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Original Article

The Effect of Cryopreservation on the Sperm Ultrastructure of Mus Musculus Albinus Strain DDY: Comparison of Nakagata vs Modified vs Kitazato Cryoprotectants

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ABSTRACT

Introduction: Sperm morphology analysis is very necessary to understand male fertility and the etiology of infertility. Currently, scanning electron microscopy (SEM) has been widely used to determine surface topology. In this study, we will compare the effects of spermatozoa cryopreservation using three different types of cryoprotectants, namely Nakagata, modification and Kitazato. The cryoprotectant compositions used are Nakagata (raffinose and skim milk), modified (glycerol and raffinose) and Kitazato (glycerol and trehalose). **Methods:** SEM analysis was carried out on 8 sperm samples before cryopreservation and after the freeze-thaw process. **Results:** The results obtain showed that cryoprotectant modification was able to protect spermatozoa morphology better than Nakagata and Kitazato. Analysis revealed damage to plasma membrane, acrosome and loss of mitochondria in all treatment groups compared to fresh sperm. SEM showed obvious signs of post-thaw damage such as missing plasma membranes, sperm showing damaged acrosomes and mitochondria in the middle showing structural disorganization. **Conclusion:** SEM revealed that cryopreservation caused ultrastructural damage to mice sperm due to freezing and thawing. These details provide valuable data for further research to minimize the damage caused by cryopreservation to mice sperm. Apart from that, further examination using TEM is recommended to obtain a more comprehensive picture.

Key words: cryopreservation, mice sperm, glycerol, raffinose, trehalose, scanning electron microscope.

INTRODUCTION

Semen cryopreservation is a widely used technique to preserve and supply sperm for breeding and maintaining genetic diversity. The standard cryopreservation protocol employs several steps (diluting of semen at 37°C with an extender and cooling to 5°C, adding and equilibrating the cryoprotectant and freezing it in liquid nitrogen at -196°C.[1] Unfortunately, cryopreservation has been proven to cause changes in sperm structure and function.[2,3] Due to thermal stress from temperature changes during cooling, freezing and thawing, as well as osmotic stress caused by the addition of high concentrations of cryoprotective agents and crystallization.[4] Cryopreservation causes protein denaturation, irreversible membrane shrinkage, and collapse.[5] Therefore, the use of phospholipids and cryoprotective agents, along with proper dilution, equilibrium, and cooling techniques, is essential to prevent cold shock, decrease crystallization, and limit damage to sperm. Moreover, there has been a reported decline in sperm fertility among various domestic livestock following artificial insemination.[6]

Successful artificial insemination requires good quality sperm structure and function with normal ultrastructural features. Meanwhile, sperm ultrastructure and the relationship between sperm structure and function are the main determinants of male fertility.[7] In order to accurately describe sperm morphology, excellent visualization tools are needed, one of which is ultrastructural observation via a scanning electron microscope. Ultrastructural damage after sperm cryopreservation has been investigated in bulls,[1] camel,[8] goat,[9] ram,[10] rabbit,[11] and human sperm.[12,13]

Sperm damage generally cannot be detected by conventional examination. Because of ultrastructural examination requires high magnification, which is impossible with a light microscope due to its low resolution. To date, no studies have explored the effects of cooling or freezing on mouse sperm ultrastructure using three distinct types of cryoprotectants. In this present research, modified cryoprotectant from a combination of permeable and nonpermeable agents (glycerol and raffinose) was used. Glycerol still serves as the golden standard of cryoprotectant for freezing mammalian semen.[14] Glycerol can diffuse through cell membranes and balance cell conditions in the cytoplasm, reducing the volume of intracellular water without causing cell dehydration,[15] Glycerol is also capable of modifying large and sharp ice crystals and flexing the cell membranes to make them stronger.[16] This research also used raffinose as a non-permeable agent which could increase the viscosity and lower the freezing point of extracellular fluid even though the cellular dehydration is quite rapid.[15] Sztein (2001) stated that the combination between glycerol and raffinose would be able to maintain the quality of mouse spermatozoa during freezing.[17,18]

Furthermore, this research will compare with currently available commercial cryoprotectants,

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namely Kitazato (glycerol and trehalose) and Nakagata cryoprotectants (raffinose and skim milk). to gain in-depth knowledge useful in enhancing the success of cryopreservation. Therefore, this study aims to assess the effect of cryopreservation on the ultrastructure of mice sperm after freezing and thawing, using three different types of cryoprotectants.

