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Influence of Intracellular Reactive Oxygen Species in Several Spermatozoa Activity in Indonesian Ongole Bull Cryopreserved Sperm

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Abstract: Certain factors may affect sperm motility and integrity in a freezing process. Reactive Oxygen Species (ROS) is essential in damaging the sperm, as the previous study revealed that ROS is a major cause damaging the membrane of spermatozoa. This study aimed to understand ROS production and its interaction within the motility, mitochondrial activity and DNA damage to the cryopreservation process in Indonesian Ongole grade bull. A total of 40 ejaculated semen samples from four different bulls had taken. The ROS intracellularly detected by 2 -7 - Dichlorodihydrofluorescein Diacetate (DCFH-DA) and propodium iodide (PI). Mito Tracker Red kit was being used to discover the mitochondrial activity, while DNA sperms' integrity was detected by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL). Examinations were done under a fluorescence microscope. Results of the study demonstrated a significant increase in ROS from fresh semen, diluted or equilibrated before being frozen (at 12°C and 4°C). ROS production showed no differences between frozen-thawed semen and thawed semen before the freezing process. A significant decrease in thawed semen had shown in membrane integrity and mitochondrial activity due to the equilibration process in which the temperature was significantly higher at 12°C and 4°C than the fresh semen temperatures, however, the diluting and freezing process had not given any significant causes to sperm DNA damage. This study concluded that ROS production with its effects might be the main factor that leads to the decreasing of sperm motility and integrity but no effect was discovered on DNA damage as the diluting and freezing process occurred. The results have proven the previous study which stated ROS has different mechanisms in affecting membrane integrity and DNA integrity. It also comes to the new theory that ROS might be affected by the chemical diluter despite the temperature of the freezing and thawing process.

Keywords: Cryopreservation, Reactive Oxygen Species, Membrane Integrity, Mitochondrial Activity, DNA Integrity

Introduction

Artificial insemination and frozen semen give advantages to the livestock industry include Indonesia. Artificial insemination is beneficial to genetic improvement and increases the cattle population in Indonesia. Despite those beneficial impacts, this technology face obstacle, which causes fertility decrease to frozen semen, as the sperm's structure is damaged (Watson, 2000). Specific mechanisms of reduced sperm fertility have not been well-known discovered in frozen sperm. Currently, researchers started observing the Reactive Oxygen Species' (ROS) roles to the sperm quality and physiology in the freezing process. Oxidative stress is a potential factor increasing the cell

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damage caused by Reactive Oxygen Species (ROS). Excessive ROS will be harmful as it is a detriment to the functional sperm (Rath *et al*., 2009). The lipid peroxidative chains continuously occur (autocatalytic) as each reaction produces the latest Reactive Oxygen Species (ROS) which means lipid peroxidation is released and led to damaging the membrane plasm of spermatozoa (Insani *et al*., 2014). High polyunsaturated fatty acids in the plasma membrane and small amount of antioxidant in the sperm cytoplasm makes them susceptible to oxidative stress and peroxidative damage (Chatterjee and Gagnon, 2001).

Quality decreased in the sperm motility and fertility caused by the oxidative damage of excessive ROS in the freezing process has previously been studied (Roca *et al*., 2005). The previous studies found that lipid peroxidation is the main factor in damaging sperm membrane by decreasing the physiological functions of preserved sperm at 4°C (Vishwanath and Shannon, 2000) and the cryopreserved frozen semen (Chatterjee and Gagnon, 2001). Oxidative stress caused significant DNA damage to both the mitochondrial and nuclear genomes of spermatozoa (Aitken *et al*., 2013). Sperm has three membrane layers, the plasm, mitochondrial and acrosome. each layer consists of high polyunsaturated fatty acids, thus easily susceptible to oxidative stress in the freezing process (Chelucci *et al*., 2015). Mitochondrial damage effects are the most detrimental, in which the sperm motility decreased with the slightness of ATP. Energy is stored in the mitochondria as a proton concentration gradient and an electric potential gradient across the membrane. These gradients are generated by electron transport maintained by the inner mitochondrial membrane and drive the synthesis of ATP. (Kasai *et al*., 2002). The sperm DNA damage caused by ROS had reported, its affect cell development after the fertilization, high rates of DNA damage in spermatozoa have been associated with impaired preimplantation development of the embryo, increased rates of early pregnancy loss and high rates of morbidity (Aitken and Baker, 2006) because the damage to sperms chromatin and membrane delays the nuclear fusion and causes embryo death (Córdova *et al*., 2002).

