Myo-inositol ameliorative effects on cryo-survival of post-thawed abnormal human sperms quality towards infertility care

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Abstract

Background
Sperm cryopreservation usually results in a significant decrease in sperm quality after thawing. The deleterious effects of freeze-thaw on abnormal sperm samples are considerably more severe. Various supplemental agents have been studied to ameliorate the negative effects due to cryopreservation. Myo-Inositol is likely to positively affect fertility. Therefore, this study aims to evaluate the beneficial effect of Myo-inositol (MI) supplementation in sperm freezing media for abnormal sperm.
Methods
Semen samples were collected from 23 male subjects that have abnormal sperm quality. Semen analysis has been conducted before freezing (pre-freeze). The samples were frozen with only a freezing medium (control) or MI-supplemented freezing medium. Semen analysis was re-evaluated after post-thaw for both groups. GoldCyto test kits were used in both post-thaw samples to evaluate the DNA fragmentation.
Results
The total motility, Progressive motility, morphology, and viability mean values of the sperms frozen with the MI-supplemented medium are greater compared to the control group ($p<0.05$). The post-thaw sample with MI supplementation (39.8%) has a higher cryo-survival rate (CSR) compared to the control group (23.4%).
Conclusion
MI supplementation in cryopreserved abnormal sperm samples significantly enhanced the post-thaw sperm motility, morphology, and vitality, and potentially increase the sperm survival rate.

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Introduction

Sperm cryopreservation is a technique that freezes the sperm in an extremely cold environment usually minus 196°C. Since the 1970s, sperm cryopreservation or freezing has been broadly utilized to treat infertile couples (Rozati et al., 2017). Sperm cryopreservation is an essential technique for managing male fertility on the assisted reproductive technique (ART) at most sperm analysis laboratories, particularly reproductive centers. It is frequently used to preserve male fertility before cytotoxic chemotherapy for cancer, radiation, or certain surgical procedures that may result in testicular failure or ejaculatory dysfunction (Donnelly et al., 2001; Shankara et al., 2019).

Although sperm cryopreservation methods have improved, detrimental effects on the sperm plasma membrane that reduces motility, morphology, viability, and acrosomes can still be

present (Akhtar et al., 2022; Ozkavukcu et al., 2008). In addition, the conventional methods, using sperm freeze solution had caused a significant decrease in sperm Progressive motility, viability, and normal morphology after conventional freezing (Le et al., 2019). Temperature fluctuations, intracellular ice formation, osmotic stress, and oxidative damage cause poor sperm quality after thawing (Rios & Botella, 2019). Cryopreservation has been shown to reduce fertilization rates by affecting the acrosome structure, acrosin activity, and Deoxyribonucleic acid (DNA) (Sun et al., 2021; Zribi et al., 2010), thus disturbing cells' genetic information (Kopeika et al., 2015).

Myo-inositol (MI) has been used to improve male reproductive function for instance, one study reported a significant increase in sperm motility during the treatment with Myo-inositol (Ghasemi et al., 2019). MI is present throughout the human male reproductive system, with concentrations rising throughout the epididymis and the vas deferens (Condorelli et al., 2012; Ghasemi et al., 2019; Hinton et al., 1980). MI is mainly synthesized by Sertoli cells and is involved in processes that include the regulation of motility, capacitation, and acrosome reaction of sperm cells (Bevilacqua et al., 2015; Condorelli et al., 2017). It seems to control seminal plasma osmolarity and volume, as well as the expression of proteins required for embryogenetic development and sperm chemotaxis, sperm motility, and acrosome response (Braich et al., 2022; Fisher et al., 2012). Researchers have attempted to exploit the antioxidative nature of MI in treating human infertility issues. It has been reported that MI is an antioxidant agent that pivotal role in the systemic treatment of male infertility and for the enhancement of the in-vitro quality of the sperm used in assisted reproductive procedures (Bevilacqua et al., 2015). A few studies have shown that MI supplementation of cryopreserved semen increased the post-thaw sperm quality (Calogero et al., 2015; Condorelli et al., 2012; Saleh et al., 2018). However, no study indicates the use of MI in cryopreservation in abnormal sperm samples. Cryopreservation often relates to a negative influence on post-thaw sperm. It is a necessary method to reduce cryodamage to abnormal sperm furthermore to boost the post-thaw quality of abnormal sperm. Therefore, this study was conducted to investigate the effect of MI-supplemented freezing media on abnormal sperm.

