EFFECTS OF STORAGE DURATION ON MITOCHONDRIAL ACTIVITY AND DNA FRAGMENTATION OF POST-THAWED SPERMATOZOA FROM SEVERAL ONGOLE GRADE BULL IN INDONESIA

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ABSTRACT

Several recent studies theoretically assumed that storage of frozen doses of semen in liquid nitrogen guarantees unlimited sperm functionality. However, several studies concluded that long-term storage causes decreased sperm quality. The purpose of this study was to evaluate the storage duration effect on mitochondrial activity and DNA integrity of Ongole Grade Bull post-Thawed spermatozoa from several Artificial Insemination Centre (AIC) in Indonesia. In this study, Ongole Grade Bull frozen semen that has been stored up to three years were investigated. According to the duration of storage, semen samples were divided into 7 groups (one month, three months, six months, nine months, one year, two years, and three years). This study used 42 straws, with 6 straws for each group were used. Samples were evaluated for sperm motility, viability, membrane integrity, mitochondria activity, and DNA fragmentation. The results showed that one-month group sperm motility was (53.80%), viability (72.75%) membrane integrity (76.35%), mitochondria activity (64.24%), and DNA fragmentation (3.82%). After 3 years of storage, the corresponding values were 39.52%, 41.56%, 48.25%, 49.12%, and 4.62%. Sperm motility and viability remained unaffected up to 6 months of storage, while membrane integrity started decreasing at 3 months (P<0.05). Mitochondria activity decreased during the 9th months (P<0.05). In contrast to the previous results, DNA fragmentation did not increase significantly after 3 years of storage (P>0.05). Thus, post-thawed motility, viability, and membrane integrity of spermatozoa from Ongole Grade Bulls started decreasing at 6 months of storage, mitochondria activity started decreasing after 9 months, while sperm DNA integrity remained unaffected for up to three years of storage.

Key words: Ongole Grade Bull, frozen semen, storage, mitochondria activity, DNA fragmentation

Introduction

Artificial insemination (AI) is an application of reproductive technology that aims to improve genetic quality and to increase the population of livestock. These goals can be achieved if supported by good quality semen because the success of AI is largely determined by the quality of frozen semen, factors affecting female fertility, and the skills of inseminators. Factors such as the processes of collecting, freezing, and storing semen can reduce motility and the integrity of the plasma membrane and can damage sperm DNA. Spermatozoa with normal morphology, intact DNA, as well as good viability and motility are spermatozoa that have high fertility (Syauqy, 2014). One of the success factors for AI is the quality of semen after thawing and its relationship with the physiological and biochemical characteristics of spermatozoa (Medeiros et al., 2002). Then, Hayashi and Ishobe (2005) reported that indicators of the quality of frozen-thawed semen including viability, motility, and sperm DNA damage are important factors to support the success of AI. Low viability and motility as well as high sperm DNA damage are significant factors because the relationship between sperm viability after thawing and conception rate has been reported in previous study (Correa, 1997). A decrease in sperm motility and fertility due to oxidative stress and the formation of reactive oxygen species (ROS) during various procedures for freezing semen and artificial insemination has been reported (Roca et al., 2005). There are various consequences of oxidative stress, including membrane damage, respiration inhibition, leakage of intracellular enzymes, damage to axonemal proteins, and damage to the mitochondrial membrane (Aitken et al., 1998). One of the main focuses of the molecular damage is on mitochondria which causes the loss of sperm motility due to changes in mitochondrial functions through ATP depletion (de Lamirande and Gagnon, 1992).

Artificial insemination centers (AIC) often produce frozen semen in a large amount due to varied cattle breeds that produce frozen semen and varied demands of breeders. Besides, several cattle breeds have high levels of semen production, especially Ongole grade bull (PO) cattle. The frozen semen mostly supplied by AIC in Java is Ongole grade bull because the population of this cattle is very dominant and this breed has a high level of semen production compared to other cattle breeds. Frozen semen is stored in a container filled with liquid nitrogen at a temperature of -196°C (Sukmawati et al., 2014). Cryopreservation at -196°C (liquid nitrogen) is a method that can be used to store spermatozoa in the long-term (Hong et al., 2009). Nonetheless, for cryopreservation to be successful, spermatozoa have to be preserved for a long time without damaging their fertility. Watson (1995) mentioned that the storage time of frozen semen does not have any effect on sperm viability. However, studies on the effect of frozen semen storage time on mitochondrial activity and sperm DNA damage are still not commonly found. It is important to examine the quality of frozen semen which has been stored for a long time.