MATERIALS AND METHODS

Ethical approval: The research protocol and ethical permit for this research were approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia under the number KET-326/UN2.F1/ETIK/PPM.00.02/2022.

Criteria for Experimental Animals

Male Mus musculus albinus strain Deutschland Denken Yoken (DDY) mice aged 12-15 weeks weighing 30-42 grams were obtained from the Faculty of Animal Husbandry, Bogor Agricultural University, Indonesia. During maintenance, mice were housed in 3-4 per cage, fed, and watered ad libitum. In this study, male mice were divided into four groups, namely Nakagata, modified cryoprotectant, Kitazato and control group (male mice which fresh sperm). Finally, 8 male mice were involved in this study.

Sperm Collection and Preparation

Fresh and frozen samples were prepared and processed separately. Sperm is taken from both cauda epididymis and then make a notch in the cauda epididymis so that the sperm can swim to the surface of the medium (Figure 1). Both the fresh sperm group and the treatment group were added with 100 µL of sperm rinse (Vitrolife Cat. No.10101, Sweden) then centrifuged at 1800 xg for 10 minutes. Discard the supernatant, and the pellet was resuspended with 100 µL EmbryoMax® Human Tubal Fluid (HTF) medium (Sigma-Aldrich Cat No.MR-070-D). Next, fresh sperm was incubated at 37°C for 30 minutes before being prepared. Meanwhile, in the treatment group, the collected samples were placed in a straw and added with cryoprotectant based on the treatment group, treatment one (P1) Nakagata cryoprotectant, treatment two (P2), modified cryoprotectant or treatment three (P3) Kitazato. Then the sample was placed 10 cm above liquid nitrogen vapor for 10 minutes. Then the straw is put into liquid nitrogen at a temperature of -196°C for storage. After 30 days, samples were thawed in a 37°C water bath for 10 seconds and evaluated for motility, cryosurvival rate, viability rate and preparation for SEM examination. Sperm cryopreservation and thawing was performed by the vitrification method using different cryoprotectants (Nakagata or Modification or Kitazato methods).[19]

Freezing Protocol

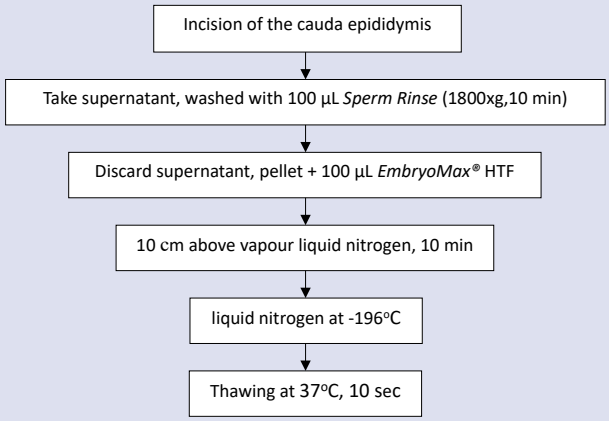

Figure 1. The mice sperm cryopreservation procedure.

