Application of Autologous Platelet-rich Plasma Exerts Cryoprotective Effects on Biological Characteristics of Human Oligoasthenoteratospermia Samples after Freezing and Thawing Procedures

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Purpose: Platelet-rich plasma (PRP) is enriched with active biological components which showed proliferative and cytoprotective properties in healing different injuries in medicinal fields. This study was designed to assess the cryoprotective effects of autologous PRP on the quality of oligoasthenoteratospermia (OAT) samples during freezing and thawing procedure.

Materials and Methods: The present study is an experimental research. Twenty OAT semen samples were obtained from individuals and prepared by discontinuous density – gradients technique DGC). The control group is sperm samples after DGC. After the procedure, the specimen was divided into four groups. The Freeze group which has no additive and the other three groups were cryopreserved with different concentrations of PRP (1×10^5/µL, 0.5×10^5/µL and 0.25×10^5/µL). Autologous PRP was provided by each participant. After thawing, sperm parameters, DNA fragmentation by sperm chromatin dispersion test (SCD), protamine deficiency by (Chromomycin A3) CMA3 staining, acrosome integrity and malondialdehyde (MDA) level were evaluated.

Results: Cryopreservation resulted in a significant decrease in all factors compared to the control group. There were no significant changes in sperm count, morphology, non-progressive motility and acrosome reaction by adding PRP as a cryoprotectant in comparison with the freeze group. PRP at all three concentrations showed a significant increase in progressive motility (3.05 ± 2.01 vs. 14.05 ± 4.13, 12.35 ± 4.90 and 12.15 ± 9.65, $P < 0.001$) and viability (36.85 ± 10.25 vs. 47.85 ± 5.86, 51.30 ± 5.54 and 50.05 ± 5.67, $P < 0.001$) compared to the sperm samples without PRP. The percentage of immotile sperms decreased at all PRP concentrations compared to the freeze group. Moreover, PRP at 1×10^5/µL concentration showed cryoprotective effects on DNA fragmentation, protamine deficiency and MDA level compared to the other three concentrations.

Conclusion: Cryopreservation and thawing procedures may exert adverse effects on biological factors of sperm samples. Therefore, adding PRP as cryoprotectant at all three concentrations especially 1×10^5/µL can be promising strategy to reduce adverse effects of cryopreservation on OAT samples.

Keywords: oligoasthenoteratospermia (OAT), platelet-rich plasma (PRP), cryoprotectant, sperm parameter, DNA fragmentation

INTRODUCTION

Cryopreservation is a known technology that is used extensively to preserve biological functions and properties of different tissues and cells for an extended period. This procedure has developed in various aspects during the last years[1]. Freezing of human spermatozoa is vastly applied in assisted reproductive technology (ART) centers to preserve the future fertility of men who have infertility due to different causes such as cancer, lupus, multiple sclerosis and ulcerative colitis diseases[2]. Moreover, this technology is used for individuals with varicocele, testicular torsion and bilateral vasectomy. Finally, cases with azoospermia, oligozoospermia and OAT and patients that undergo procedures like percutaneous epididymal sperm aspiration (PESA) or testicular sperm extraction (TESE) because of ejaculatory dysfunction or spinal cord injury may benefit from this technique[3].

Structural and functional properties of sperm are impaired during freezing and thawing procedures which decreases sperm motility, viability, mitochondrial membrane potential and DNA integrity[4]. Reactive oxygen species (ROS) generation is one of the leading causes of cryo-damage. Although ROS at physiological levels is essential for several sperm functions, high levels of this component have negative effects on sperm biological characteristics. Sperm cell is susceptible to oxidative stress due to plenty of plasma membrane polyunsaturated fatty acids[5]. It was reported that cryopreservation technique resulted in oxidative stress owing to a disturbance of balance between ROS generation and total antioxidant capacity (TAC). Furthermore, MDA is a byproduct of lipid peroxidation induced by oxidative stress and affects sperm parameters such as viability, motility and morphology[6]. To decrease adverse impacts of cryopreservation, optimization of freezing technique, carrier and medium have great importance [4]. Many studies improved the freezing results by adding different antioxidant supplements to the cryopreserved sperm medium. Based on these studies peptides,

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Table 1. The characteristics of the samples before and after density – gradients (DGC) technique.