It is necessary to observe the quality of frozen semen to ensure that the spermatozoa to be used are still in the best condition because fertilization can only be carried out if the spermatozoa from the frozen semen are viable and motile as well as have good morphology and DNA membrane integrity. This study was aimed to determine the effect of storage time of Ongole Grade Bull semen obtained from several artificial insemination centers in Indonesia on motility, viability, plasma membrane integrity, mitochondrial activity, and sperm DNA damage.
Materials and Methods
Research Materials
The main material of this study was Ongole grade bull frozen semen that has been stored for up to three years. According to the duration of storage, semen samples were divided into 7 groups (one month, three months, six months, nine months, one year, two years, and three years). This study used 42 straws, with 6 straws for each group were used. straw samples of PO bulls from 6 healthy Indonesian Ongole Grade Bulls aged 6 to 8 years with normal reproductive organs obtained from three different Indonesian Artificial Insemination Centers (AIC). TUNEL kit purchased from Sigma-Aldrich Chemicals (Cat No.: 12 156 792 910), Mitotracker Red Purchased from Invitrogen (MP 07510).

Assessment of motility of spermatozoa
Analysis of spermatozoa motility was performed to find out the number of spermatozoa moving forward. The motility test started by dropping 20 µL semen using a micropipette on an object-glass, then covered with a cover glass. The percentage of sperm motility was assessed from 0 to 100% in an estimate from five view points by comparing the number of spermatozoa moving forward. The sperm motility was evaluated using a microscope with a magnification of 100x and equipped with a heating table (37°C).

Assessment of viability of spermatozoa
Sperm viability testing used eosin nigrosin staining. The sperm viability (%) was observed based on live spermatozoa and their intact membrane structure, thus preventing stain to enter the sperm membrane. On the other hand, the membrane structure of dead spermatozoa no longer functions so the stain can enter the sperm membrane. The procedure started by dropping a 1 drop of semen on an object-glass. Two drops of eosin-nigrosin staining reagent were added, then stirred until the mixture was homogenous. Smear preparation was made using another object-glass. Fixation was done on Bunsen, then the smear preparation was observed using a microscope with a magnification of 400x.

Assessment of membrane integrity of spermatozoa
The membrane integrity of spermatozoa was analyzed using a hypo-osmotic swelling test (HOST) (Gangwar et al., 2018) with a slight modification, using a solution with a composition of 0.9 g fructose and 0.49 g sodium citrate dissolved with distilled water until the volume was 100 ml. 200 µl of the solution was added to 20 µl of semen, then incubated at 37°C for 45 minutes. Next, smear preparation was made on an object-glass; the preparation was then immersed in a methanol solution for 10 minutes, rinsed in running water, then dried. The next was an examination of at least 100 spermatozoa under a microscope with a magnification of 400x. Membrane-intact spermatozoa were characterized by the swollen tail while sperms with damaged membranes were characterized by straight tails.

Assessment of sperm mitochondrial activities
Sperm mitochondrial activity was examined using Mitotracker Red staining (Sigma-Aldrich, USA). To prepare the stock solution, the Mitotracker Red product was dissolved using dimethylsulfoxide (DMSO) until achieving a final concentration of 1 mM; Molecular weight (MW) could be seen on the product label. The Mitotracker Red stock solution was then stored at a temperature of -20°C and protected from light. One mM Mitotracker Red stock solution was dissolved using buffer media or growth media. The final concentration to be used was 25-500 nM. The solution was then centrifuged to obtain cell pellets and remove the supernatant. Spermatozoa cells were then incubated using the Mitotracker red solution (37°C) for 15-45 minutes in dark conditions. Once the staining was done, the cells were once again centrifuged to obtain cell pellets and the cells were resuspended using buffer media. The samples could be analyzed directly under a fluorescence microscope or laser-scanning confocal microscope (BioRad MRC-1024). Sperm cells with active mitochondrial activity were marked by bright-red color on the sperm neck.