A study adding the antioxidant to the thawed semen materials and its advantages to sperm quality had reported (Funahashi and Sano, 2005). However, oxidative stress specific mechanism has not been observed though it is important in antioxidant research plan against the damages. Therefore, this study objective was to detect ROS production intracellularly in Indonesian Ongole bull sperms in the freezing process with its impact on decreased sperm motility, membrane sperm damage, mitochondrial activity and DNA damage.

Materials and Methods

Research Materials

A total of 40 semen samples were collected from four different Indonesian Ongole breed bulls aged 4 to 6 years old in health conditions with normal reproductive organs.

Chemicals

2-7-dichlorodihydrofluorescein diacetate (DCFH-DA), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit and certain chemicals purchased from Sigma-Aldrich (Australia). The Mito Tracker Mitochondrion-Selective Probes purchased from Thermo Fisher Scientific (Australia).

Semen Collection and Cryopreservation

A total of 40 ejaculated semen samples (10 semen samples per bull) were collected by artificial vagina method from four different Indonesian Ongole bulls aged 4 to 6 years old in health conditions with normal reproductive organs. The bulls were treated in Balai Pengembangan Bibit, Pakan Ternak and Diagnostik Kehewanan (BPPPTDK Yogyakarta, Indonesia). The semen samples have to meet the standard procedure within 70% motility rates. Collected semen samples were placed into two different aliquots, as the first aliquot was to evaluate the fresh semen and the second aliquot was being used for cryopreservation (at -196°C using liquid nitrogen). Evaluations were performed on the fresh semen, diluted semen and frozen semen (post thawing). Semen was diluted into two different stages of dilution using soy skim-lecithin-based diluent. First diluent (A) as an antibiotic buffer (skim + penicillin + streptomycin) 13.5 mL added with 1.5 mL soybean lecithin. The second diluent (B) consisted of antibiotic buffer (skim + penicillin + streptomycin) 10.8 mL added with 1.5 mL soybean lecithin, 2.4 mL of glycerol and 0.3 gram of glucose (to gain 60 mil sperm cells motility per ml). The buffer was maintained in a range between 6.8 - 7.0 with 7.0 in the last extender. Equilibrate the diluted semen in the equilibration room at 4°C for 4 h, packed to 0.25 mL straw, pre-freeze using liquid nitrogen at -20°C for 9 min and stored in liquid nitrogen (-196°C).

Sperm Membrane Integrity. The membrane integrity of spermatozoa was analyzed using a Hypo-Osmotic Swelling Test (HOST) according to Gangwar *et al*., (2018). For the HOST steps, 10 µL of semen were diluted to 100 µL of HOST solution (a mixture of 0.9 fructose, 0.49 g of citrate sodium and distillate water to reach the final volume of 100 mL) and incubated for 30 min at 37°C. The solution was smeared on a slide, dried and fixated in methanol solution for 10 min, rinsed in running water and dried. A total of 200 spermatozoa were calculated under a light microscope with a 400x magnification. Normal membrane plasma of spermatozoa shows swelling or curling-tail, otherwise, a damaged membrane showed a straight-tail.

Sperm Acrosomal Integrity. The Giemsa staining method was used to examine the acrosomal integrity of spermatozoa (Almadaly *et al*., 2014). Dripping semen on an object-glass began the staining process. Smear preparation was done and warmed on a warming plate at

37°C. The sample was fixed in methanol for 10 min before being washed under running water. The sample was stained for 3 h in a staining jar using a Giemsa solution that contained 3 mL absolute giemsa, 2 mL PBS and 35 mL distilled water. It was then cleaned in running water and dried once more. A light microscope with a magnification of 400x was used to examine 200 cells. Purple heads were seen in spermatozoa with intact acrosomes, while pale lavender heads were found in those with damaged acrosomes. The number of spermatozoa with an intact acrosome was divided by the total number of spermatozoa and multiplied by 100% to determine acrosomal status.

