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Effect of Glutathione Supplementation in Cryoprotectant Modification on Tyrosine Phosphorylation, Acrosin Expression and Acrosome Reaction of Post-Thawing Spermatozoa Quality

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ABSTRACT

Background: Tyrosine phosphorylation, acrosin, and acrosome reaction play an important role in fertilisation. However, cryopreservation causes changes in tyrosine phosphorylation, acrosin expression, and acrosome reaction which affect the quality of spermatozoa. Cryoprotectant media added with antioxidants is needed to protect Spermatozoa from the effects of cryopreservation so that the quality of spermatozoa can be maintained. **Objectives**: This research examined the effect of glutathione (GSH) supplementation in cryopreservation media on tyrosine phosphorylation, acrosin expression, and acrosome reaction. In this research, pure modified Cryoprotectant (CPA) was compared with CPA supplemented with GSH in three different concentrations. **Materials and Methods:** The research sample was male mus musculus albinus strain Deutchland Denken Yoken (DDY). Mice spermatozoa was cryopreserved and several parameters were measured including tyrosine phosphorylation, acrosin expression, and acrosome reaction. **Results:** The addition of GSH to the modified CPA increased tyrosine phosphorylation, acrosin expression, and acrosome reaction (maintaining acrosome integrity). The group with 1.00 mM GSH obtained the highest results among the other groups. Significant increases were found in tyrosine phosphorylation, acrosin expression, and acrosome reaction after the addition of 1.00 mM GSH. **Conclusion:** Glutathione supplementation in modified CPA can increase tyrosine phosphorylation, acrosin expression, and acrosome reaction of frozen-thawed spermatozoa. Treatment using GSH at a dose of 1.00 mM is the most effective and modification of CPA with the addition of glutathione can improve the tyrosine phosphorylation, acrosin expression and acrosome reaction in cryopreserved spermatozoa.

Keywords: spermatozoa cryopreservation, CPA modification, glutathione (GSH), tyrosine phosphorylation, acrosin expression, acrosome reaction.

INTRODUCTION

Spermatozoa cryopreservation, a common technique to maintain spermatozoa quality, produces oxidative stress that can damage the structure and function of spermatozoa.[1] Cryopreservation can change certain elements of spermatozoa, such as ions, lipids, proteins, and carbohydrates.[2] Changes in proteins can affect tyrosine phosphorylation and acrosin expression while damage to the acrosome can affect the acrosome reaction.

Cryopreservation exposes spermatozoa to frequent stress, resulting in changes in capacitation. Prior to fertilisation, spermatozoa require a period of adjustment in the female reproductive tract, and undergo a series of physiological and biochemical changes collectively referred to as capacitation. Research results from several laboratories show that protein tyrosine phosphorylation (PTP) is one of the most important intracellular signalling events regulating spermatozoa function, and is an important indicator in capacitation. Cryopreserved spermatozoa usually show a different PTP pattern from normal in vitro capacitated spermatozoa.[3]

Cryopreservation can also damage the acrosome membrane. The degree of damage to the inner acrosome membrane and/or equatorial segment is an important factor in the loss of acrosin molecules from the spermatozoa acrosome during cryopreservation procedures.[4] In addition, according to Neild et al. (2005) for successful fertilisation, spermatozoa must have an intact acrosome in order to perform the function of the acrosome reaction at the right time, which is to release enzymes and facilitate the spermatozoa in penetrating the zona pelusida.[5]

In cryopreservation, the occurrence of excess free radicals is a threat to spermatozoa, causing harmful damage.[6] The addition of antioxidants in the cryopreservation media is necessary to protect spermatozoa from free radicals. The antioxidant used in this study is glutathione. The addition of glutathione in the cryopreservation media produces good quality frozen semen because glutathione functions as an antioxidant compound that prevents lipid peroxidation reactions in the plasma membrane of spermatozoa during the process of freezing semen, so that the plasma membrane remains intact.[7] Thus, spermatozoa that have an intact plasma membrane are able to properly regulate the traffic in and out of all substrates and electrolytes at the cellular level, so that metabolic processes including fructolysis and the Krebs cycle can take place properly. This metabolic process produces ATP which contains energy so that the motility and viability of spermatozoa can be maintained. An intact cell plasma membrane will also protect the acrosome vesicle below the cell

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plasma membrane at the tip of the spermatozoa head from mechanical damage, so that the acrosome vesicle remains intact and the percentage value of the Acrosome Hood Intact increases.[8]

In this research, the cryopreservation medium used was a combination of glycerol and raffinose because optimal results would not be achieved without the addition of pCPA. While glycerol, a permeable CPA, can protect spermatozoa, it can be toxic. Rafinose (C18H32O16) is a high molecular weight non-permeable CPA that is unable to penetrate the plasma membrane. Instead, raffinose stabilises the lipid bilayer and forms hydrogen bonds between hydrophilic sites.[9]

Efforts to improve the quality of spermatozoa continue to be made so that the fertilisation process can run well. Addressing the problem, this study examined the effect of GSH addition at different concentrations in CPA modification on tyrosine phosphorylation, acrosin expression, and acrosome reaction.

MATERIALS AND METHODS

Ethics approval

The research protocol and ethical clearance for this research were approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia under the number KET-380/UN2.F1/ETIK/PPM.00.02/2022.