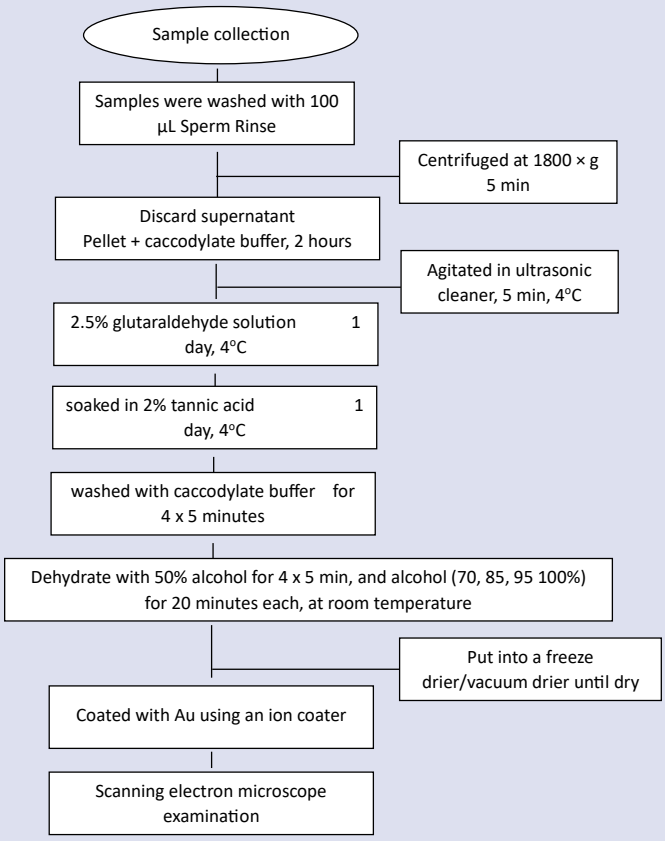

Figure 2. Scanning Electron Microscope Protocol.

Scanning Electron Microscope (SEM)

SEM analysis was carried out on sperm samples obtained before cryopreservation (fresh sperm), and after freezing-thawing using Nakagata, modified and Kitazato cryoprotectants (Figure 2). Sperm samples (n = 2 each group) were washed with 100 µL Sperm Rinse (Vitrolife Cat.No.10101, Sweden) and centrifuged twice at 1800 × g, 5 min. The supernatant was removed and the pellet formed by spermatozoa was soaked in caccodylate buffer for 2 hours, then agitated in an ultrasonic cleaner for 5 minutes, carried out at 4°C. Then the sample was placed in a 2.5% glutaraldehyde solution for 1 day. After that, the sample was soaked in 2% tannic acid for 1 day then washed with caccodylate buffer for 4 x 5 minutes. Next, the samples were dehydrated (at room temperature) with 50% alcohol for 4 x 5 minutes, alcohol (70, 85, 95 and 100%) for 20 minutes each. Next is the drying stage, the sample is soaked in tert butanol for 2 x 10 minutes, then frozen in the freezer until frozen and put into a freeze drier/vacuum drier until dry. Then the sample is attached to the specimens stub as needed. Finally, the sample was coated with gold (Au) using an ion coater. Then it was observed using a scanning electron microscope (Zeiss SUPRA 55VP, Oberkochen, Germany). The preparation process and SEM analysis were carried out in the SEM ZOO advanced characterization laboratory at the National Research and Innovation Institute, Cibinong, Indonesia

Statistical analysis

Expressed as mean ± Standard Deviation and analyzed using Statistical Package for the Social Sciences (SPSS) software version 22, IBM (IBM Corporation, Armonk, New York, United States). Comparisons between sample groups were analyzed using the t test and ANOVA test at a significance level of 0.05.