Assessment of sperm DNA damage
Sperm DNA damage was examined using the TUNEL assay (Sigma-Aldrich, USA). Dried spermatozoa samples were fixed using a fresh fixation solution for 1 hour at a temperature of 15 to 25°C. The samples were then rinsed using PBS, after which the samples were incubated in permeabilization solution for 2 minutes on ice (2 to 8°C). The slides were rinsed twice with PBS. For the negative control, 50 µl of Label solution was added, while for the positive control, the samples were incubated using recombinant DNase (3000 U/ml–3 U/ml in 50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA) for 10 minutes at a temperature of 15 to 25°C to induce DNA strand separation. This step was done before the labeling procedure. Then the area around the sample was dried. The labeling procedure was performed by adding 50 µl TUNEL reaction mixture to the sample. Then the slides were incubated in a dark and humid condition for 60 minutes at a temperature of 37°C. These slides were then rinsed three times with PBS. The samples could directly be analyzed under a fluorescence microscope or laser-scanning confocal microscope (BioRad MRC-1024) at a wavelength maximum of 580 nm (red).

Data analysis
Data on various parameters were subjected to one-way ANOVA, using a completely randomized design. Duncan’s multiple range test was applied for multiple means comparisons, where necessary. The data were in the form of motility, viability, plasma membrane integrity, mitochondrial activity, and DNA damage of spermatozoa from the straw samples of Ongole grade bulls.

Results and Discussion
The effect storage duration of semen in liquid nitrogen on the motility, viability, membrane integrity, mitochondrial activity and DNA damage of Ongole grade bull spermatozoa have been presented in Table 1. Based on the evaluation of frozen semen are shown in Table 1, the overall parameters of semen characteristics were considered as standard. The results showed that one-month group sperm motility was (53.80%), viability (62.75%) membrane integrity (76.35%), mitochondria activity (64.24%), and DNA fragmentation (3.82%). After 3 years of storage, the corresponding values were 39.52%, 41.56%, 48.25%, 49.12%, and 4.62%. Sperm motility and viability remained unaffected up to 6th months of storage, while membrane integrity started decreasing at 3rd month (P<0.05). Mitochondria activity decreased during the 9th months (P=0.05). In contrast to the previous results, DNA fragmentation did not increase significantly after 3 years of storage (P=0.05). Thus, post-thawed motility, viability, and membrane integrity of spermatozoa from Ongole grade bulls started decreasing at 6th months of storage, mitochondria activity started decreasing after 9th months, while

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sperm DNA integrity remained unaffected for up to three years of storage.

The research data showed that the mean sperm motility stored for one to three years was 46.58±1.66% (Table 1), meaning that the frozen semen was still good to be used for artificial insemination. Sperm motility remained unaffected up to 6th months of storage. Sperm motility after thawing is at least 40%; if less than 40%, the frozen semen is not suitable for insemination. Besides, the results of the statistical test showed that different storage times had a significant effect (P<0.05) on sperm motility of Ongole grade bull. These results indicate that the quality of Ongole grade bull cattle semen stored in liquid nitrogen has decreased but it still meets the SNI standard (2008) that frozen semen thawed at a temperature of 37°C for 30 seconds must show motility of at least 40%. Sperm motility and membrane integrity are generally considered to be fairly reliable indications of semen fertility (Hafez and Hafez, 2000; Jannet et al., 2008). In this study, mean post-thawed sperm motility of 39.52±1.65% were recorded in samples stored for 3 years.

The sperm viability of the Ongole grade bull can be seen in Table 1. Based on the results of the statistical tests, it can be known that different storage times of frozen semen had a significant effect (P<0.05) on the sperm viability of Ongole grade bull. The mean sperm viability of frozen semen stored under three years in each of the artificial insemination centers was 59.02±1.14%. This indicates that frozen semen could still be used. Sperm viability remained unaffected up to 6th months of storage. This regards the fact that insemination requires at least 40% to 50% of viable and motile spermatozoa. Approximately 30% of sperm will die during a freezing process and the viable sperms are very sensitive to the environment and have a short life cycle after thawing (de Lamirande and Gagnon, 1992). Spermatozoa will have good viability during a freezing process if plasma membrane integrity is maintained, allowing for a good metabolic process in spermatozoa. Damage to the plasma membrane during the freezing and thawing processes is caused by lipid peroxidation in spermatozoa that have been stored for a long time, lowering viability and affecting the preservation of semen for artificial insemination. Decreasing viability and motility after a period of storage in liquid nitrogen in the present study were caused by the effect of genetic and cryopreservation. Genetic damage has been suggested to the sperm after several long storages at liquid nitrogen. (de Lamirande and Gagnon, 1992).