Criteria for Experimental Animals

Samples of this research were male Mus musculus albinus strain Deutchland Denken Yoken (DDY) mice aged 12-16 and females aged 8-10 weeks with a weight of 30-45 grams obtained from the Faculty of Animal Husbandry, Institute Pertanian Bogor, Indonesia. The mice were first acclimatized for 5 days at the Animal Research Facilities-Institute of Medical Education and Research (ARF-IMERI) Jakarta, Indonesia, where 2-4 mice were placed in one cage. The cleanliness of the cage was maintained and light was set 12 hours on and 12 hours off. Mice were fed and provided with ad libitum water. The male mice were divided into five groups: control group without treatment (fresh), control group (CPA modification without the addition of GSH), treatment group 1 (CPA modification with the addition of 1.00 mM GSH), treatment group 2, (CPA modification with the addition of 1.50 mM GSH), and treatment group 3 (CPA modification with the addition of 2.00 mM GSH).

Spermatozoa Collection and Preparation

Euthanasia was performed by anaestheting the mice before performing cervical dislocation. The cauda epididymis of each male was extracted through a careful incision in the skin and peritoneum. The extracted cauda epididymis was then placed in 500 µL Spermatozoa Rinse (Vitrolife Cat.No.10101, Sweden) within a tube (Biologix Cat. No.80-0015, USA). Following an incision in each cauda epididymis, spermatozoa cells were allowed to flow out and swim to the surface of the media, with a waiting period of approximately 20-30 minutes. The resulting spermatozoa suspension was divided into tubes (Biologix Cat. No.80-0015, USA) containing approximately 200 µL of the suspension to be processed in simple washing. Spermatozoa preparation involved centrifugation at 300 g for 15 minutes, yielding the final spermatozoa sample for further analysis.

Spermatozoa Cryopreservation

Spermatozoa cryopreservation by vitrification was categorized into different groups: control group without GSH addition and treatment groups with CPA modification added with different GSH concentrations of 1.00 mM, 1.50 mM, and 2.00 mM. The spermatozoa cryopreservation was conducted through the following steps:

Cryopreservation Medium

Modification method : Cryopreservation medium was prepared by dissolving 3.6 grams of raffinose (Nakagata, 2000)[10] (Sigma Aldrich Cat. No. 83400) in 15 mL of distilled water at 60°C until dissolved. Then, 0.6 grams of skim milk (Sigma Aldrich Cat No.70116) was added. Following centrifugation at 12000 rcf for 30 minutes, the supernatant was carefully collected. Subsequently, 2 mL of glycerol (Sigma Aldrich Cat. No. C6039) was added to the collected supernatant. A volume of 1.5 mL of the resulting solution was then transferred into a tube (Biologix Cat. No. 80-0015, USA) and stored at 4^0 C. The 1.00 M GSH solution was prepared by dissolving 3.07 grams of GSH in 10 mL of distilled water. The addition of GSH to the modified CPA was done by mixing it in doses of 1.00 mM, 1.50 mM, and 2.00 mM GSH. Following this, 200 µL of the resulting solution was transferred into tubes (Biologix Cat. No.80-0015, USA) and stored at 4°C for further use.

Freezing/Vitrification and Thawing

The pellet obtained from spermatozoa preparation with CPA (200 µL) was gently mixed. Subsequently, 10-20 µL of the pellet and CPA mixture was aspirated into the straw. Freezing of the spermatozoa in the straw was done by exposing it to the steam over liquid nitrogen at a distance of approximately 5 cm from the surface for 10 minutes and straw was carefully placed into the liquid nitrogen. Straw packaging was removed and straws were immersed in a water bath at 37° C for 10 seconds. After thawing, water on the straws was dried using a soft tissue, and both ends were cut. The thawed spermatozoa suspension was carefully extracted and placed in a tube (Biologix Cat. No. 80-0015, USA). Centrifugation was carried out at 1800 RPM for 15 minutes. Then, the supernatant was discarded. After that, further analysis were performed

Examination of Tyrosine Phosphorylation using the Western Blot method

The incubated samples were centrifuged at 13,000 RPM for 5 minutes. The supernatant was discarded, then the pellet was added 20 µg SDS Extraction Buffer (2% SDS, 10% sucrose, 0.1875M Tris pH 6.8, protease inhibitor). The sample was then heated on a heating block at 100°C for 5 minutes. The sample was centrifuged at 13,000 RPM for 10 minutes. Then, the supernatant was taken and transferred into a new tube while the pellet was discarded. The protein sample was stored at-20°C.

The isolated proteins (20 µg) were separated on 15% SDS-PAGE and then transferred to 0.45 µm HybondTM polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Germany, Cat. No. A10229666). The membrane was blocked with 5% Bovine Serum Albumin (BSA) for 1 hour at room temperature. Then, it was incubated with primary antibody Monoclonal Anti Phosphotyrosine Antibody Mouse (Sigma, US, Cat. No. P3300) overnight at 4oC. The next day, the membrane was washed with TBST 1x for 5 minutes for 3 times and incubated with secondary antibody Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology, Singapore, Cat. No. 7074) (for protein tyrosine phosphorylation) for 1 hour at room temperature. The membrane was then washed again with TBST 1x for 5 minutes for 3 times. Visualisation was performed with ECLTM Primer Western Blotting Detection Reagent (GE Healthcare, UK, Cat. No. 9812291). Antigen antibody reactions were detected using Uvitec Cambridge The FireReader imaging systems. Band intensity was measured with Image J software (NIH). The same membrane was then stripped using RestoreTM Western Blot Stripping Buffer (Thermoscientific, Cat. No. 2160887) and reprobed with α-tubulin antibody (Cell Signaling Technology, Singapore, Cat. No. 2144) as loading control.