Sperm parameters	Before DGC	After DGC
Count (106)	14.25 ± 1.2(7-13)	11.80 ± 1.9(8-14)
PMS (%)	15.18 ± 1.20(12-19)	15.25 ± 1.51(13-19)
NPMS (%)	30.10 ± 1.41(17-28)	22.10 ± 5.10(19-30)
NMS (%)	55.13 ± 1.20(35-52)	62.65 ± 4.66(54-70)
Viability (%)	50.30 ± 1.45(20-67)	52.60 ± 12.26(22-69)
Morphology (%)	1.30 ± 0.65(1-3)	1.85 ± 0.74(1-3)

fatty acids and plant extracts etc. reduce adverse effects of cryo-damage and apply beneficial effects on sperm quality[7-9].

Recently, PRP in several medical fields especially reproduction developed and achieved considerable attention due to its potential therapeutic effects[10]. These protective effects are associated with a variety of cytokines contents including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factors β (TGF-β), insulin-like growth factor I (IGF-I), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and peptide hormones[11]. Furthermore, granules of platelet enrich with factors such as adenosine triphosphate (ATP), ions (calcium and zinc), histamine, serotonin and superoxide dismutase (SOD) play key roles in regulating different mechanisms to preserve homeostasis of cells and tissues. Plenty of These factors show substantial functional effects on sperm quality and function. It was reported that TGF- β and VEGF increase sperm motility[12]. Additionally, VEGF removes ROS production through nuclear factor (erythroidderived 2)-like2 (Nrf2) pathway activation. IGF-I ameliorates sperm parameters (motility and viability) and maintains the integrity of mitochondrial and plasma membrane of cryopreserved sperm[13]. Zinc and calcium are essential ions for sperm capacitation and acrosome reaction[14,15]. Moreover, NGF, ATP, zinc ions, SOD and platelet-activating factors reduce cryo-injury and enhance sperm quality during freezing and thawing procedures. PRP elevates the level of antioxidant enzymes such as SOD and catalase in damaged tissues[16-19]. Therefore, due to the detrimental effects of cryo-injury on cryopreserved sperm quality and the protective effects of PRP, the main aim of this study is to investigate the impacts of autologous PRP on sperm parameters (count, motility, viability and morphology), DNA fragmentation and protamine deficiency, acrosome reaction and MDA levels after human sperm cryopreservation.

MATERIALS AND METHODS

Study participants

The present study is an experimental research. OAT sperm samples were obtained from 20 individuals aged 20 to 40, who were referred to the Yazd Reproductive Sciences Institute for infertility assessment and consent forms were completed by all cases. Individuals who have Sperm concentration less than 16 million, <42% total motility, and <4% normal morphology based on WHO guidelines 2020 for sperm processing were involved in the present study. The ethics committee of Yazd Reproductive Sciences Institute approved this study (IR.SSU.RSI.REC.1401.016).

Sperm samples preparation

Samples were provided by masturbation in a sterile specimen container after 2-7 days of sexual abstinence. The samples were incubated at 37°C for 10 to 30 min. For OAT samples, preparation was performed by discontinuous density–gradients technique. A 15 ml conical tube was used to prepare single layer 40 gradient medium (Perception, SAGE, USA) and then 1ml of semen sample was added on top of the density medium. Samples were centrifuged (Eppendorf, North America) with 300×g for 20 min. The supernatant was removed and samples were centrifuged twice followed by adding

Table 2. Comparison of sperm parameters in different groups.