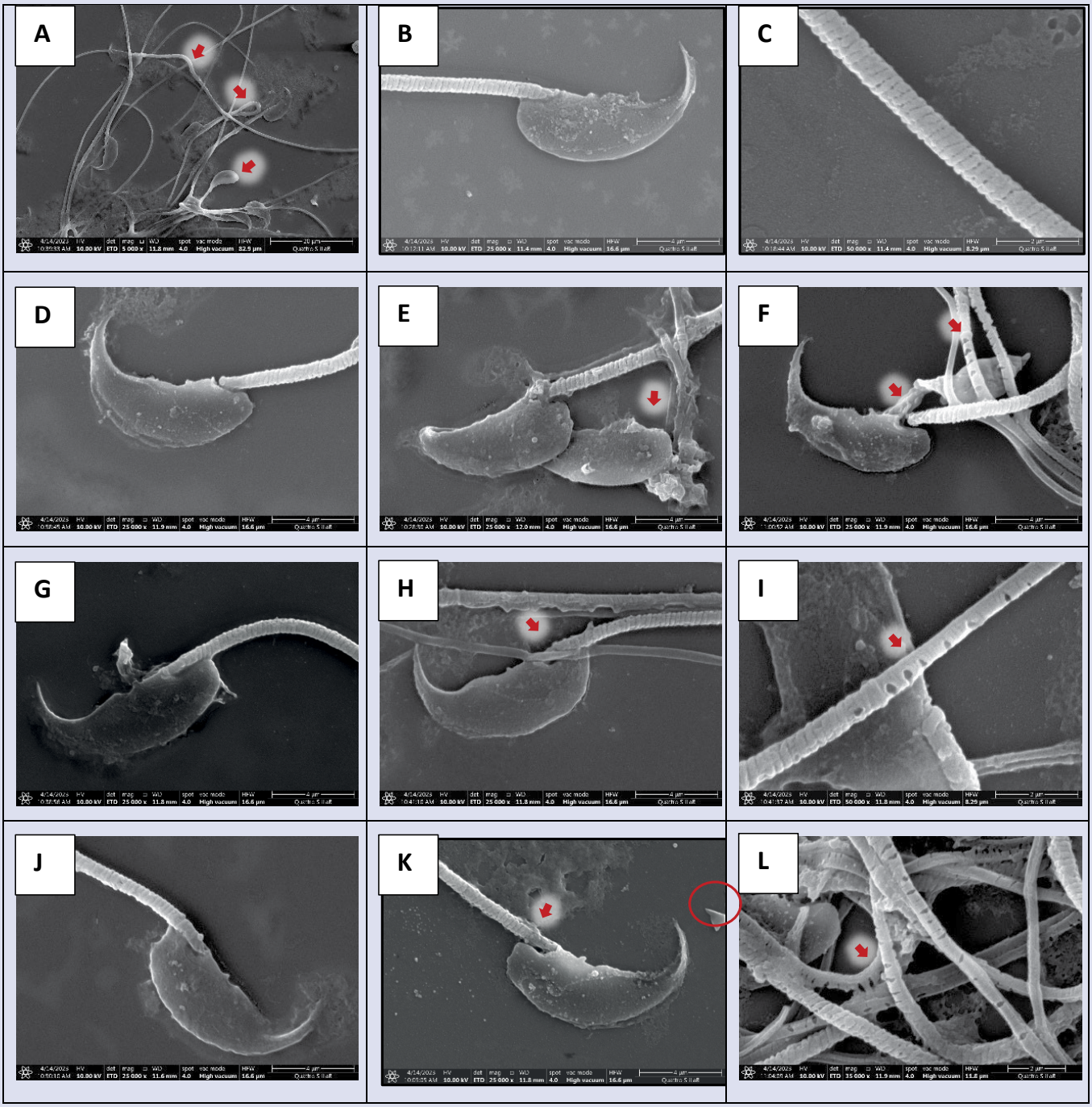

Figure 3. Differences in spermatozoa ultrastructure by using a scanning electron microscope. Fresh spermatozoa (A-C) were compared after cryopreservation by using Nakagata (Figures D-F), modified cryoprotectant (G-I), and Kitazato (J-L). (A) Typical morphology of spermatozoa, but the head is not hook-shaped and the tail is bent or curled in fresh spermatozoa (arrow). (B) Sperm shows a slightly rough or cracked surface; the anterior acrosomal segment, equatorial region, and post-acrosomal in the sperm head. (C) Sperm showed acrosomal reaction characteristic (triangle) (D) Normal ultrastructure upon application of Nakagata cryoprotectant. (E) Disconnection of the head and tail of the spermatozoa. (F) Deficiency of plasma membrane and mitochondria. (G) Ultrastructure of normal spermatozoa upon cryoprotectant modification. (H) Mitochondrial loss (arrow). (I) Loss of plasma membrane (arrow). (J) Normal ultrastructure of Kitazato cryoprotectant. (K) Loss of mitochondria in the midpiece, loss of the acrosome marked by a break in the tip of the spermatozoa head (circle sign). (L) Loss of plasma membrane in the tail of spermatozoa is indicated by a hole due to the cryopreservation process.

RESULTS

Fresh Sperm Ultrastructure

Before cryopreservation, sperm cells showed normal morphology although sperm abnormalities were found in the fresh group (arrow sign) (Figure 3A). Three well-differentiated regions can be detected in the sperm head: the anterior acrosomal, the equator region, and the post-acrosomal (Figure 3B). Tail structure of mice spermatozoa (Figure 3C).