Acrosin Expression

Immunocytochemistry (ICC) techniques were applied to detect the localization of acrosin expressions. The spermatozoa concentration was adjusted to 7x10 ^{5} spermatozoa/ml, centrifuged with cytospin (Sigma Chemical Co., Madrid, Spain), and smeared on the fixed slides in a 3% buffered formalin solution at room temperature for 20 minutes. After that, the slides were permeabilized with a solution of 0.2 M HCL and 0.1% Triton X-100 for another 20 minutes. To block unspecific protein binding, the slides were incubated with Background Sniper for 15 minutes. Then, a primary anti-acrosin antibody (Santa Cruz, CA, USA) was applied at a 1:50 dilution and left to incubate at 4°C overnight. The next day, Trekkie Universal Link (Starr Trek HRP Detection System, Biocare Medical) was used as a secondary antibody, followed by HRP, and incubated for 20 minutes. Finally, DAB chromogen was added, and the slides were dehydrated with alcohol and ethanol, and cleared with xylol. The results were calculated as the percentage of acrosin expression (%).

Acrosome reaction evaluation

Staining of spermatozoa was done with Fluorescein Isothiocyanate-Conjugated Pisumsativum (FITC-PSA) (Sigma, USA). Spermatozoa was washed, fixed with 50 µl of 1-3% paraformaldehyde (PFA), and incubated for 30 minutes. Then, 500 µl of PBS 7.4 was added and centrifuged, and pellets were taken. Spermatozoa suspension of 50 µl was applied to glass slides and allowed to dry for 90 minutes at -4oC. Then, the slides were dried and incubated with 10 drops of FITC-PSA in a dark room for 1 hour. The slides were washed with ddH2O for 5-7 times, after which DAPI was dripped and incubated for 10 minutes. The slides were washed again with ddH2O for 5-7 times. The slides were covered with glass cover. Using a fluorescence microscope (Olympus BX51, Tokyo, Japan), spermatozoa were randomly differentiated at 1000× magnification according to their acrosome fluorescence pattern. Only the fluorescence of the equatorial segment was considered as an acrosome reaction.

Statistical Analysis

The data was analysed using IBM SPSS Statistics version 26 to assess data normality. Paired t-tests were employed for two groups of related normal data (before and after), while the Mann-Whitney test was utilized for two groups of abnormal data. Post-thawing treatment data was evaluated for homogeneity. The analysis proceeded with the One-Way ANOVA test, followed by the LSD post hoc test if the data was homogeneous and showed statistical significance. If the data was non-homogeneous, Kruskal-Wallis test would be applied. Post Hoc analysis was conducted using independent t-tests for normal data and Mann-Whitney tests were used for abnormal data indicating statistical significance.

RESULTS

Effects of Glutathione Supplementation in Cryopreservation Media on Tyrosine Phosphorylation

The results showed the detection of tyrosine phosphorylation in the control and treatment groups with cryoprotectant modification and glutathione addition with bands detected, which were around ~73, ~52, and ~27 kDa (Figure 1). To ensure the accuracy of the striping results, the membrane was re-labelled with α-tubulin monoclonal antibody as a loading control (Figure 1). Furthermore, to measure the density of tyrosine phosphorylation bands of western blot results, ImageJ software was used.

From the results of the study, the average density ratio of tyrosine phosphorylation bands in the fresh (unfrozen) spermatozoa group (control group) (K0) was 2.35 ± 1.54%. Then, it increased in spermatozoa cryopreservation using cryoprotectant modification alone (GSH 0) (K1) by 2.90 ± 1.43%, then increased in spermatozoa cryopreservation using cryoprotectant modification plus GSH 1. 00 mM (P1) by 4.78±1.47%, then decreased in spermatozoa cryopreservation using modified cryoprotectant plus GSH 1.50 mM (P2) by 3.76±2.11%, and decreased again in spermatozoa cryopreservation using modified cryoprotectant plus GSH 2.00 mM (P3) by 1.50±0.47% (Figure 2). Statistical tests showed that there was no significant difference in the density ratio of tyrosine phosphorylation bands between the groups.

Acrosin Expression

The functionality of post-thawed spermatozoa was evaluated through observation of the acrosin enzyme found in the acrosome located in the head of the spermatozoa. (Figure 3).

