.95 ± 1.63[a]	5.54 ± 1.61[a]	4.33 ± 1.61[a]	4.96 ± 1.42[a]
2	Madura cattle	4.65 ± 1.70[a]	5.24 ± 1.71[a]	4.34 ± 1.09[a]	4.90 ± 1.07[a]

Note: P0: thawing in water at 37°C for 30 seconds duration, P1: 28°C for 30 seconds, P2: 28°C for 45 seconds and P3: 28°C for 60 seconds, Different notations on the same line show a noticeable difference ($p<0.05$)

The results of analysis of variance showed that temperature and duration thawing did not have a significant effect ($p>0.05$) on chromatin damage in Bali cattle and Madura cattle. The results of this study showed that the average DNA damage in the two breeds of cattle was less than 6%, this indicates that frozen post-thawing semen is still suitable for insemination. This is in accordance with the statement of Ardhani et al. (2020) that the DNA damage of Bali cattle spermatozoa showed an average yield of 1.60±0.21%. These results mean that Bali cattle semen is eligible for artificial insemination because the spermatozoa DNA is located on the inside of the spermatozoa, its position is protected by the plasma membrane and acrosome. This is in accordance with the opinion of Priyanto (2014) that DNA damage or decrease occurs when there has been damage to the plasma membrane and acrosomal hood that affects the DNA integrity of spermatozoa.

The quality of chromatin in the cell nucleus greatly determines the status of DNA that is tightly bound to protamine which functions as a protector for nuclear DNA (Saili et al., 2006). The process thawing is one of the critical points for DNA damage to spermatozoa. When thawing, spermatozoa will be extreme temperature changes causing cell damage, decreasing motility, viability, plasma membrane integrity, and damaging spermatozoa DNA.

CONCLUSION

It can be concluded that the results of the four treatments are above the Indonesian National Standard (SNI). Liquefaction using water temperature 28°C with durasi 45 seconds is the best methodto get the quality ofspermatozoa post thawing.

CONFLICT OF INTEREST

The authors whose names are listed have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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