Effect Cryopreservation on Sperm Ultrastructure

Sperm showed signs of damage after freezing-thawing in all treatment groups Nakagata (Figure 3E, F), modified cryoprotectant (Figure 3H, I), and Kitazato (Figure 3K, L). Some sperm cells showed with rough or cracked surfaces, and other cells even showed loss of plasma membrane and mitochondria. After cryopreservation, several detached heads and coiled tails were observed. The sperm showed an uneven surface, membrane damage and loss of mitochondria were detected in the middle part of the sperm (Figure 3E, H, K). At the sperm head, the plasma membrane showed perforation. In the middle part, loss of mitochondria can be distinguished. In addition, there is a loss of the acrosome which is marked by a break in the tip of the spermatozoa head (circle sign) (Figure K). Loss of plasma membrane in the tail of the spermatozoa is indicated by a hole due to the cryopreservation process (Figure 3I, L).

In Figure 3F, H and K, SEM micrographs show different types of sperm with thin necks. If the neck and middle part of the tail are very thin, then the so-called cellular membrane maybe lose there and reveal bundles of fibrils and mitochondria. The thin areas showed a decrease in the number of mitochondria (Figure 3H and K), which is a possible cause sperm motility reduction or even the inability sperm movement.

Scanning electron micrograph of spermatozoa with open neck and central portion of tail (Figure 3F). Scanning electron micrographs of spermatozoa show a much thinner central portion of the tail and an absence of mitochondria.

The results of ultrastructural examination of normal sperm decreased in all treatment groups which compared to fresh sperm (Table 1). The mean ultrastructure of fresh sperm 78.5%. However, the percentage of normal spermatozoa was reduced after cryopreservation by using Nakagata (41.5%), modified (63.5%) and Kitazato (58%) cryoprotectants. There was a significant difference in the Nakagata group compared to fresh spermatozoa ($p = 0.049$). One way ANOVA statistical test revealed that there were significant differences in each group ($p = 0.028$).

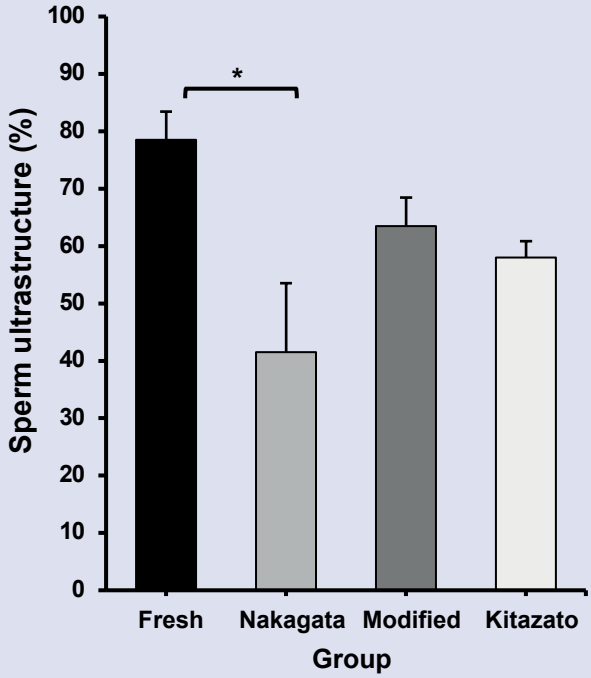

Table 4. Ultrastructure analysis of sperm.

DISCUSSION

The implantation of electron microscope techniques to assess sperm allows the observation of injury cell structures after freezing and thawing process. To advance the conservation procedure, mice sperm samples first underwent an exposure protocol with liquid nitrogen vapor for 10 minutes before being placed in liquid nitrogen.[1] Then evaluated using SEM, to disclose any morphological damage associated with the cryopreservation method compared to fresh sperm cells. In addition, to compare the effect of sperm cryopreservation using modification versus Nakagata or Kitazato. We will also evaluate structural integrity while reassessing what is known about mice sperm morphology.

The use of SEM for diagnostic purposes is still relatively rare. With higher resolution compared to light microscopy and wider magnification range, SEM can be a useful additional tool in diagnosing difficult cases related to male infertility.[20] Study Nussdorfer et al demonstrated potential defects in semen that are not visible on light microscope yet clearly visible on SEM.[20] Overall, our findings verify the suggestion that SEM analysis is a practical and valuable method in clinical practice. In our study, SEM micrographs presented some unusual deformations on the spermatozoa surface or absence of mitochondria, which were not visible when standard microscopy was used.