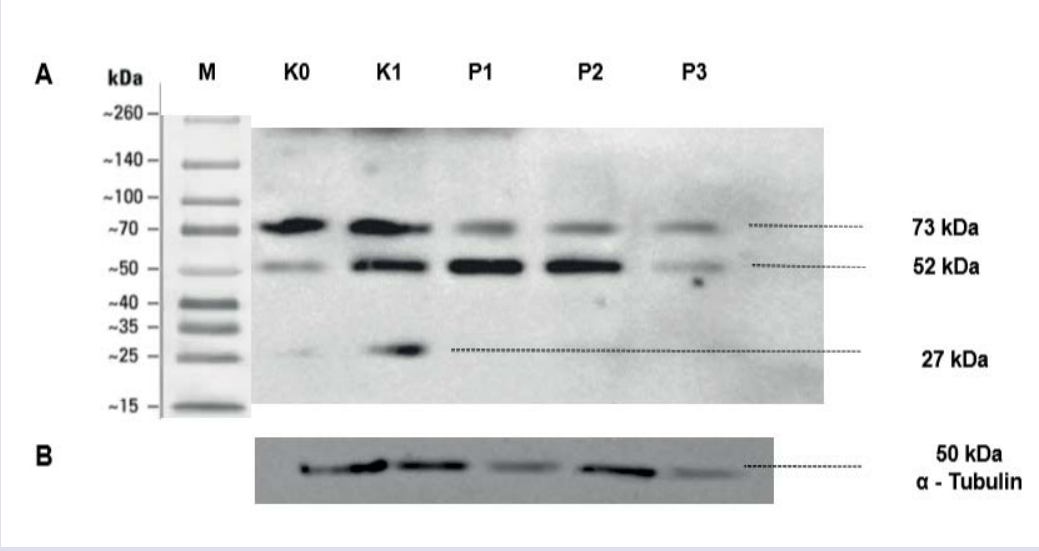

Figure 1. Western blot analysis of control and treatment groups with cryoprotectant modification and glutathione addition on tyrosine phosphorylation. (A) The band thickness of proteins with molecular weights of ~73, ~52, and ~27 kDa, respectively, is shown. (B) To ensure the same amount of protein loading in all lanes, the membranes were stripped with α-tubulin antibody. M = Marker, K0 = Fresh; K1 = modified cryoprotectant without GSH; P1 = with the addition of 1.00 mM GSH; P2 = 1.50 mM GSH; and P3 = 2.00 mM GSH.

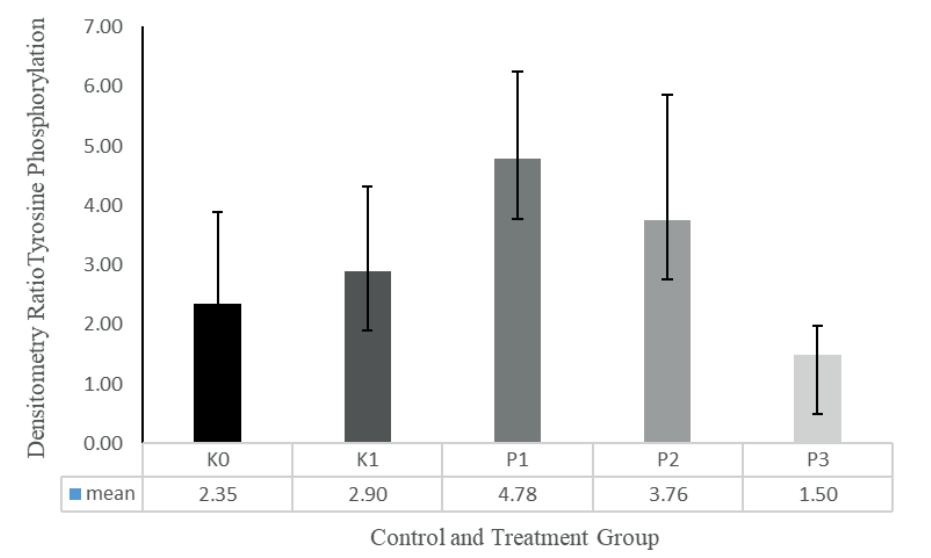

Figure 2. Densitometric Ratio of Tyrosine Phosphorylation. K0 = Fresh; K1 = modified cryoprotectant without GSH; P1 = with the addition of 1.00 mM GSH; P2 = 1.50 mM GSH; and P3 = 2.00 mM GSH.

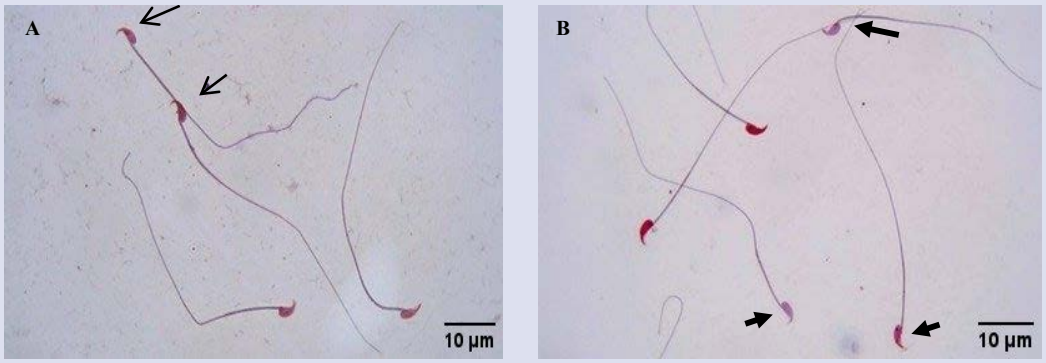

Figure 3. Acrosin immunocytochemical preparations of spermatozoa A) before cryopreservation and B) after thawing. Thin arrow: acrosin positive spermatozoa (strong brown); thick arrow: acrosin positive (weak and medium brown). Magnification = 1000x