The results showed that different storage times had a significant effect (P<0.05) on the plasma membrane integrity of Ongole grade bull. The mean plasma membrane integrity was 63.50±1.24%. The results indicated that the frozen semen of the Ongole grade bull could still be used for artificial insemination. Membrane integrity started decreasing during the 3rd month. This is in line with a statement of (Anwar et al., 2015). That a well-maintained plasma membrane allows for a good metabolic process in spermatozoa, which has a positive effect on sperm motility during storage. This shows that plasma membrane integrity determines the life and death of spermatozoa. This way, the percentage of intact plasma membrane should be similar to the percentage of viable spermatozoa (Rizal et al., 2003). Plasma membrane functions to maintain the integrity of the membrane, create a dynamic surface between cells, and protect against extreme environments. Damage to the membrane in the head causes the enzyme which functions for fertilization to be released and causes spermatozoa to lose fertility. Meanwhile, damage to spermatozoa in the tail causes the aspartate aminotransferase enzyme to be released. The aspartate aminotransferase enzyme functions to break down Adenosine Triphosphate (ATP) into Adenosine Diphosphate (ADP) and Adenosine Monophosphate (AMP), causing spermatozoa to lose the ability to move (de Lamirande and Gagnon, 1992). Evaluation of the intact plasma membrane using the hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the plasma membrane. Membrane integrity is a condition that indicates that the physiological function of the membrane is maintained as a control for ion transport, preventing fluids outside the cells from entering the cells. According to Sukmawati et al. (2014) damage to the plasma membrane will disrupt metabolic processes, cause ATP to not be normally synthesized, and decrease the motility and viability of spermatozoa.

Mitochondria in spermatozoa are necessary for the production of cellular energy for sperm motility via oxidative phosphorylation, the major generator of ATP through the electron transport chain. Furthermore, mitochondria can regulate cell apoptosis by releasing cytochrome C (Scarlett and Murphy, 1997) and other apoptosis-inducing factors (Susin et al., 1999), suggesting a strong link between apoptosis and mitochondria (Zamzami et al., 1996). Apoptosis is a critical process during spermatogenesis and many fertile men have a disproportionately high number of terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling-positive spermatozoa representing fragmented nuclear DNA (nDNA) (Varum et al., 2007). Thus, abnormal mitochondrial DNA may have consequences for male infertility by decreased ATP production or abnormal apoptosis. Mitochondria are the source of sperm energy, and damage to their structure during the cryopreservation process is associated with reduced post-thaw including sperm viability and motility. The results showed that different storage times had a significant effect (P<0.05) on the mitochondrial activity of Ongole grade bull. The mean plasma membrane integrity was 63.50±1.24%.

Damage to the chromatin deoxyribose nucleic acid (DNA) of spermatozoa is a crucial factor that may cause infertility. The sperm head has a nucleus that contains DNA, i.e. an important component in the fertilization process. All the genetic information which is passed from one generation to the next is contained in the DNA strands in the sperm nucleus (Sali et al., 2006). Gametes can survive at −196°C for an indefinite time although it may cause DNA damage accumulation that cannot be repaired by enzymes. Examination of sperm DNA damage is an important parameter in making a diagnosis of male fertility, including spermatozoa from frozen semen (Erenpreisa et al., 2003). The results of statistical tests showed that different storage times had no significant effect (P>0.05) on the sperm DNA of Ongole grade bull. Damage to the sperm DNA of Ongole grade bull had a mean of 4.16±1.34%. In contrast to the previous results, DNA fragmentation did not increase significantly after 3 years of storage. Fragmentation of DNA was shown to interact negatively with sperm viability (Cortes-Gutierrez et al., 2008). Because the sperm DNA is located inside the spermatozoa, meaning that it is protected by the plasma membrane and acrosome. Sperm DNA damage or decline occurs when the plasma membrane and acrosome hood are damaged, affecting the integrity of the sperm DNA. The quality of chromatin in the cell nucleus greatly determines the status of DNA-bound protamine which functions to protect DNA (Varum et al., 2007)
Table 1: Values for various parameters of post-thaw semen quality of Ongole grade bulls at different storage intervals in liquid nitrogen (Mean ±SE)