Scanning electron microscope assessment showed the sperm head detached from the Scanning electron microscopy (SEM) revealed that the sperm head becomes detached from the rest of the cell after thawing, potentially due to ice crystal formation during freezing. SEM is more sensitive to defects in the neck and tail compared to TEM examinations.[12] SEM also has a high intensification feature like TEM but is limited to cell surface evaluation. Acrosome abnormalities are clearly visible in the form of cracks or peeling on SEM. Low temperature increases cytoplasmic Ca^{2+} levels, reactions such as capacitation, ion leakage, and exocytosis differ from acrosomal contents.[21] Acrosome changes are also impacted after freezing and thawing, suggesting that the defects observed after thawing are caused by a pathological mechanism instead of induced physiological process.[12]

Fixation mainly maintains the morphology of cellular structures as close as possible to their original condition. In this research, Glutaraldehyde was used as the fixative agent for it provides the most optimal protection for the sperm plasma membrane. Glutaraldehyde buffer – the most common fixative in electron microscopy was used to gain fast and reliable fixation of cells linking their proteins with dialdehyde.[22]

When mice spermatozoa are frozen to -196°C, the presence of glycerol in the extender appears to be important for maintaining sperm properties in camels.[8] Although cooling is not the most destructive technique, SEM images show ultrastructural changes that affect sperm quality after freezing and thawing. This may explain the poor outcomes obtained when sperm is preserved by cryopreservation.

The plasma membrane surrounding the sperm head is most susceptible to cryodamage to cryodamage with varying degrees of undulation, swelling, or loss affecting approximately 50%, of the total sperm population after equilibration and freezing.[1] We know that the structure of the plasma membrane is the most affected due to cryopreservation. The plasma membrane plays a crucial role in regulating various sperm functions; controlling the movement of sodium and potassium ions,[23]

and calcium.[24] The sperm plasma membrane also has receptors that mediate binding and fusion of sperm zones, such as PH-20,[25] and other receptors that regulate sperm capacitation. In addition, an intact plasma membrane is also required for fusion with the outer acrosomal membrane and induction of the acrosomal reaction.[26] Therefore, it is very important to maintain the integrity of the sperm plasma membrane. Rapid changes in osmolarity often occur during freeze-thaw causing deformation of the membrane structure.[12] Tail defects after cryopreservation have been reported previously, and plasma membrane damage in this region was suggested as a possible cause of these defects.[8]

Mitochondrial structure is also affected by the freeze-thaw process. SEM images show loss of mitochondria in the mid piece of frozen sperm during freezing and thawing that will decrease the mitochondrial membrane potential (MMP) of sperm. As it causes damage to the mitochondrial membrane potential which affects the sperm motility. This damage can directly impacts the DNA through changes in the inner and outer membranes or indirectly through DNA fragmentation.[28] ATP synthesis which refers to the ability of sperm cells to produce ATP can be impaired, thereby affecting sperm motility.

Interestingly, our research was the use of three different types of cryoprotectants. When using Nakagata (raffinose) cryoprotectant, more abnormal morphologies were found compared to modification (glycerol, raffinose) and Kitazato (glycerol, trehalose). We assume that the raffinose composition contained in Nakagata cryoprotectant alone is not able to protect the structure of spermatozoa due to cold shock in the cryopreservation process. Therefore, in this study, we combined raffinose with glycerol as a cryoprotectant modification. A mixture of permeable and nonpermeable materials (as a vitrification medium) provides better results compared to a single cryoprotectant to reduce spermatozoa damage due to cryopreservation.[29] Although in reality, the use of modified cryoprotectants also results in a decrease in normal morphology due to cryopreservation. In the cryoprotectant modification, more morphological abnormalities were found in the principal piece, namely the helical structure of the tail became hollow and not solid. However, no abnormalities were found in the midpiece area or even causing the head and tail of the spermatozoa to be severed. Compared with Nakagata and modifications, Kitazato cryoprotectant is still maintaining the structure of spermatozoa stored frozen. Therefore, we assume that the sugar content of trehalose is still better than raffinose. But when glycerol is combined with raffinose, the results are almost as good as trehalose. However, more comprehensive ultrastructural studies may help in understanding the mechanisms underlying the failure of the cellular response to cryopreservation. Therefore, optimizing protocols and maintaining the viability of thawed samples can improve current cryopreservation techniques while also addressing specific challenges to prevent cryoinjury due to intracellular ice formation.[30]