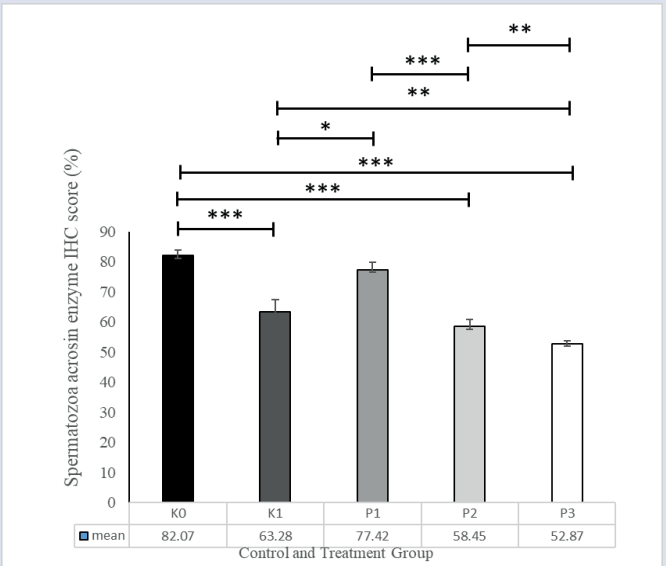

Figure 4. IHC score of acrosin enzyme in spermatozoa head (acrosome) of fresh and post thawing samples. K0 = Fresh; K1 = modified cryoprotectant without GSH; P1 = with addition of 1.00 mM GSH; P2 = 1.50 mM GSH; and P3 = 2.00 mM GSH. * p value < 0.05, ** p value < 0.01, *** p value < 0.001

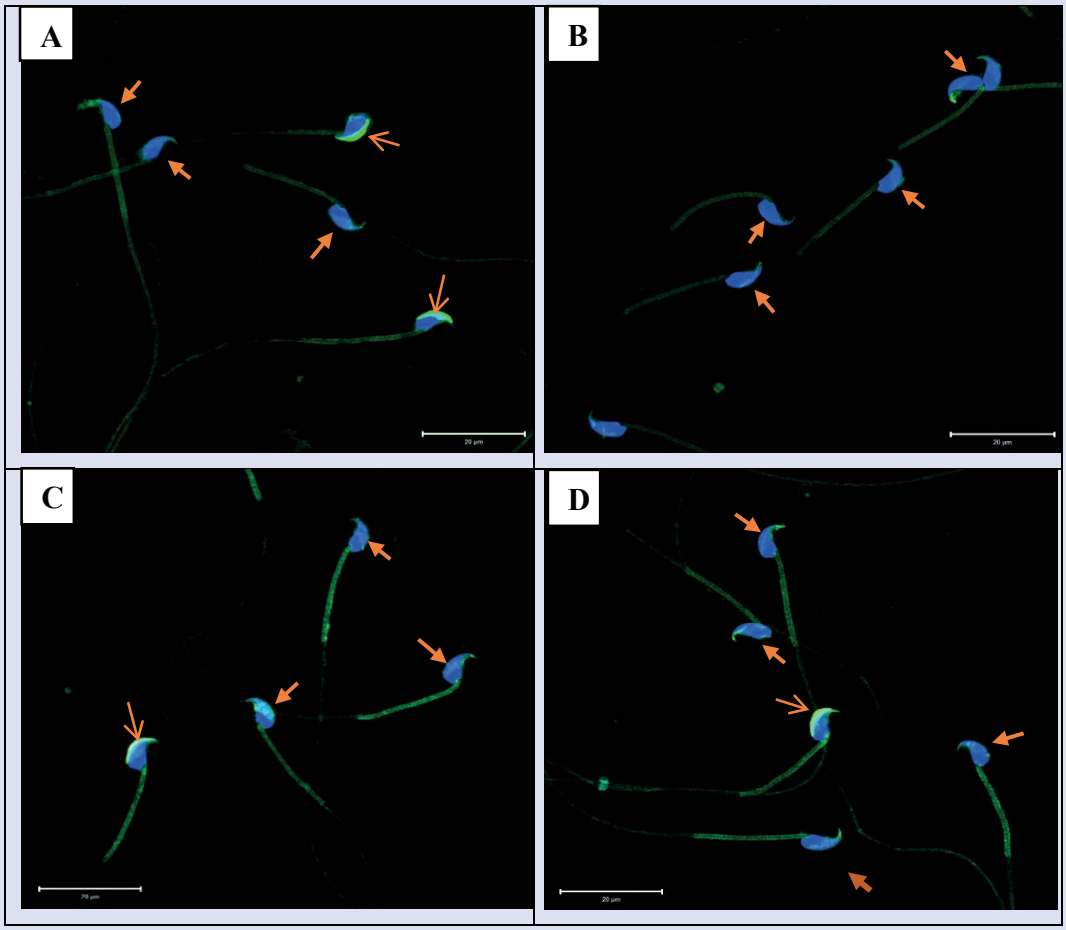

Figure 5: Spermatozoa acrosome reaction preparation A) before cryopreservation and B, C and D) after thawing. Thin arrow: negative acrosome reaction (intact acrosome); thick arrow: positive acrosome reaction (intact acrosome). Magnification = 63x