<table>
<thead>
<tr>
<th>Storage duration</th>
<th>Motility (%)</th>
<th>Viability (%)</th>
<th>Membrane integrity (%)</th>
<th>Mitochondrial activity (%)</th>
<th>DNA damage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>53.80±1.22a</td>
<td>72.75±1.56a</td>
<td>76.35±0.84a</td>
<td>64.24±1.46a</td>
<td>3.82±0.72a</td>
</tr>
<tr>
<td>3 months</td>
<td>51.60±1.56a</td>
<td>70.95±1.22a</td>
<td>74.55±0.96a</td>
<td>62.76±2.12a</td>
<td>3.85±1.22a</td>
</tr>
<tr>
<td>6 months</td>
<td>50.55±0.86a</td>
<td>69.62±1.35a</td>
<td>67.24±1.22a</td>
<td>60.96±1.52a</td>
<td>3.96±1.56a</td>
</tr>
<tr>
<td>9 months</td>
<td>46.23±2.12a</td>
<td>59.26±0.86a</td>
<td>63.56±1.55a</td>
<td>56.52±0.93a</td>
<td>4.08±0.86a</td>
</tr>
<tr>
<td>1 year</td>
<td>43.58±1.98a</td>
<td>55.78±0.52a</td>
<td>61.72±2.13a</td>
<td>53.16±1.74a</td>
<td>4.32±1.47a</td>
</tr>
<tr>
<td>2 years</td>
<td>40.76±2.26a</td>
<td>43.23±1.56a</td>
<td>52.83±0.75a</td>
<td>51.48±2.36a</td>
<td>4.45±2.32a</td>
</tr>
<tr>
<td>3 years</td>
<td>39.52±1.65a</td>
<td>41.56±0.94a</td>
<td>48.25±1.25a</td>
<td>49.12±1.65a</td>
<td>4.62±1.24a</td>
</tr>
<tr>
<td>Mean</td>
<td>46.58±1.66</td>
<td>59.02±1.14</td>
<td>63.50±1.24</td>
<td>57.17±1.68</td>
<td>4.16±1.34a</td>
</tr>
</tbody>
</table>

abcde Different lowercase superscripts in the same column show the significant difference (P <0.05)

Fig. 2: Morphology of Pasundan bull sperm after incubation in extreme acidic and alkali media. (A) sperm with normal morphology, (B) sperm with the abnormal head shape, (C) sperm with acrosome loose, (D) sperm with proximal cytoplasmic droplet, (E) sperm with coiled tail, (F) loose head sperm (G) sperm with the distal cytoplasmic droplet.

Fig. 3. Frozen-thawed Pasundan bull sperm with major defect morphology after short time incubation at media with extreme pH media, AHS= abnormal head shape, AA= Abnormal acrosome, ND= neck defect, PCD= proximal cytoplasmic droplet. (B). Frozen-thawed Pasundan bull sperm with minor defect morphology after short time incubation at media with extreme pH media, SLT= single loop tail, HL= Head loose, DCD= distal cytoplasmic droplet. Data presented in average and standard deviation, bars with a symbol above show a significantly different (P<0.05).
Sperm DNA integrity canal so vary due to the type of extender used or the breed of the bull (Waterhouse et al., 2010). The thawing process is also one of the critical points for sperm DNA damage. During the thawing process, spermatozoa are faced with extreme temperature changes which may damage cells, reduce motility, viability, and plasma membrane integrity, as well as damage sperm DNA reactive oxygen species (ROS) produced from cryopreservation can also induce damage to sperm thawed. Long-term storage of spermatozoa may also affect their potential for fertilizing (Haugan et al., 2007). When DNA fragmentation values were more than 2.1%, Boe-Hansen et al. (2008) reported a decrease in litter size.

Conclusion

The results of the present study showed that post-thawed motility, viability, and membrane integrity of spermatozoa from Ongole grade bulls started decreasing at 6th months of storage. mitochondria activity started decreasing after 9th months, while sperm DNA integrity remained unaffected for up to three years of storage in liquid nitrogen. Moreover, fertility rates for semen samples stored for different durations in liquid nitrogen need to be investigated extensively.

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References


Anwar P, YS Ondho and D Samsudewa (2015) The quality of plasma membranes integrity and intact acrosome of Bali bull spermatozoa were preserved at 5 ° C in sugarcane water extract diluent with the egg yolk addition. Agromedia. 35(1): 53-63.


Hafez ESE and B Hafez (2000). Reproduction in Farm Animals. 7th ed, Lippincott, USA.