Sperm parameters	Control	Freeze	Freeze + (1×10^5/µL) PRP	Freeze + (0.5×10^5/µL) PRP	Freeze + (0.25×10^5/µL) PRP
Count (106)	11.80 ± 1.9	5.80 ± 2.3[a]	6.70 ± 2.6	6 ± 2.27	6.65 ± 1.84
	MD = 12.5 IQR = 3	MD = 5 IQR = 4	MD = 5.50 IQR = 5	MD = 5 IQR = 3	MD = 6.5 IQR = 3
PMS (%)	15.25 ± 1.51 MD = 15 IQR = 2	3.05 ± 2.01[a] MD = 3 IQR = 3	14.05 ± 4.13[b] MD = 14.5 IQR = 8	12.35 ± 4.90[b] MD=11.50 IQR=9	12.15 ± 9.65[b] MD = 9 IQR = 14
NPMS (%)	22.10 ± 5.10 MD = 21.50 IQR = 9	11.95 ± 5.08[a] MD = 11.50 IQR = 10	15.40 ± 5.98 MD = 15.50 IQR = 10	15.90 ± 5.50 MD = 17.50 IQR = 10	16.35 ± 5.78 MD = 17.50 IQR = 9
NMS (%)	62.65 ± 4.66 MD = 62 IQR = 7	85 ± 5.04a MD = 85.50 IQR = 9	70.5 ± 7.59[b] MD = 69.50 IQR = 11	71.75 ± 7.73[b] MD = 72 IQR = 12	71.50 ± 9.22[b] MD = 71.50 IQR = 14
Viability (%)	52.60 ± 12.26 MD = 55 IQR = 11	36.85 ± 10.25[a] MD = 35 IQR = 18	47.85 ± 5.86[b] MD = 48.50 IQR = 10	51.30 ± 5.54[b] MD = 51 IQR = 7	50.05 ± 5.67[b] MD = 50 IQR = 9
Morphology (%)	1.85 ± 0.74 MD = 2 IQR = 1	0.90 ± 0.78[a] MD = 1 IQR = 2	1.45 ± 0.82 MD = 2 IQR = 1	1.30 ± 0.92 MD = 1 IQR = 1	1.50 ± 0.76 MD = 1 IQR = 1

Data presented as Mean ± Standard deviation. Kruskal–Wallis test was performed followed by Mann-Whitney test.

MD: Median, IQR: Interquartile Range, PRP: platelet-rich plasma, NMS: nonmotile spermatozoa; NPMS: nonprogressive motile spermatozoa; PMS: Progressive motile spermatozoa

[a]: ($p < 0.001$) ($p = 0.00$) significant difference versus Control group.

[b]: ($p < 0.001$) ($p = 0.00$) significant difference versus Freeze group.