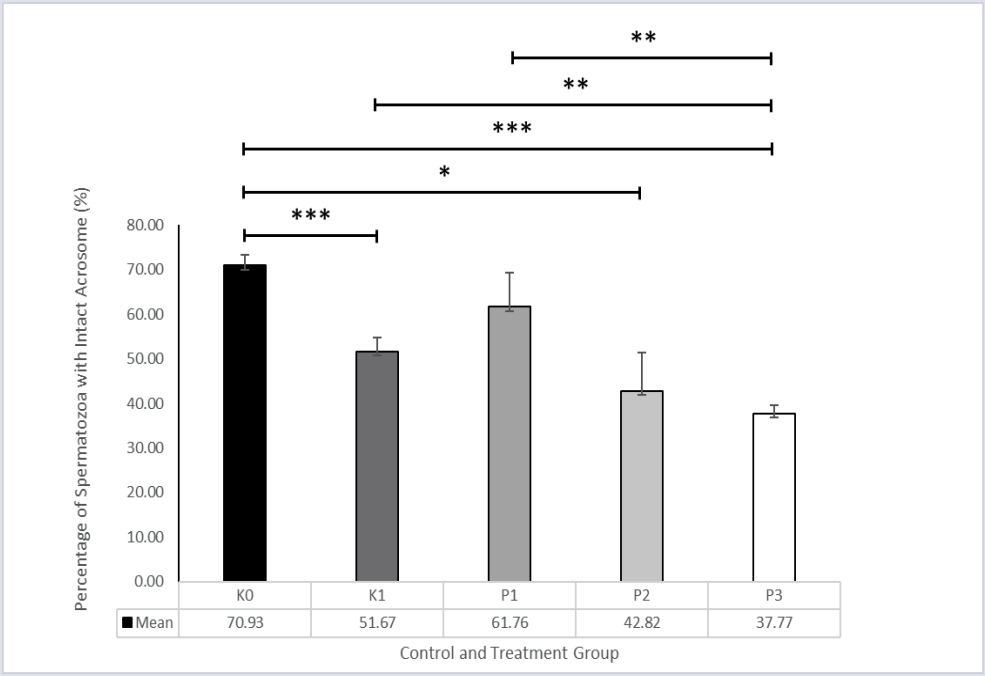

Figure 5. Percentage of Spermatozoa with Intact Acrosomes from fresh and post thawing samples. K0 = Fresh; K1 = modified cryoprotectant without GSH; P1 = with the addition of 1.00 mM GSH; P2 = 1.50 mM GSH; and P3 = 2.00 mM GSH. * p value < 0.05, ** p value < 0.01, *** p value < 0.001

The calculation of the IHC score of spermatozoa immunocytochemical preparations revealed a decrease in the IHC score after thawing (Figure 4). The availability of acrosin enzyme on the spermatozoa head in the acrosome section decreased, which was represented by the IHC score. Group P1 is a group of post-thawing spermatozoa that has the ability to maintain the availability of acrosin enzyme with the highest IHC score of 77.42 ± 2.54%. Based on statistical tests, there were significant differences between groups, both with fresh and post-thawing samples ($p < 0.05$).

Analysis of the Effect of Adding Different Doses of Glutathione to Modified Cryoprotectant on Acrosome Reaction

In the acrosome reaction parameter, spermatozoa with intact acrosomes (Figure 5) and acrosomes that have acted (Figure 5) were observed. In this study, it was found that group P1 was the treatment group most capable of maintaining the acrosome integrity of spermatozoa in the cryopreservation process. The addition of GSH antioxidant especially at a dose of 1.00 mM (Group P1) significantly affected the level of spermatozoa acrosome preservation during the cryopreservation process, as indicated by the p value of 0.035. While the P3 group had the lowest percentage of spermatozoa with intact acrosomes, even lower than the cryoprotectant modification group without the addition of glutathione.

DISCUSSION

In this research, the addition of GSH to the modified cryoprotectant aims to improve the quality of freezing-thawed spermatozoa. Spermatozoa quality is generally reduced during the freezing-thawing process and there is an increase in ROS. In this study, cryoprotectant modification was used by adding GSH with various concentrations, namely GSH 1.00 mM, GSH 1.50 mM and GSH 2.00 mM.

The results showed the detection of tyrosine phosphorylation in the control and treatment groups with cryoprotectant modification and the addition of glutathione. The density of tyrosine phosphorylation bands on western blot showed that proteins phosphorylated tyrosine residues have molecular weights of 73 kDa, 52 kDa and 27 kDa. At 27 kDa is the ADAM2 protein which plays a role in the fertilisation process such as the penetration of spermatozoa into the egg which is proteolysed from its initial 46 kDa. At 52 kDa ~ 55 kDa is the HSP90 protein (related to capacitation) and 73 kDa is the CD44 protein in humans as a hyaluronic acid receptor. Based on the results of statistical tests, it was found that there was no significant difference in the density ratio of tyrosine phosphorylation bands between the control and treatment groups.