Sperm Mitochondrial Activities. Sperm mitochondrial activity was examined using Mitotracker Red Mitochondrion-Selective Probes (Thermo Fisher Scientific, Australia). To prepare the stock solution, the Mito Tracker 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 Mito Tracker Red solution was then stored at a temperature of -20°C and protected from light. 1 mM Mito Tracker 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 Mito Tracker red solution (37°C) for 15-45 min in dark conditions. Once the staining was done, the cells were once again centrifuged to obtain cell pellets and the cells were suspended using buffer media. The samples could be analyzed directly under a fluorescence microscope or laser-scanning confocal microscope (Bio Rad MRC-1024). Sperm cells with active mitochondrial activity were marked by bright red colour on the sperm neck.

Sperm DNA fragmentation. DNA fragmentation was examined by using the terminal deoxynucleotidyl transferase dUTP nick end labeling *(*TUNEL*)* assay or in Situ Cell Death Detection Kit, TMR Red version 12th (Sigma-Aldrich, USA). The samples of semen were smeared to an object glass, dried and fixated for an hour at 15-25°C and rinsed with Phosphate Buffer Saline (PBS). The stained samples were added 0.1% Triton X-100 in sodium citrate 0.1% for two min at 2-8°C and rinsed with PBS twice. The negative control was made by adding 50 µL label solution and the positive control was made by incubating the samples with DNAse recombinant to induct DNA separation. The stained samples and controls were dried and mixed with TUNEL assay reaction at 37°C for 60 min and rinsed with PBS three times. The results were examined using a laser-scanning confocal microscope at a wavelength of 517 nm. Spermatozoa DNA fragmentation showed as a green fluorescent.

Estimation of sperm ROS. The sperm ROS activity was examined using 2-7-dichlorodihydrofluorescein diacetate DCF-DA (Sigma-Aldrich, Australia). The samples were centrifugated using media buffer and growth media to obtain the cell pellets and supernatant removal; incubated with an additional 20 µM DCFDA at 37°C for 30 min under dark conditions. After staining, centrifugated the cell pellets and resuspended with buffer media. The samples were analyzed under a fluorescence microscope or confocal laser scanning microscope (BioRad MRC-1024). The highest rate of ROS will be stained as light-green in the acrosome.

Statistical Analysis

Analysis of variance and correlation coefficient was performed by SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) as the analysis tool. Duncan's multiple range test was used for treatment comparisons. The data were in the form of motility, viability, plasma membrane integrity, acrosome integrity, mitochondrial activity and DNA damage of spermatozoa.

Results

Sperm Motility, Membrane Integrity and Acrosomal Integrity

Results study revealed a decrease of sperm motility (post-thawing) by 33% compared to fresh semen. A similarity result to membrane integrity (26%) and acrosome integrity (24%) comparison. A decrease occurred in the equilibration process (at 12°C and 4°C). Motility, membrane integrity and acrosome integrity rates significantly decreased after equilibrated at 12°C ($P<0,05$), 4°C ($P<0,05$) in dilution process compared to fresh semen (Table 1).

Mitochondrial Activities

Percentages between mitochondrial activity in fresh semen, equilibration semen and frozen-thawed semen are given in Table 1. Fresh semen consisted of 68.60±2.94% cells with normal mitochondrial activity. The rates significantly decreased to 25.4±2.15% ($P<0,05$) of the frozen-thawed semen result. The sperm proportion and mitochondrial activity decreased in the equilibration process at 12°C and 4°C (Table 1). Examination of the spermatozoa mitochondrial activity could be seen in the Fig. 1.

DNA Damage

The sperm DNA damage varies between 1.4 to 3.8% in a total average of 2.32%, while the sperm DNA damage in frozen-thawed semen 3.82% (range between 1.8-4.6). Differed to motility and cellular integrity, the sperm's DNA integrity was not significantly affected in the freezing and thawing process. In Table 1, an increase occurred in DNA damage of equilibration at 12°C and 4°C. DNA damage levels were increasing along with the decreased equilibration temperature, though it revealed no differences compared to fresh semen ($P>0,05$). Sperm DNA Fragmentation results using the TUNEL assay method could be seen in the Fig. 2.

Intracellular ROS

The post-thawing ROS production was significantly higher compared to fresh semen (P<0,05) with the proportion of the dead cells included, although it was not significantly different from semen before the freezing process (equilibration at 4°C). A total average of fresh semen 48.45±2.40 and 28.9±1.80% of post-thawing semen (dead cells included). In the equilibration or freezing process, sperm ROS production increased along with the rising of equilibration temperatures at 12°C and 4°C (Table 1). The sperm proportion and ROS were significantly higher in the equilibration process compared to fresh semen. Intracellular ROS results using the 2-7-dichlorodihydrofluorescein diacetate DCF-DA method could be seen in the Fig 3.