Materials and methods

Ethical consideration.

This study was approved by the Malaysia Research Ethics Committee (MREC), National Medical Research Register (NMRR: NMRR-20-151-52655-IIR), and the IIUM ethics committee (IREC: KAHS 143/2020). All participants were informed, and written consent was obtained if the subjects are willing to participate in this study without any coercion.

Preparation of Myo-inositol (MI) supplementation.

Myo-inositol (MI) powder (product no. I7508-50G; Sigma Aldrich Co, St. Louis, MO, USA) at 500 mg was weighed using a digital scale with 0.01 g accuracy (Mettler, Toledo, USA) and dissolved in 5 ml of sperm washing media (Fertipro, Belgium). The prepared solution was stored in a refrigerator between 2°C to 8°C temperature and kept away from direct light.

Sample collection.

The study was conducted at the Cytology Laboratory, Pathology Department of Sultanah Bahiyah Hospital, Alor Setar, Kedah, Malaysia. The sperm samples (n=23) were collected between June 2020 to June 2021. Abnormal sperm samples were collected from men who visit the infertility clinic for a routine checkup including oligozoospermia, asthenozoospermia, teratozoospermia, and either combination. Semen samples with azoospermia and leukocytopenia were excluded from this study. Patients must refrain from sexual activity for 48 to 72 hours before semen analysis. Masturbation was used to acquire a sperm sample, which was collected in a sterile container. The sperm parameters assessment was done before and after freezing which includes sperm count, motility, morphology, and viability.

Semen analysis.

Semen samples were analyzed before the initiation of sperm cryopreservation. The sperm specimens were left at room temperature for at least 20 minutes before being assessed. Semen samples were analyzed for sperm concentration, motility, morphology, and viability after

liquefaction. Sperm count and motility were evaluated by using the Makler counting chamber (Sefi Medical Instruments, Haifa, Israel).

Sperm cryopreservation.

After the pre-freeze semen analysis, the sample was split into two equal aliquots (0.5 mL each). One aliquot of semen contains 0.5 mL of sperm freezing media (Fertipro, Belgium) and 10 µL/mL of MI (1 mg/mL) whereas the second aliquot of semen consists of 0.5 mL of sperm freezing media and 10 µL/mL of sperm washing (Fertipro) as a control. Both aliquots were incubated for at least 10 minutes at room temperature before cryopreservation. The rapid freezing technique was used to preserve the sperm. Sperm freezing medium (Fertipro) was warmed up to room temperature for at least 30 minutes before cryopreservation. The freezing medium was dropped into each aliquot and mixed thoroughly to obtain 1:1 dilution to avoid osmotic stress. The sample was loaded into a cryovial which was labeled with the subject's name, type of sample, and identification number. The vial was hung in liquid nitrogen vapor for 30 minutes and submerged in a liquid nitrogen tank (at -196°C) for two months to complete the experiment.

Post-thaw sperm recovery assessment.

The storage sample was taken out of the storage tank to thaw after two months of cryopreservation. The cryovial was left at room temperature for at least 20 minutes. Both samples of control and supplemented were assessed again to quantify sperm count, motility, morphology, and vitality according to the World Health Organization, 2010 criteria. DNA fragmentation index was also evaluated for both samples.

Diff Quik staining.