Trehalose is a natural sugar that contains two d-glucose units in an α, α-1,1 linkage. It can protect proteins and cell membranes from denaturation or inactivation caused by various stress conditions, including heat, cold, dehydration, desiccation, and oxidation.[31] It has been established that the antioxidant properties of trehalose extender may be related to its effectiveness in membrane cryopreservation.[32] The cryoprotective effect of trehalose may be due to the increased fluidity of sperm membranes before freezing. Additionally, it maintains the structural integrity of cells and protects them from low molecules (ethylene glycol) by inducing osmotic stress during the freezing and thawing processes.[30] Trehalose also increases membrane integrity, motility, viability as well as reducing morphological abnormalities in post-thaw male sheep sperm.[32] Malo et al stated that trehalose improved spermatozoa viability and in vitro fertilization parameters in cryopreserved boar spermatozoa. It was described that a trehalose concentration of 50 mM produced the uppermost percentage of membrane intact sperm in rams.[33]

In addition, Buyukleblebici also revealed the beneficial cryoprotective effects of 25 mM trehalose and 3% ethylene glycol on acrosome morphology, and 3% glycerol on bovine semen membrane integrity.[34] In our study, the best ultrastructural cryoprotective effect was found at 10% Glycerol +18% Raffinosa + 3% skim milk. The glycerol content provides greater protection for the sperm plasma membrane.[35] Therefore, modification and Kitazato, which both contain glycerol, produce better sperm plasma membranes than Nakagata (raffinose only, without glycerol).

However, the combination of trehalose and glycerol showed the best viability and motility in boar sperm,[36] and is able to increase motility, acrosome integrity, and mitochondrial membrane potential (MMP).[37] In line with Aboagla and Terada's research on goat spermatozoa. The cryoprotective effect of trehalose may be due to increased fluidity of sperm membranes before freezing.[38] Uysal and Bucak demonstrated that trehalose had greater cryoprotective effects on motility, anomalous morphology, viability, and membrane integrity in ram sperm.[39] Similarly, in our study, the cryoprotectant Kitazato, a combination of glycerol and trehalose, protected the ultrastructural morphology of frozen-thawed mice sperm.

Raffinose is often used in mice sperm cryopreservation to create a hypertonic condition before freezing, while glycerol is used to block intracellular ice formation. Meanwhile, trehalose is able to provide better optimistic protection to the plasma membrane than raffinose.[40] Previous research suggests that trehalose is able to interact with specific membrane phospholipids by making the medium hypertonic, causing cellular osmotic dehydration before freezing and reducing the ice crystals formation.[41] In this study, the combination of glycerol and raffinose resulted in better protection of the ultrastructural morphology of mice sperm compared to commercial Kitazato. We observed that 10% glycerol and 18% raffinose provided the highest protection for the ultrastructural morphology of cryopreserved mice sperm. Thus, it can be postulated that the modified cryoprotectant, glycerol 10% and raffinose 18%, is able to overcome the limitations of the Nakagata cryoprotectant which only consists of raffinose and can act as a cryoprotectant in cryopreserved mice sperm.

In conclusion, this research is the first in investigating the ultrastructure of mice sperm and the ultrastructural alterations induced by freeze-thaw cycles when employing Nakagata, modified, or Kitazato cryoprotectants. Utilizing scanning electron microscopy (SEM), specific types of sperm damages were observed, which were not visible in conventional semen analysis, highlighting alterations that occur during the freezing and thawing processes. Further research should be performed to minimize the damage to sperm plasma membranes, acrosomes, and mitochondria in these processes. This research explores cryoprotectants that can be used in the cryopreservation of mice sperm, where the cryoprotectant modification group yielded the most effective preservation of sperm ultrastructural integrity after freezing and thawing. The results of this research enhance the genetic health and sustainability of various species which can be adapted for human assisted reproductive technologies to increase the success rates of in vitro fertilization by using modified cryoprotectants. Future researchers are encouraged to employ transmission electron microscopy (TEM) to conduct deeper, high-magnification analysis of cellular components, including the cytoskeleton, membrane systems, organelles, and specialized structures within differentiated cells.

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