Parameter Correlation

The correlation between ROS with sperm motility and other functional sperm integrity metrics during the freezing and dilution process was proven (Table 2). The rate of ROS was negatively correlated with sperm motility (r = -0.75) and mitochondrial activity (r = -0.84) in the freezing or equilibration process. The mitochondrial activity was positively correlated with motility and negatively related to DNA damage in the equilibration process. There is no correlation between ROS and DNA damage to the frozen-thawed semen.

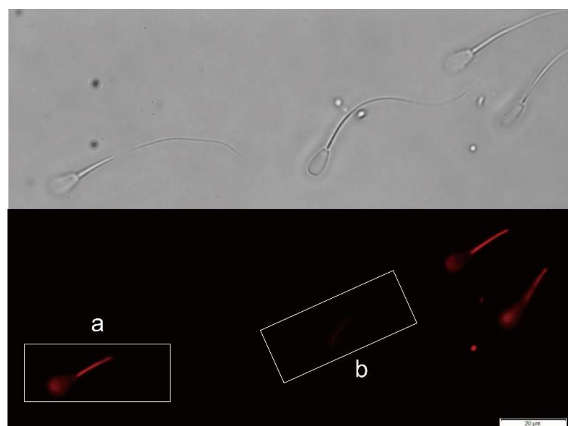

Fig. 1: Examination of the spermatozoa mitochondrial activity using the Mito Tracker red staining method, (a) the bright red neck spermatozoa is indicating mitochondrial activity. whereas (b) the colourless neck spermatozoa is showing the absence of mitochondrial activity

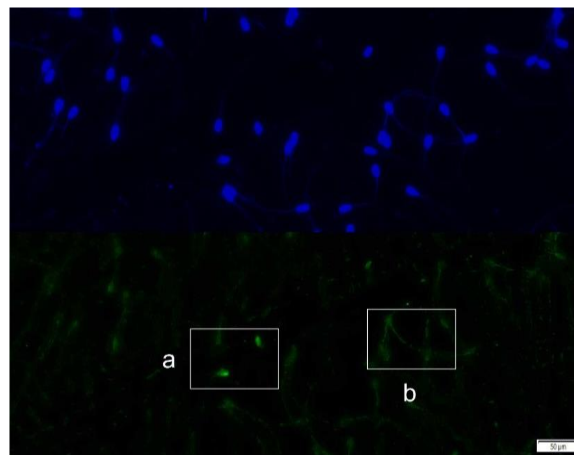

Fig. 2: DNA Fragmentation results using the TUNEL assay method, spermatozoa in a bright green fluorescence showed damaged (fragmented) DNA (a), while spermatozoa in a dull green fluorescence showed normal DNA (b)

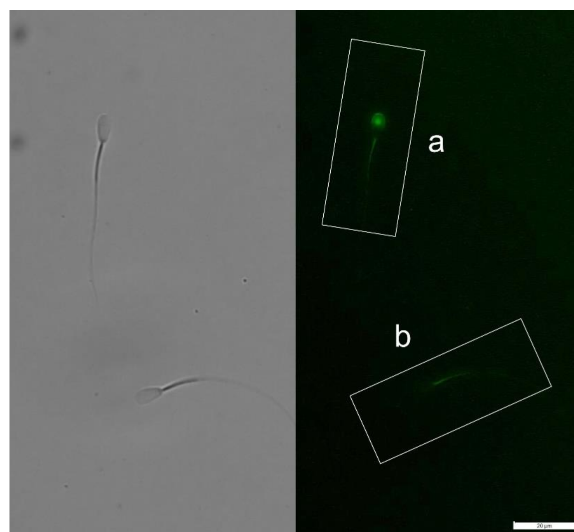

Fig. 3: Intracellular ROS results using the 2-7-dichlorodihydrofluorescein diacetate DCF-DA method, haed of spermatozoa in a bright green fluorescence showing ROS activity (a), while spermatozoa in a dull green fluorescence showing the absence of ROS active

Table 1: Ongole grade bull spermatozoa quality parameters before and after Cryopreservation