Smear 5-10 µl of the semen sample on the glass slide and let the slide dry at room temperature. Then, the slide was fixed with fixative, triarylmethane for 15 seconds. Then the slide was immersed for 10 seconds in rapid stain solution 1, and 5 seconds in rapid stain solution 2 before running in tap water for 10 seconds. The slide was then dried vertically on absorbent paper. The stained slide was examined under a bright-field optics microscope at 100x magnification with immersion oil. The number of normal and abnormal sperm is calculated in approximately a total of 200 sperm per slide.

Sperm vitality.

The eosin-nigrosine staining technique was used for sperm vitality by using the Vital Screen kit (FertiPro, Belgium). The sample was mixed thoroughly, and then 50 µl of semen in a test tube was stained with 2 drops of eosin for 30 seconds and 3 drops of nigrosine for 30 seconds. Put 20 µl of the semen stain mixture on a slide and immediately covered it with a coverslip on top which was assayed using a light microscope for determining the percentage of live sperm. A total of 100 sperms were counted for each case. White or unstained sperm were classified as live sperm, whereas pink or red sperm were classified as dead sperm.

DNA fragmentation test.

The GoldCyto Sperm DNA kit (GoldCyto Biotech Corp, Hong Kong, China) was used for sperm DNA fragmentation analysis. Eppendorf tube containing agarose gel was floated in water at 90°C to 100°C for 5 minutes until the agarose gel dissolved. The agarose tube was transferred to a temperature-controlled water bath at 37°C and left for 5 minutes until the temperature was even. 30 µl of sperm sample was immersed in agarose microgel and mixed well immediately, and 20 µl of the mixture was placed onto the pretreated coated glass slide and a coverslip was placed to spread to the edge of the slide. Then, the slide was placed on a cold plate for 5 minutes at 4°C to solidify with the spermatozoa embedded within. The coverslip was gently removed when the agarose polymerized and cooled at 4°C, and the slide was horizontally dipped in a denaturation solution for 7 minutes at room temperature. After that, the slide was placed horizontally in a lysis solution and incubated for 25 minutes. Following that, the slide was rinsed with distilled water for 5 minutes to remove the lysis solution. The slide was fixed in 70%, 90%, and 100% ethanol baths for 2 minutes each and dried at room temperature before staining. The slide was stained with Wright's solution and cleaned with water after drying. Spermatozoa with a big halo or medium are considered as having no DNA fragmentation whereas spermatozoa with a small halo and without a halo or degraded are considered as DNA fragmentation. DNA fragmentation index (DFI) was determined by multiplying a total of

100 sperms that were counted with the number of sperm with fragmented DNA divided by the number of sperm counted. According to the manufacturer's guidelines, a sperm DFI score of more than 30% was deemed abnormal.

Analysis of data.

IBM SPSS 22.0 was used to analyze the data (IBM Corp., Armonk, NY, USA). The pre-freeze and post-thaw semen analysis findings are shown as the mean standard error of the mean (SEM). The Shapiro-Wilk normality test was used to assess the normality of the data. All data were evaluated using the independent samples, while Mann-Whitney U Test was applied to determine the significance level since the data is not normally distributed. A p-value less than 0.05 is considered a statistically significant difference.

Results

In this study, a total of 23 surplus semen samples with abnormal sperm quality (lower than the WHO reference limit) were obtained for data analysis. The results of sperm count, total motility, progressive motility, morphology, and viability before and after freezing, and with and without MI supplementation are summarized in Table 1. The post-thaw sperm concentration showed that the sperm concentration was higher in the sample with 1 mg MI supplementation (8.3 ± 1.6) than in the control (7.6 ± 1.5). However, there is no significant difference as the p-value is greater than 0.05.