Protein tyrosine phosphorylation (PTP) is one of the most important intracellular signalling events regulating spermatozoa function, and is an important indicator in capacitation. Various factors that affect PTP are cholesterol efflux, influx of HCO3, increased intracellular Ca2+, cAMP and reactive oxygen species (ROS). cAMP/PKA and extracellular signal regulated kinases (ERKs) are important signalling pathways involved in PTP. Cryopreservation exposes spermatozoa to frequent stressors, leading to changes in capacitation (cryo-capacitation). Capacitation in cryopreserved spermatozoa usually shows a PTP pattern that differs from normal in vitro spermatozoa capacitation.[3]

Research conducted by Sepideh J. et al reported that tyrosine phosphorylation in human spermatozoa was similar to that of mice[11] and boar.[12] Studies conducted on mouse spermatozoa using phosphotyrosine antibodies identified three proteins of 52, 75 and 95 kDa respectively. The 95 kDa protein showed an increase in immunoreactivity with the antibody after spermatozoa capacitation and interaction with oocyte ZP protein.[11]

Research conducted by Visconti PE et al. in humans showed the presence of four sets of proteins phosphorylated on tyrosine residues with different molecular weights. These proteins have molecular weights ranging from 95/94±3 kDa, 46±3 kDa, 25±7 kDa, to 12±2 kDa. The results indicate tyrosine phosphorylation of several proteins that play an important role in spermatozoa motility. Tyrosine phosphorylation of these proteins may affect their function and activity in regulating spermatozoa motility.[13]

In this research, cryopreserved spermatozoa had increased tyrosine phosphorylation possibly related to oxidative stress and environmental changes during the cryopreservation process. Oxidative stress can increase tyrosine phosphorylation because it causes changes in the activity of enzymes involved in the tyrosine phosphorylation process. In addition, the cryopreservation process can cause damage to cell membranes and organelles that can trigger cellular responses, including increased tyrosine phosphorylation. This is consistent with the statement that cryopreservation induces protein damage[14] and increases protein tyrosine phosphorylation in spermatozoa.[15]

In this research, the increase in tyrosine phosphorylation in spermatozoa cryopreserved with glutathione may be due to the protective mechanism of antioxidants against oxidative stress that occurs during the cryopreservation process. Oxidative stress can cause damage to spermatozoa cells, including changes in certain proteins that can trigger tyrosine phosphorylation. The administration of glutathione aims to protect spermatozoa cells from damage caused by oxidative stress. Thus, when spermatozoa cells are protected by the antioxidant glutathione, they may experience an increase in tyrosine phosphorylation in response to environmental changes and oxidative stress that occur during the cryopreservation process. [16,17]

A study conducted by Nadeem Shah et al. (2017) found that exogenous glutathione supplementation at a concentration of 0.5 mM decreased tyrosine phosphorylation of spermatozoa proteins especially p30, decreased spermatozoa cells showing positive immune reactivity to tyrosine phosphorylated proteins, caused a decrease in intact Acrosome Reaction of spermatozoa, and increased the percentage of spermatozoa showing high trans-membrane mitochondrial potential and a characteristic reduction in DNA fragmentation in freeze-thawed Hariana bull spermatozoa.[18]

Cyclic adenosine monophosphate (cAMP) can activate protein kinase A (PKA), which has an important role in the regulation of tyrosine phosphorylation in spermatozoa. Under normal conditions, increased levels of cAMP can activate PKA, which in turn regulates tyrosine phosphorylation on various protein targets in spermatozoa. This tyrosine phosphorylation can affect processes such as capacitation, acrosome reaction, and spermatozoa motility.[19]

In this research, the calculation of the IHC score of spermatozoa immunocytochemical preparations revealed a decrease in the IHC score after thawing. The availability of acrosin enzyme on the spermatozoa head in the acrosome section decreased, which was represented by the IHC score. Group P1 is a post-thawing spermatozoa group that has the ability to maintain the availability of acrosin enzyme with the highest IHC score and there were significant differences between control and treatment groups

This decrease in acrosin during cryopreservation can be caused by several factors, including extreme temperature changes, the effect of the cryoprotectant used, the occurrence of ice crystals, and osmotic stress. However, in the group given the antioxidant glutathione 1.00 mM, there was an increase in acrosin because glutathione is an antioxidant that can protect the acrosin enzyme from damage caused by free radicals during the cryopreservation process. Thus, the addition

of glutathione can minimise the damage to the acrosin enzyme and increase its activity after the cryopreservation process.

Acrosin is an enzyme found in spermatozoa, and its role is mainly related to egg penetration during the fertilisation process. The enzyme is produced by spermatozoa cells in the form of a precursor called proacrosin. Proacrosin is then activated into acrosin during the spermatozoa capacitation process, which is the process by which the spermatozoa undergo biological changes to enable egg penetration. The main function of acrosin is to help the spermatozoa penetrate the protective layer of the egg, called the zona pellucida. Acrosin contains proteolytic activity, which means that it can break down proteins in the zona pellucida, facilitating the penetration of spermatozoa into the oocyte for fertilisation.[20,21]

In addition to its key role in fertilisation, acrosin expression and activity may indicate spermatozoa quality. Disruptions in acrosin production or activation can potentially affect the ability of spermatozoa to fertilise the egg. Therefore, acrosin is one of important aspects considered in fertility research and evaluation. Changes in acrosin expression and activity can be affected by a variety of factors, including the health condition of the spermatozoa, exposure to environmental factors, and certain pathological conditions.[21]

At thawing, acrosin protein is the only acrosome protein which decreases. Acrosin-deficient male mice are still able to bind the zona pellucida but cause delayed fertilisation[22] and this, most likely, is due to delayed dispersion of acrosome proteins during the acrosome reaction.[23]

In this research, the group added with GSH 1.00 mM had the highest percentage of acrosin compared to the control and other treatment groups. In contrast to research conducted by Efren Estrada A et al (2015) explained that the addition of 2 mM GSH to cryopreservation media significantly increased acrosin activity, in line with higher spermatozoa survival rates.[24]

In this research, the addition of GSH antioxidant especially at a dose of 1.00 mM (Group P1) significantly affected the level of spermatozoa acrosome preservation during the cryopreservation process. The P3 group had the lowest percentage of spermatozoa with intact acrosomes, even lower than the cryoprotectant modified group without the addition of antioxidant.