Characteristics	Fresh semen	Equilibration (12°C)	Equilibration (4°C)	Frozen-thawed semen
Motility	79.00±0.50^{a}	70.15±1.45^{b}	65.13±1.25^{b}	53.63±1.00^{c}
Membrane Integrity	63.65±0.86^{a}	54.59±0.62^{b}	51.69±0.72^{b}	47.48±0.94^{bc}
Acrosome Integrity	61.82±0.62^{a}	52.35±0.43^{b}	48.31±0.53^{b}	46.97±0.58^{b}
Mitochondrial activities	68.60±2.94^{a}	66.80±3.25^{a}	53.70±2.45^{b}	25.4±2.15^{c}
DNA damage	2.32±0.92^{a}	2.82±0.75^{a}	3.15±0.52^{a}	3.82±0.72^{a}
Intracellular ROS (Dead Cells Included)	47.45±2.40^{a}	50.72±2.65^{a}	58.76±3.84^{b}	59.65±1.80^{b}

^{a,b,c} Different lowercase superscripts in the same row show the significant difference (P<0.05)

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Table 2: Relationship between ROS, motility, Mitochondrial activity and DNA damage of spermatozoa in fresh, before freezing and frozen-thawed PO bull semen

Bet between parameters	Correlation coefficient			
	Fresh semen	Equilibration (12°C)	Equilibration (4°C)	Frozen-thawed semen
ROS and motility	-0.59	-0.65	-0.75*	-0.26*
ROS and mitochondrial activity	-0.78	-0.81	-0.84	-0.30*
Mitochondrial activities and Motility	0.81	0.87	0.92	0.86
ROS and DNA damage	0.61	0.69	0.77	0.35*
Mitochondrial activities and DNA damage	-0.55	-0.64*	-0.66*	-0.25*
Motility and DNA damage	-0.29	-0.35	-0.48*	-0.26
Membrane Integrity and Mitochondrial activities	0.71	0.77	0.81	0.76

*Values in the same row show the significant difference compared to fresh semen (P<0.05)

Discussion

The post-thawing semen fertility rates might be agitated by intracellularly and extracellularly damage during cryopreservation. Several factors were correlated with cry damage, thereby the possible major cause was one mediated by ROS (Chatterjee and Gagnon, 2001; Guthrie and Welch, 2006). The biochemical and mechanical factors had proven their roles in inducing the ROS production in sperm. A previous study reported the sperm quality decreased as related to the increasing ROS on cryopreservation (Chatterjee and Gagnon, 2001).

This study revealed the sperm ROS was significantly increasing in fresh semen and during diluted or equilibrated before the freezing process at 12°C and 4°C, while in frozen-thawed semen had not significantly found the difference of ROS production compared to the diluted semen before the freezing process. The ROS production was increasing along the equilibration process at 4°C. Metabolic activity, which is relatively stable in the freezing process at 4°C, has a major role in increasing sperm ROS. The decreased ROS comparison between frozen-thawed semen and diluted semen before the freezing process might be affected by metabolic activity decrease and the living cells. Therefore, the free radicals are found in living cell's metabolic activity. Kadirvel *et al*. (2009) stated a slight difference in the frozen semen study that was no increasing ROS in the fresh semen process to frozen-thawed semen. Those might be caused by a different diluent used. ROS production in cryopreservation and antioxidant decreased enzyme in the freezing process (Bilodeau *et al*., 2000) were causing biochemical damage in sperm cell functions (Chatterjee and Gagnon, 2001). Thus concluded the diluent has substantial roles in protecting from cryodamage (Bucak *et al*., 2008).

Cryopreservation significantly decreased the mitochondrial activity (P<0,05). The decreased mitochondrial activity sequentially occurred in the equilibration (at 12°C and 4°C), freezing and thawing process. The results correlated to the previous study as found in humans (Paasch *et al*., 2004), wild boar (Guthrie and Welch, 2006) and buffalo (Martin *et al*., 2004). As ROS is produced intracellularly in mitochondrial, this caused the electron transport process disrupted (Halliwell and Gutteridge, 2015). ATP which is produced in mitochondrial needs an optimal mitochondrial activity to transport electrons and this could be disrupted by ROS production. Besides, mitochondrial activity and sperm motility mostly affected by ROS production (Sawyer *et al*., 2003). ROS is a crucial factor in the physiological process as capacitation, acrosomal mechanism activity and signaling fertility mechanisms (Bansal and Bilaspuri, 2011). ROS production and its impact correlated with the motility decrease and functional sperm integrity in the dilution process at 4°C, however, ROS production had a slight effect on the thawing process (Kadirvel *et al*., 2009).