The results indicated that the post-thaw mean of total motile (TM) sperm in MI-supplemented samples (14.4 ± 1.3) is higher compared to the control (8.5 ± 0.9. There was a significant difference in TM mean between pre-freeze and both post-thaw groups ($p < 0.001$). Furthermore, the progressive motility (PM) for MI-supplemented samples (1.8 ± 0.5) showed a significant difference in comparison with the control (0.3 ± 0.2) with a p-value is 0.007. In addition, Table 2 shows the post-thaw cryo-survival rate (CSR) of total motility (TM) exhibited a higher survival rate in MI supplemented sample (39.8%) compared to the control (23.5%). The post-thaw cryo-survival rate (CSR) of progressive motility (PM) indicated a higher survival rate in MI supplemented sample (12.3%) compared to the control (2.1%).

Moreover, Table 1 also shows the result of the mean of morphology showed that MI-supplemented samples (11.8 ± 1.1) and control samples (7.4 ± 1.3) which the former better than the latter. Figure 1 shows the representation of the abnormal sperms that have been stained with Diff Quik staining before and after the cryopreservation process.

The result of sperm viability in the post-thaw, the MI-supplemented sample (30.5 ± 3.3) had higher sperm viability compared to the control sample (20.6 ± 2.4). Based on the findings, it shows a significant difference ($p<0.05$). Figure 2 depicts the vitality/viability of the sperm that has been stained with eosin-nigrosin.

The post-thaw result of deoxyribonucleic acid (DNA) fragmentation is presented in Table 3. Samples supplemented with 1 mg/mL Myo-Inositol showed a reduced percentage of sperm with DNA fragmentation after freezing as compared to the control even though no statistical differences were found between the groups (18.4 ± 2.4 versus 24.6 ± 2.9). Figure 3 illustrates the DNA fragmentation of the post-thaw sample treated with and without MI.

Discussion

The process of sperm freezing and thawing is linked with various degrees of sperm cryodamage, which results in diminished post-thaw sperm recovery. Advancements have been made in sperm cryopreservation over the last several decades. The freezing and thawing techniques would always result in sperm degradation, which would have unfavorable repercussions. Recent advances have been made in sperm cryopreservation approaches. However, the cryopreservation process still has a detrimental impact on sperm quality. It has been reported that the sperm cryopreservation process damages its quality by the alterations of sperm function that are associated with reduced sperm viability and motility, and DNA fragmentation (Ponchia et al., 2021). Several supplements have been studied to ameliorate the degree of cryodamage of human sperm cryopreservation and enhance the post-thaw

Table 1: The comparison of sperm parameters before and after freezing in abnormal sperm samples supplemented with and without Myo-inositol.

Parameters*	Pre-freeze	Post-thaw		P-value		
		Control	MI	a	b	c
Concentration	9.8 ± 1.8	7.6 ± 1.5	8.3 ± 1.6	0.48	0.32	0.59
Total Motility (TM)	36.2 ± 3.6	8.5 ± 0.9	14.4 ± 1.3	< 0.001	< 0.001	< 0.001
Progressive motility (PM)	14.6 ± 3.8	0.3 ± 0.2	1.8 ± 0.5	< 0.001	< 0.001	0.01
Morphology	16.4 ± 1.9	7.4 ± 1.3	11.8 ± 1.1	0.02	< 0.001	0.01
Viability	56.8 ± 3.5	20.6 ± 2.4	30.5 ± 3.3	< 0.001	< 0.001	0.02

*The values are shown as the mean and standard error of the mean (SEM). Statistical significance was determined by p-values of 0.05. a: pre-freeze vs post-thaw in the control group; b: pre-freeze vs post-thaw in the MI group; c: post-thaw in the MI group vs. the control group. Sample size=23 equally apportioned to both treatments

Table 2. The comparison of cryo survival rate (CSR) of pre-freeze and post-thaw sperm motility in abnormal sperm samples with and without MI supplementation.