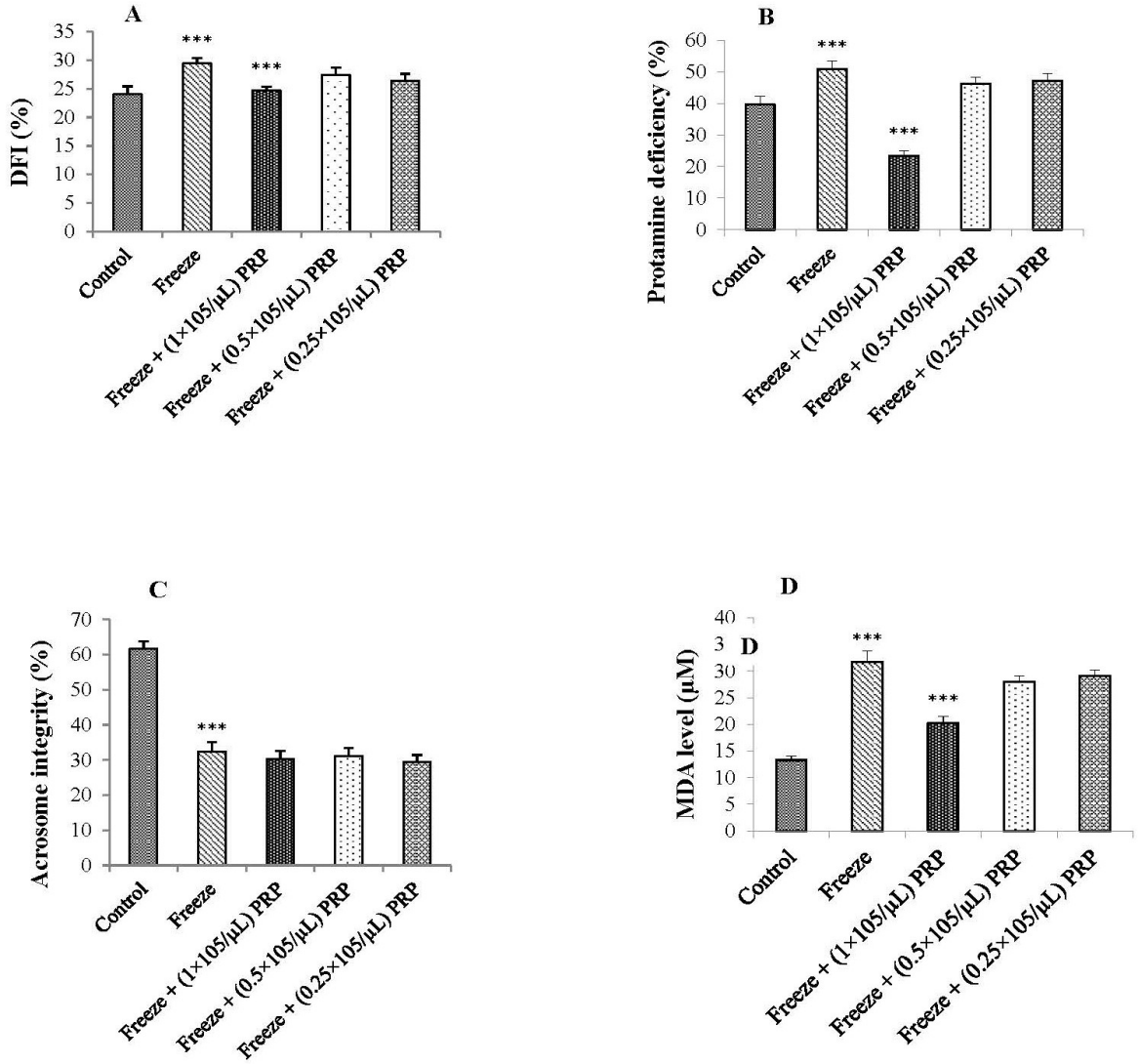

Figure 1. Comparison of DNA fragmentation **(A)**, protamine deficiency **(B)**, acrosome integrity **(C)** and MDA level **(D)** in different group. Data presented as Data presented as Mean ± Standard deviation. Kruskal–Wallis test was performed followed by Mann-Whitney test.
PRP: platelet-rich plasma, MDA: Malondialdehyde
(***$p < 0.001$) ($p = 0.00$) significant difference versus Control group.
(***$p < 0.001$) ($p = 0.00$) significant difference versus Freeze group.

5ml Hams F10 (Biochrome, Berlin, Germany) to sediment. Sperm samples macroscopic (liquefaction time, volume, appearance, and viscosity) and microscopic (sperm count, motility, morphology and viability) parameters were assessed before and after discontinuous density – gradients preparation according to the World Health Organization (WHO) (2020)[20]. The characteristics of the samples before and after processing were reported in Table 1.