This study concluded that ROS might affect motility and mitochondrial activity in the dilution and equilibration process (Table 2). The high mitochondrial activity had a positive correlation with sperm motility. However, the ROS correlation was negative during the equilibration (at 12ºC and 4ºC) or dilution process. Previous studies discovered the dynamic relation between sperm motility, mitochondria activity and ROS in humans (Wang *et al*., 1997) and wild boar (Guthrie and Welch, 2006). According to the current and relevant studies, it has concluded that they have an identical mechanism as motility decreased has correlated to oxidative stress. Frequently used mechanism refers to lipid peroxidation in the sperm membrane (Aitken and Baker, 2006). One of the peroxidation effects is increasing the membrane permeability or decreasing sperm membrane integrity. These could affect the cells' ability to control ions which roles to manage the sperm movement. Motility decreased after ROS binding was caused by ATP slightness in mitochondrial activity without the following increase to lipid peroxidative. Thus, the ROS might negatively impact the sperm motility through mitochondrial functions decreased. Gravance *et al*. (2000) has concluded that the decreased sperm motility is caused by a disrupted mitochondria activity.

Mitochondria are located in the spermatozoa midpiece and produce accessible energy to the tail of sperm, thus facilitating efficient propulsion for the sperm both to reach the oocyte and to penetrate its zona pellucida (O'Connell *et al*., 2002). The decrease in the motility of spermatozoa after freezing and thawing can be around 24-64% (Ozkavukcu *et al*., 2008). The decrease in motility is related to mitochondrial activity (Conell *et al*., 2002). Mitochondrial damage disrupts Adenosine Triphosphate (ATP) production. Mitochondria are the cell's main source of oxidative energy in the production of ATP via the electron transport system (Silva and Gadella, 2006). Following the above-mentioned study, we found in bovine spermatozoa that motility and mitochondrial activity were almost equally vulnerable to the freezing and thawing process because values were nearly identical and highly correlated with each other.

There is a correlation between progressive sperms motility and mitochondrial activity, both in bulls and wild boar cryopreservation (Martin *et al*., 2004; Guthrie and Welch, 2006). Membrane damage and motility-loss in cryopreservation are related to the decreasing mitochondrial activity caused by mechanical and chemical factors. The mechanical factor is mainly caused by temperature change and diluters as the chemical cause. Alvarez and Storey (1993) proposed two different hypotheses regarding cryodamage to the membrane, it might be caused by lipid peroxidative and ROS stress induction. They stated that oxidative stress contributes to cryodamage higher than lipid peroxidative. Membrane damage and lipid peroxidation were separated mechanisms that have no correlation in between (Brouwers and Gadella, 2005). Our study discovered that the decreased mitochondrial activity more contributed to decreased sperm motility in the post-thawing semen, while the equilibration or dilution process correlated to oxidative damage in decreased semen motility.

Chromatin changes and decreased DNA integrity of mammalian sperm had been studied earlier (Agarwal and Said, 2003). Although the mechanism of the increased DNA nuclear damage has not been clearly explained, a potential explanation is the excessive ROS production can cause DNA damage (Aitken and Baker, 2006). In previous research models, there was a substantial obstacle in mitochondrial function found in DNA fragmentation which is expected as the factor in decreasing the mitochondrial activity. This was followed by the high production of ROS (Henkel *et al*., 2004). In the dilution and frozen-thawed process, we had observed DNA damage in the Ongole breed was not affected by cryopreservation. Marin *et al*., (2004) and Isachenko *et al*., (2004-) reported the sperm DNA integrity was not affected during cryopreservation.

Conclusion

ROS production and its effect might be the main factor in decreasing sperm motility and cellular integrity in diluting and freezing process. However, ROS has no effect on DNA damage under the same conditions.

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Author's Contributions

Kurniawan Dwi Prihantoko: Contributed on the original ideas of the research, research Design, data collection, analysis and interpretation and manuscript writing.