Parameters*	Pre-freeze	Post-thaw		CSR	
		Control	MI	a	b
Total Motility (TM)	36.2 ± 3.6	8.5 ± 0.9	14.4 ± 1.3	14.4 ± 1.3	23.4%
Progressive motility (PM)	14.6 ± 3.8	0.3 ± 0.2	1.8 ± 0.5	1.8 ± 0.5	2.1%

*The values are shown as the mean and standard error of the mean (SEM).
The CSR = post-thaw value / pre-freeze value x 100; presented in percentage,
a: control vs pre-freeze, b: MI vs pre-freeze.
Sample size = 23; equally apportioned for all treatments

Table 3: The comparison of sperm parameters before and after freezing in abnormal sperm samples supplemented with and without Myo-inositol.

Parameter*	Post-thaw		
	Control	MI	P-value
DNA fragmentation	24.6 ± 2.9 (11-70) (n=23)	18.4 ± 2.4 (6-54) (n=23)	0.07

*The values are shown as the mean and standard error of the mean (SEM).
Statistical significance was determined by p-values of 0.05
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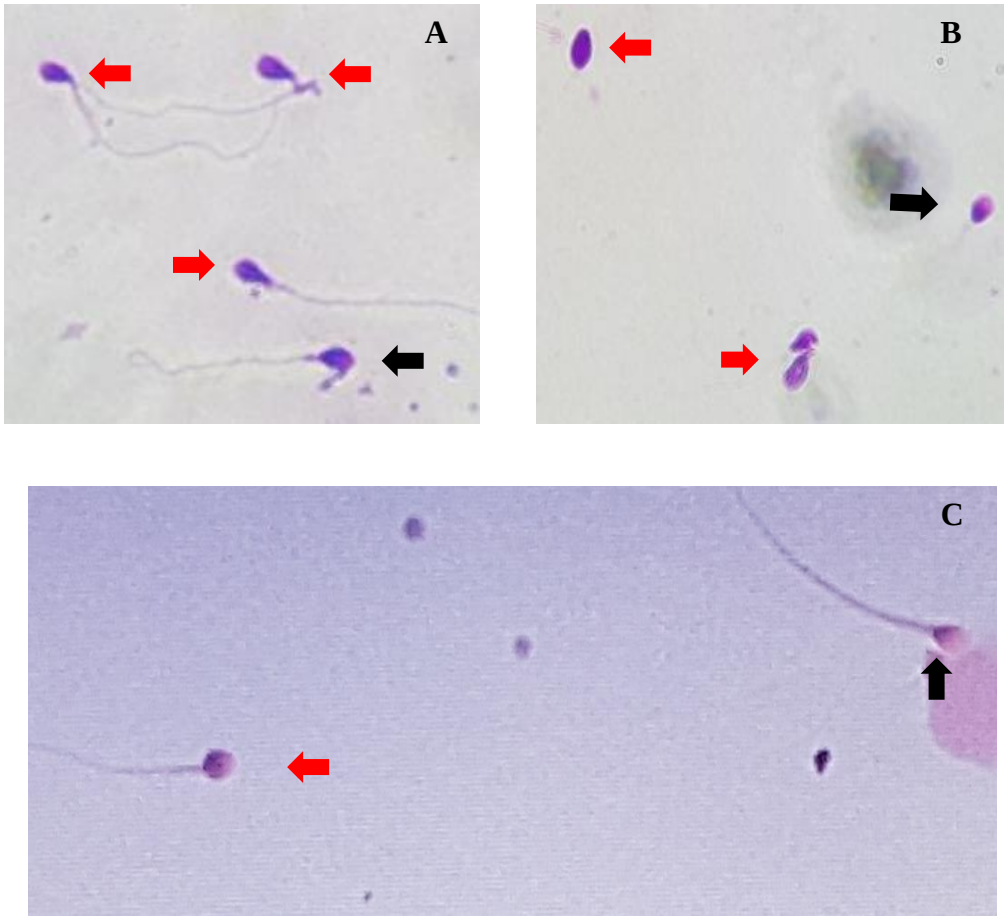

Figure 1: Sperm morphology; (A) Pre-freeze, (B) Post-thaw without MI (control), (C) Post-thaw supplemented with MI. The red arrows point to the abnormal sperm while the black arrows point to the normal sperm.