PRP preparation

Blood samples were obtained from each individual who had OAT sperm analysis. To prepare Autologous PRP, PRP kits (Rooyagen, Tehran, Iran) were used. Preparation was performed according to the kit instruction. Anticoagulant solution (1.5 mL) (Arya Mabna Tashkhis, Iran) was mixed with blood (8.5 mL) and centrifuged at 1600 ×g for 10 min. Then, the supernatant was centrifuged at 3500 ×g for 5 min. To count platelets, cell analyzer was applied. For activation of PRP and releasing growth factors, freezing and thawing procedures were performed at -80 and room temperature (RT), respectively for 30 min before using the prepared PRP. 1×105, 0.5×10^5 and 0.25×10^5 platelet per µl was poured into each prepared sperm sample[21].

Sperm rapid-freezing and thawing

Sperm samples were divided into four different groups: Sperm samples and different concentrations of PRP with freezing medium and sperm samples and freezing medium without PRP as freeze group. 250 µl of semen was gradually mixed with an identical volume of PRP and then the 500µl of sperm freezing medium (Kitazato, Japan) was added to the mixture. Cryovials were placed above liquid nitrogen (LN2) vapor for 30 min at 3–5 cm above the surface of liquid nitrogen and then were immersed immediately in LN2 tank. After twenty days, cryovials were placed at 37 °C water bath to

Table 3. Presentation of median and Interquartile Range of sperm parameters in different groups.

Parameters	Control	Freeze	Freeze + (1×10^5/µL) PRP	Freeze + (0.5×10^5/µL) PRP	Freeze + (0.25×10^5/µL) PRP
DNA fragmentation (%)	MD = 23 IQR = 5	MD = 28.50 IQR = 7	MD=25 IQR=3	MD = 25 IQR = 9	MD = 25 IQR = 6
Protamine deficiency (%)	MD = 38.50 IQR = 19	MD = 49 IQR = 19	MD=24 IQR=11	MD = 43 IQR = 18	MD = 4 IQR = 19
Acrosome integrity (%)	MD = 3 IQR = 11	MD = 31 IQR = 17	MD=29.50 IQR=14	MD = 30 IQR = 14	MD = 30 IQR = 17
MDA level (µM)	MD = 12.50 IQR = 4	MD = 30.50 IQR = 14	MD=19.50 IQR=8	MD = 28.50 IQR = 5	MD = 28.50 IQR = 6

MD: Median, IQR: Interquartile Range, PRP: platelet-rich plasma, MDA: Malondialdehyde

perform thawing procedure. Hams F10 medium (1mL) with human serum albumin (10%) was used. Samples were centrifuged at 300×g for 5 min and after removing the supernatant the pellet was used for sperm parameters assessment[22].

Sperm parameters evaluation

10µl of sperm sample was placed on the Makler chamber slide and sperm number in vertical and horizontal squares was counted. Then, the mean of two was recorded. To assess sperm motility, a phase-contrast microscope with ×400 magnifications was used to count 200 sperm and then, according to WHO 2010 [20] progressive motile sperm (PMS), non-progressive motile sperm (NPMS) and non-motile sperm (NMS) percentages were reported. Eosin-nigrosin staining was performed for evaluation of sperm viability. The smear was prepared on a slide, light microscopy at a magnification of ×1000 was used. Colourless spermatozoa are considered as viable, while pink or red colour counted as dead spermatozoa. Diff-Quik staining kit (Dian bioassay, Avicenna, Tehran, Iran) was used to evaluate the sperm morphology. Two hundred sperms were investigated by using light microscopy at a magnification of ×1000. Normal spermatozoa percentages were reported [23].

Sperm chromatin dispersion (SCD) test

Sperm DNA fragmentation assessment was done according to the Halo sperm kit (SDFA kit, manufactured in Tehran, Iran) instruction. 50µl of sperm suspension was added to the low-melting agarose. After that, 20µl of this mixture was loaded on the pre-coated slide and coverslip was placed on it to spread the droplet. Slides were incubated at 4°C for 5 min. Then, coverslip was removed and the staining procedure was carried out. Light microscopy was applied to assess 200 spermatozoa at a magnification of ×1000. The size of halo is an indicator of the DNA fragmentation statue. Large or medium size of the halo does not show DNA fragmentation, while a small or absent of halo shows DNA fragmentation. Spermatozoa with DNA fragmentation percentage were recorded[24].