The decrease in the percentage of intact acrosomes can be caused by the cryopreservation technique used, cooling speed, cryoprotectant concentration and oxidative stress can also affect the success in maintaining the acrosome structure. Changes in temperature that are too fast or too slow during the cryopreservation process can also cause damage to the acrosome. Likewise, the concentration of cryoprotectant used in the cryopreservation process can also affect the integrity of the acrosome. During the cryopreservation process can increase oxidative stress in spermatozoa, which can cause damage to cell structures including the acrosome, the addition of antioxidants, namely glutathione in cryoprotectant modifications can reduce oxidative stress and maintain the integrity of the acrosome which can increase fertilisation success. This is in accordance with the statement of Neild et al. (2005) that for successful fertilisation, spermatozoa must have an intact acrosome to be able to perform the function of the acrosome reaction at the right time, release enzymes and facilitate spermatozoa in penetrating the zona pelusida.[5]

Acrosome integrity is key to successful fertilisation. Only spermatozoa with intact acrosomes are able to penetrate the zona pellucida and fuse with the oocyte plasma membrane, so acrosome integrity is an important indicator of the fertility of frozen spermatozoa.[25]

Under physiological conditions, spermatozoa cells produce ROS at low and controlled levels and these ROS, as second messengers, have many roles in various spermatozoa functions (e.g. capacitation, acrosome reaction, and fertilisation).[26] Cryopreservation can cause premature capacitation and spontaneous acrosome reaction. Early capacitation caused by cryopreservation decreases the ability of spermatozoa in the female reproductive tract accompanied by the loss of the acrosome reaction.[27]

Excessive ROS formation or a low concentration of antioxidants in semen leads to oxidative stress (OS), which results in the production of oxygen-derived oxidants, and in turn has adverse effects on spermatozoa)[28,29] OS can impair many spermatozoa functions at the plasma membrane (e.g. motility, viability, acrosome reaction and spermatozoa-oocyte fusion) and DNA integrity (e.g. fertilisation, embryo development, implantation and maintenance of pregnancy)[26, 28, 30]

To neutralise the effects of OS and improve the quality of spermatozoa, antioxidants should be added. In this research, the addition of antioxidant GSH 1.00 mM showed the highest percentage of acrosome reaction. GSH is one of the antioxidants added to various semen specimens. It is a tripeptide thiol (ÿ-glutamyl cys teinyl glycine) with several biological functions that is widely found in the animal body, not only in somatic cells but also in gametes. This thiol plays an important role in the antioxidant process of endogenous and exogenous compounds, as well as in the maintenance of intracellular redox conditions. GSH is a natural reservoir of redox power, which can be rapidly utilised to defend cells against oxidative stress.[31] t is synthesised from the amino acids glutamate, cysteine, and glycine. Its reductive power is used to retain thiol groups in intracellular proteins and other molecules. It acts as a physiological reservoir of cysteine and is involved in the regulation of protein synthesis, cellular detoxification, and leukotriene synthesis. Glutathione's protection against oxidative damage is provided by its sulphydryl (SH) group, which can be presented in the form of reduced glutathione (GSH) and oxidised glutathione (GSSG). The attack of GSH against ROS is supported by interactions with enzymes, such as gluta thione reductase and glutathione peroxidase (GPx)[31]

Spermatozoa capacitation and acrosome reaction are two important steps in the fertilisation process. The presence of a normal acrosome in the spermatozoa cell is essential for the acrosome reaction, necessary for fertilisation to occur at the correct time.[32] Therefore, evaluation of the acrosome reaction is crucial in assessing the ability of spermatozoa to fertilise.[33]

This research is different from research conducted by R. Masoudi et al. (2019) which found that the use of glutathione dose of 4 mM with high glutathione concentration showed higher acrosome integrity than other groups, indicating that the addition of this antioxidant can be useful in improving acrosome integrity.[34] Similarly, research conducted by Gadea et al. (2013) found that the use of glutathione doses of 5 mM with high glutathione concentrations affected the percentage of viable spermatozoa with plasma membrane disorders and also the percentage of viable spermatozoa with intact acrosomes.[35]

High doses of antioxidants can have adverse effects, where antioxidants may scavenge even the physiologically necessary levels of free radicals, causing detrimental effects on cell survival. Therefore, it is necessary to use precise dose of antioxidants to gain the most optimal results.

CONCLUSION

Glutathione supplementation in modified CPA can increase tyrosine phosphorylation, acrosin expression and acrosome reaction of frozen-thawed spermatozoa. In general, treatment using GSH at a dose of 1.00 mM was considered the most effective, and modification of CPA with the addition of glutathione can improve the tyrosine phosphorylation, acrosin expression and acrosome reaction in cryopreserved spermatozoa.

ABBREVIATION

ARF-IMERI, Animal Research Facility – Institute of Medical Education and Research; DDY, Deutchland Denken Yoken; ROS, Reactive Oxygen Species. GSH, Glutathione; CPA, Cryoprotectant.

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CONFLICT OF INTERESTS

The author declares no conflict of interest.

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