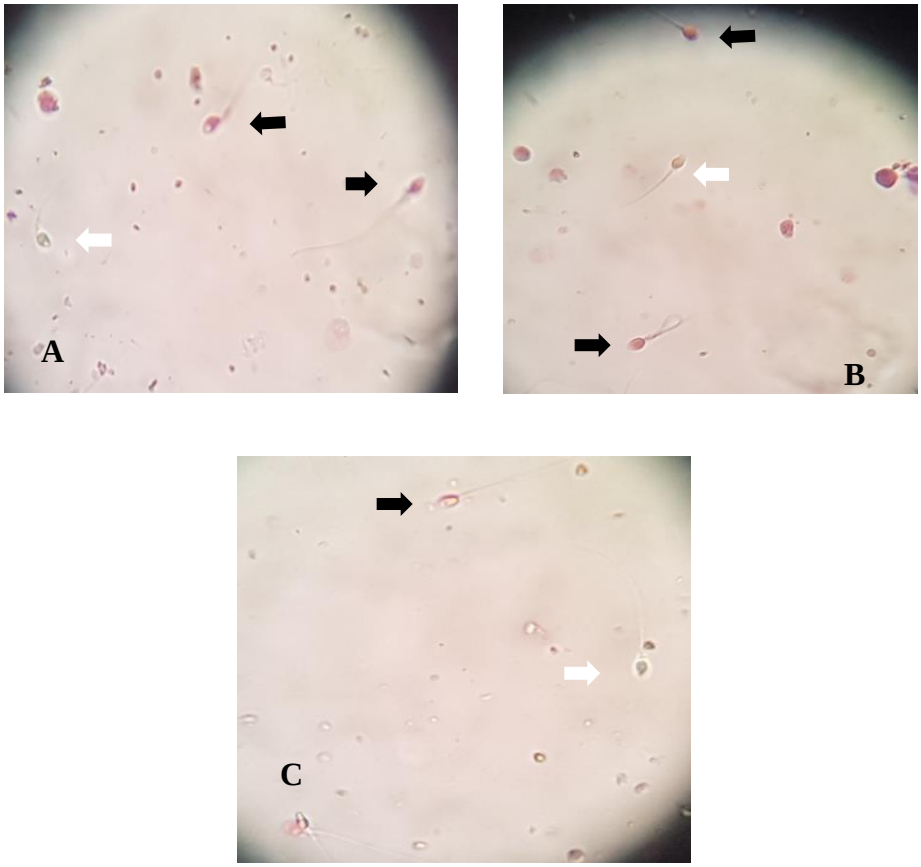

Figure 2: The morphology of the sperm vitality; (A) Pre-freeze, (B) Post-thaw (control), (C) Post-thaw supplemented with MI. Living sperm are pointed with white arrows while dead sperm are pointed with black arrows.