Assessment of protamine deficiency by CMA3 staining

To evaluate sperm protamine deficiency rate, chromomycin A3 (CMA3) staining was carried out. For each sample smear was prepared and dried in air. Carnoy's solution (methanol/glacial acetic acid, 3:1) was used as fixative and slides were kept at 4°C for 10 min. Staining of smears was done by CMA3 solution (Sigma-Aldrich, USA) for 10 min. Two hundred sperms were assessed by using a fluorescent microscope (Olympus BX5) at ×100 magnification. Bright yellow spermatozoa were indicators of CMA3+ cells, while spermatozoa with dull yellow colour were indicators of CMA3− cells. CMA3+ spermatozoa percentages were recorded[25].

Sperm MDA levels

A TPR kit (Teb Pazhoohan Razi, Tehran, Iran) was provided to measure the levels of MDA in sperm. 100µl of sample was added to the 100µl of standard solution in a sterile microtube. 100 µL of reagent (R4) and 200 µL of chromogen were added to the mixture. The samples were exposed to the hot water and ice for 1h and 10 minutes, respectively. Ultimately, after samples were centrifuged, 200 µL of the samples were poured to the plate wells and the absorption rate was recorded by a plate reader device at 530–540 nm[26].

Sperm acrosome reaction

Smears were prepared and dried in air and fixed with 95% ethanol for 10 min. Staining was done by using PSA-FITC (Sigma-Aldrich, USA) (solution and slides were kept at 4°C for 1 h. slides were washed in distilled water and 200 spermatozoa were evaluated by a fluorescent microscope (Olympus BX5) (×100). Sperm with reacted acrosome shows a single fluorescent band in the tropics or no stain. Acrosome-reacted sperm percentages were reported[27].

Statistical analysis

Data were analyzed by The Statistical Package for the Social Sciences (SPSS) version 20 (IBM, California, United States). Data were expressed as mean ± St. Deviation. Normal distribution of data was checked with Kolmogorov-Smirnov test and all date distribution was not normal. Kruskal–Wallis test was performed followed by Mann-Whitney test. Mann-Whitney test was used four times to compare different groups (Freeze vs Control, Freeze + (1×10^5/µL) PRP, Freeze + (0.5×10^5/µL) PRP and Freeze + (0.25×105/µL) PRP vs Freeze). Therefore, $P < 0.01$ was considered statically significant. Median and Interquartile Range of DNA fragmentation, Protamine deficiency, Acrosome integrity and MDA level were reported in **Table 3**.

RESULTS

Effects of PRP on sperm parameters (count, motility, viability and morphology)

Sperm count significantly changed in the Freeze group compared to the Control group (**Table 2**, $P < 0.001$). Adding PRP as a cryoprotectant did not alter sperm count compared to the Freeze group. The mean percentage of PMS, NPMS and NMS remarkably changed in the Freeze group compared to the Control group (**Table 2**, $P < 0.001$). Moreover, the percentage of PMS increased in three concentrations of PRP compared to the Freeze group (**Table 2**, $P < 0.001$). For NPMS, no significant changes were observed in the PRP groups compared to the Freeze group. Different concentrations

of PRP decreased the percentage of NMS compared to the Freeze group (**Table 2**, $P = 0.00$). The freezing procedure resulted in a significant decrease in the mean percentage of viable spermatozoa in comparison with the Control group (**Table 2**, $P < 0.001$). There was a significant increase in the percentage of viable sperm after using different concentrations of PRP compared to the Freeze group (**Table 2**, $P < 0.001$). The percentage of sperm normal morphology significantly decreased during cryopreservation procedure versus the Control group (**Table 2**, $P < 0.001$). PRP with different concentrations did not significantly preserve sperm normal morphology compared to the Freeze group.