Asmarani Kusumawati: Contributed on the original ideas of the research, coordinate the research funding, data collection and manuscript preparation.

Mulyoto Pangestu: Contributed data collection and analysis, methods preparation and manuscript preparation.

Diah Tri Widayati and Agung Budiyanto: Contributed research ideas, research preparation and manuscript preparation.

Conflict of Interest

The author(s) declared no potential conflicts of interest to the research, the authorship and/or publication of this article.

References

Agarwal, A., & Said, T. M. (2003). Role of sperm chromatin abnormalities and DNA damage in male infertility. Human reproduction update, 9(4), 331-345. doi.org/10.1093/humupd/dmg027

Aitken, R. J., & Baker, M. A. (2006). Oxidative stress, sperm survival and fertility control. Molecular and cellular endocrinology, 250(1-2), 66-69. doi.org/10.1016/j.mce.2005.12.026

Aitken, R. J., Bronson, R., Smith, T. B., & De Iuliis, G. N. (2013). The source and significance of DNA damage in human spermatozoa; a commentary on diagnostic strategies and straw man fallacies. MHR: Basic science of reproductive medicine, 19(8), 475-485. doi.org/10.1093/molehr/gat025

Almadaly, E., Farrag, F., Shukry, M., & Murase, T. (2014). Plasma membrane integrity and morphology of frozen-thawed bull spermatozoa supplemented with desalted and lyophilized seminal plasma. Global Vet, 13, 753-766. doi.org/10.5829/idosi.gv.2014.13.05.8684

Alvarez, J. G., & Storey, B. T. (1993). Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: Glycerol and other polyols as sole cryoprotectant. Journal of andrology, 14(3), 199-209. doi.org/10.1002/j.1939-4640.1993.tb00383.x

Bansal, A. K., & Bilaspuri, G. S. (2011). Impacts of oxidative stress and antioxidants on semen functions. Veterinary medicine international, 2011. doi.org/10.4061/2011/686137

Bilodeau, J. F., Chatterjee, S., Sirard, M. A., & Gagnon, C. (2000). Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Molecular Reproduction and Development: Incorporating Gamete Research, 55(3), 282-288. doi.org/10.1002/(SICI)1098-2795(200003)55:3%3C282::AID-MRD6%3E3.0.CO;2-7

Brouwers, J. F., Silva, P. F., & Gadella, B. M. (2005). New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze-thawing. Theriogenology, 63(2), 458-469. doi.org/10.1016/j.theriogenology.2004.09.046

Bucak, M. N., Ateşşahin, A., & Yüce, A. (2008). Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze–thawing process. Small ruminant research, 75(2-3), 128-134. doi.org/10.1016/j.smallrumres.2007.09.002

Chatterjee, S., & Gagnon, C. (2001). Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. Molecular Reproduction and Development: Incorporating Gamete Research, 59(4), 451-458. doi.org/10.1002/mrd.1052

Chelucci, S., Pasciu, V., Succu, S., Addis, D., Leoni, G. G., Manca, M. E., ... & Berlinguer, F. (2015). Soybean lecithin–based extender preserves spermatozoa membrane integrity and fertilizing potential during goat semen cryopreservation. Theriogenology, 83(6), 1064-1074. doi.org/10.1016/j.theriogenology.2014.12.012

Córdova, A., Pérez-Gutiérrez, J. F., Lleó, B., García-Artiga, C., Alvarez, A., Drobchak, V., & Martín-Rillo, S. (2002). In vitro fertilizing capacity and chromatin condensation of deep frozen boar semen packaged in 0.5 and 5 mL straws. Theriogenology, 57(8), 2119-2128. doi.org/10.1016/S0093-691X(02)00701-X

Funahashi, H., & Sano, T. (2005). Select antioxidants improve the function of extended boar semen stored at 10 C. Theriogenology, 63(6), 1605-1616. doi.org/10.1016/j.theriogenology.2004.06.016

Gangwar, C., Saxena, A., Patel, A., Singh, S. P., Yadav, S., Kumar, R., & Singh, V. (2018). Effect of reduced glutathione supplementation on cryopreservation induced sperm cryoinjuries in Murrah bull semen. Animal reproduction science, 192, 171-178. doi.org/10.1016/j.anireprosci.2018.03.005

Gravance, C. G., Garner, D. L., Baumber, J., & Ball, B. A. (2000). Assessment of equine sperm mitochondrial function using JC-1. Theriogenology, 53(9), 1691-1703. doi.org/10.1016/S0093-691X(00)00308-3

Guthrie, H. D., & Welch, G. R. (2006). Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. Journal of animal science, 84(8), 2089-2100. doi.org/10.2527/jas.2005-766

Halliwell, B., & Gutteridge, J. M. (2015). Free radicals in biology and medicine. Oxford university press, USA. ISBN-10: 9780198717485.

Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., ... & Kruger, T. F. (2004). Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertility and sterility, 81(4), 965-972. doi.org/10.1016/j.fertnstert.2003.09.044

Insani, K., Sri, R., Agung, P., & Aries, S. (2014). Kadar MDA spermatozoa setelah pembekuan. Jurnal Biotropika, 3(2).

Isachenko, V., Isachenko, E., Katkov, I. I., Montag, M., Dessole, S., Nawroth, F., & Van Der Ven, H. (2004). Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity and fertilization ability. Biology of reproduction, 71(4), 1167-1173. doi.org/10.1095/biolreprod.104.028811

Kadirvel, G., Kumar, S., & Kumaresan, A. (2009). Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. Animal reproduction science, 114(1-3), 125-134. doi.org/10.1016/j.anireprosci.2008.10.002

Kasai, T., Ogawa, K., Mizuno, K., Nagai, S., Uchida, Y., Ohta, S., ... & Hoshi, K. (2002). Relationship between sperm mitochondrial membrane potential, sperm motility and fertility potential. Asian journal of andrology, 4(2), 97-104. http://www.asiaandro.com/archive/1008-682x/4/97.htm?mpclwjsbcesrixsb

Martin, G., Sabido, O., Durand, P., & Levy, R. (2004). Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biology of Reproduction, 71(1), 28-37. doi.org/10.1095/biolreprod.103.024281

O'connell, M., Mcclure, N., & Lewis, S. E. M. (2002). The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Human reproduction, 17(3), 704-709. doi.org/10.1093/humrep/17.3.704

DOI: 10.3844/ajavsp.2022.11.18

Ozkavukcu, S., Erdemli, E., Isik, A., Oztuna, D., & Karahuseyinoglu, S. (2008). Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. Journal of assisted reproduction and genetics, 25(8), 403-411. doi.org/10.1007/s10815-008-9232-3

Paasch, U., Sharma, R. K., Gupta, A. K., Grunewald, S., Mascha, E. J., Thomas Jr, A. J., ... & Agarwal, A. (2004). Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. Biology of reproduction, 71(6), 1828-1837. doi.org/10.1095/biolreprod.103.025627

Rath, D., Bathgate, R., Rodriguez-Martinez, H., Roca, J., Strzezek, J., Waberski, D., ... & Ziecik, A. J. (2009). Recent advances in boar semen cryopreservation. Control of pig reproduction VIII, 66, 51-66.

Roca, J., Rodríguez, M. J., Gil, M. A., Carvajal, G., Garcia, E. M., Cuello, C., ... & Martinez, E. A. (2005). Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. Journal of andrology, 26(1), 15-24. doi.org/10.1002/j.1939-4640.2005.tb02867.x

Sawyer, D. E., Mercer, B. G., Wiklendt, A. M., & Aitken, R. J. (2003). Quantitative analysis of gene-specific DNA damage in human spermatozoa. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 529(1-2), 21-34. doi.org/10.1016/s0027-5107(03)00101-5.

Silva, P. F. N., & Gadella, B. M. (2006). Detection of damage in mammalian sperm cells. Theriogenology, 65(5), 958-978. doi.org/10.1016/j.theriogenology.2005.09.010

Vishwanath, R., & Shannon, P. (2000). Storage of bovine semen in liquid and frozen state. Animal reproduction science, 62(1-3), 23-53. doi.org/10.1016/S0378-4320(00)00153-6

Wang, A. W., Zhang, H., Ikemoto, I. anderson, D. J., & Loughlin, K. R. (1997). Reactive oxygen species generation by seminal cells during cryopreservation. Urology, 49(6), 921-925. doi.org/10.1016/S0090-4295(97)00070-8

Watson, P. F. (2000). The causes of reduced fertility with cryopreserved semen. Animal reproduction science, 60, 481-492. doi.org/10.1016/S0378-4320(00)00099-3