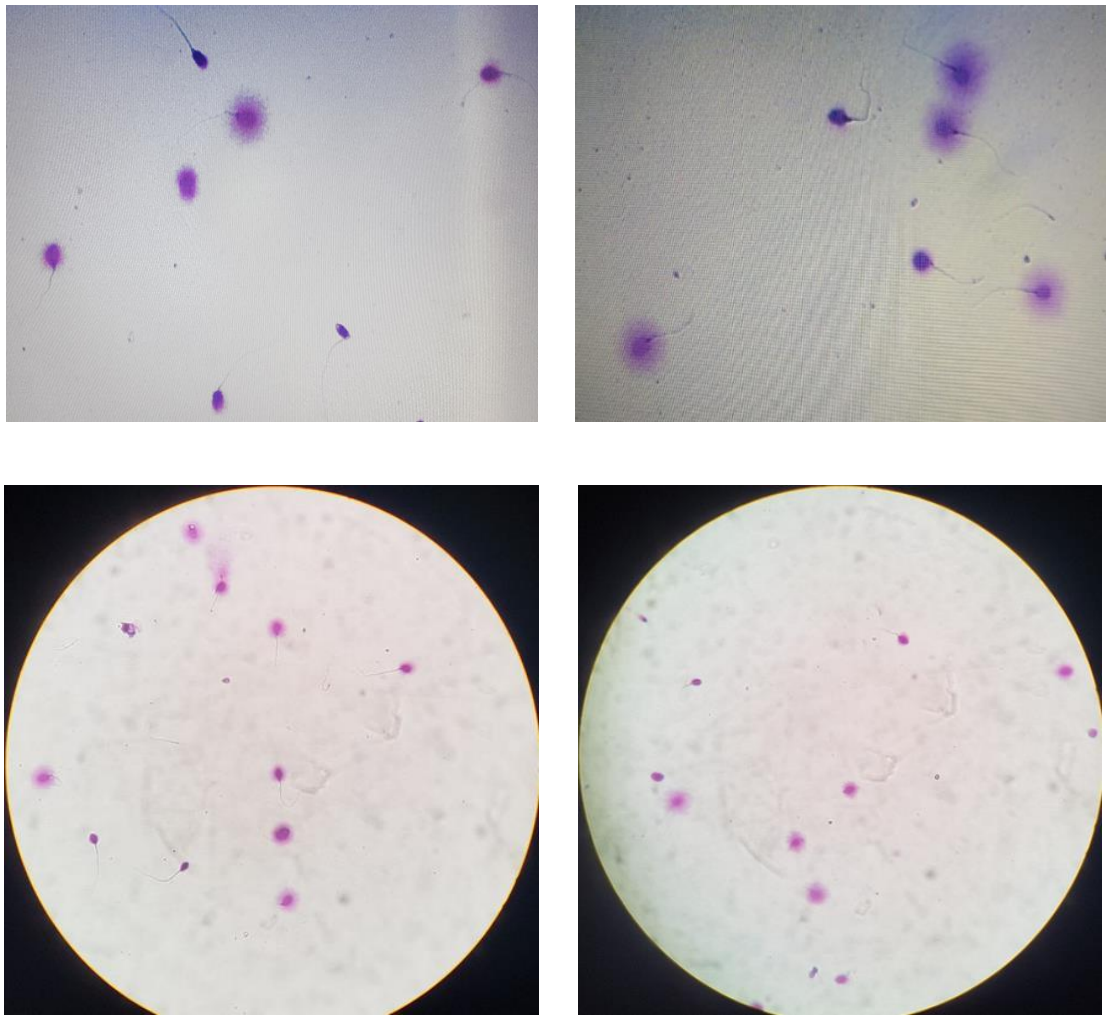

Figure 3: DNA fragmentation of the post-thaw samples; (A) Control sample, (B) Supplemented with MI.

quality, such as vitamin E (Taylor et al., 2009), glutathione (Gadea et al., 2011)22, melatonin (Karimfar et al., 2015), and Myo-Inositol (Saleh et al., 2018).

The results of this study found that post-thaw sperm cryopreservation from infertile men resulted in a substantial decrease in sperm motility, morphology, and viability. This research found that samples frozen with MI supplementation in a freezing medium showed substantially greater improvement in sperm motility, morphology, and viability than the control samples. MI is well tolerated by

spermatozoa in vitro, resulting in an increase in sperm total and progressive motility in both fresh and thawed samples, suggesting that MI may be used as an antioxidant in laboratory practice and for the successful and safe generation of sperm samples in ART (Palmieri et al., 2016). Cryopreservation causes mitochondrial damage, which disrupts adenosine triphosphate (ATP) and affects sperm motility (Donnelly et al., 2001). In this study, the post-thaw motility result (14.4 ± 1.3 and 1.8 ± 0.5) indicates that MI supplementation potentially improves mitochondrial function and reduces the abruption of ATP because mitochondrial function consists of organelles that provide the energy needed for sperm activity (Condorelli et al., 2017). Similar findings were shown by a product, Andrositol® that contains MI could, improves sperm motility and morphology in asthenospermic men with metabolic syndrome (Montanino et al., 2016).

The post-thaw result in Table 1 showed that the MI-supplemented sample positively impacted sperm morphology more than the control sample. The application of 1 mg/mL MI to sperm freezing media before cryopreservation reduced the rate of sperm morphological deterioration. MI may decrease the impact of cryopreservation on morphology by decreasing the amorphous material surrounding sperm cells (Calogero et al., 2015). MI was also found to play a role as an antioxidant agent in the oligoasthenoteratospermia (OATS) sample which can reduce ROS (reactive oxygen species) production during the cryopreservation process. It has been proven possible to ameliorate the effect on the sperms' inner membrane and mitochondria (Colone et al., 2010). Alterations in cellular osmolality caused by extracellular ice formation during cryopreservation induce morphological changes.4 Based on this finding; MI supplementation may decrease the rate of sperm morphological damage by decreasing extracellular ice formation. This indicates that the MI-supplemented sample had considerably better post-thaw morphology than the control group.

Sperm survival (sperm viability) is one of the essential characteristics to consider when evaluating cryopreservation outcomes. The eosin-nigrosine stain has been used to determine whether immotile sperm was dead or alive. The percentage of live sperm in the MI-supplemented sample was significantly higher than in the control sample after thawing (Table 1). In the control group, the viability reduced after thawing showed that sperm cell membrane damage occurred because of physical and chemical stress during cryopreservation. MI supplementation in sperm freezing ameliorates the sperm plasma membrane integrity, contributing to higher live sperm counts. It has been reported that MI supplementation can improve membrane integrity and viability, indicating that MI is protective against oxidative stress (Qamar et al., 2019). In addition, MI supplementation can enhance semen parameters in oligoasthenozoospermia groups and is a helpful therapy in enhancing the chance of reproductive outcomes (Gulino et al., 2016). The supplementation of MI to the freezing media before cryopreservation resulted in a higher increase in survival rates than in samples that did not have MI supplementation (Table 2). This rise was observed in both motility groups. This finding is consistent with recent research, which found an increase in CSR in samples treated with MI before cryopreservation (Saleh et al., 2018).

DNA fragmentation test was used to examine the effects of MI supplementation on abnormal sperm quality by administering MI to sperm freezing media before freezing. Results showed that there was no significant difference between both groups even though the p-value was low ($p=0.07$) (Table 3). Despite this, the results suggest that MI has a good impact on low sperm quality by reducing DNA fragmentation when compared to the control. Cryopreservation has previously been shown to harm sperm DNA integrity due to oxidative stress during the freezing and thawing processes (Kalthur et al., 2011). Our study showed that adding MI into sperm freezing media can lower the DNA fragmentation rate of abnormal sperm quality. Adding MI into the freezing media could be an effective way to lower the incidence of DNA fragmentation and improve fertility outcomes. This finding is comparable to the studies using normal samples, which found that adding MI to the freezing media before cryopreservation can reduce the DNA breakdown after thawing (Kalthur et al., 2011). MI acts as an antioxidant agent that may defend sperm DNA from ROS

that induces DNA instability (Mohammadi et al., 2019).

Conclusion

In conclusion, MI-supplemented freezing media resulted in a significant improvement in sperm parameters on cryopreserved abnormal sperm quality. The supplementation of sperm freezing medium with 1 mg/ml of Myo-inositol (MI) can enhance the post-thaw ameliorative effects on human sperm parameters including sperm motility, morphology, viability, and DNA fragmentation. Based on the findings, MI has a promising effect on improving the cryopreservation technique for abnormal sperm quality. The next step for future studies can explore the effects of MI supplementation in freezing media on assisted reproductive technology (ART) outcomes.

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