The effects of PRP on sperm DNA fragmentation percentage

Freezing caused a significant increase in the percentage of sperm with fragmented DNA versus the Control group (**Figure 1**, $P < 0.001$). PRP at a concentration of 1×105/µL PRP noticeably decreased the percentage of sperm with fragmented DNA (**Figure 1**, $P < 0.001$).

The effects of PRP on sperm protamine deficiency

The mean percentage of sperm with protamine deficiency increased during cryopreservation procedure (**Figure 1**, $P < 0.001$). 1×105/µL concentration of PRP decreased protamine deficiency compared to the freeze group (**Figure 1**, $P < 0.001$).

The effects of PRP on sperm acrosome integrity

The percentage of sperm with intact acrosome significantly decreased in the Freeze group compared to the Control group (**Figure 1**, $P < 0.001$). PRP did not increase the percentage of sperm that did not undergo acrosome reaction and remained intact.

The effects of PRP on sperm MDA level

Cryopreservation procedure caused a significant increase in sperm MDA levels versus sperms that have not undergone freezing technique (**Figure 1**, $P < 0.001$). Moreover, PRP at concentrations of 1×10^5/µL, showed a significant decrease in sperm MDA levels (**Figure 1**, $P < 0.001$).

DISCUSSION

Male factor infertility refers to noticeable changes in one or more sperm parameters including count, motility and morphology according to the WHO criteria. Male infertility may manifest by OAT which is characterized by low sperm count and motility and a high percentage of abnormal sperms. Cryopreservation of samples with low quality for a long time may reduce the quality further. Therefore, applying cryoprotectants with fewer possible adverse effects is noteworthy. This study was designed to evaluate the efficiency of autologous PRP as a cryoprotectant on biological characteristics of OAT samples during the freezing procedure.

In the present study, freezing procedure decreases sperm parameters and other factors significantly compared to the control group. Moreover, no significant changes were observed in sperm count, morphology, non-progressive motility and acrosome reaction by adding PRP as cryoprotectant in comparison with the freeze group. PRP at all three concentrations showed a significant increase in progressive motility and viability compared to the sperm samples without PRP. The percentage of immotile sperms decreased at all PRP concentrations compared to the freeze group. In addition, 1×10^5/µL concentration of PRP showed cryoprotective effects on DNA fragmentation, protamine deficiency and MDA level compared to the other three concentrations.

Sperm parameter such as motility is the most important determining factor for sperm quality as well as contributing agent in the fertilization process. Based on previous outcomes and similar to our results, motility and viability of post-warmed sperm decreased compared to the pre-freeze group. The main reasons that alter these parameters are destructive mechanisms that occur during freeze-thaw process including cell membrane impairment, inhibition of glycolysis pathway and generation of ATP[4]. Adding various components with beneficial properties to the freezing medium as protectant ameliorates detrimental consequences of freeze-thaw process. In the current study, PRP in all three concentrations showed protective effects on sperm motility and viability against freezing due to the several biologically active contents that PRP possesses. FGF, one of the PRP substances, exerts its impacts by elevating FGFR phosphorylation in sperm flagella and activating different signaling pathways including kinase which is regulated by extracellular signals and protein kinase B. Ultimately, these underlying mechanisms significantly enhance the percentage of sperm progressive motility[28]. It was reported that another component of PRP, VEGF preserves sperm motility in vitro which depends on concentrations of PRP[29]. VEGF affects biological characteristics of sperm and reduces injuries by suppressing oxidative stress via NF-E2-related factor 2 (NrF2) pathway[13]. Serotonin that is found in PRP can ameliorate sperm curvilinear velocity[30]. Platelets alpha granules contained TGF-β which has favorable impacts on sperm quality like movement and vitality[12]. All the mentioned reasons that increase motile sperm are in agreement with research conducted by Hern´andez-Corredor et al.[31] concluded that PRP recovers sperm motility and morphometric factors, which confirmed the results of this study. Badar et al. demonstrated that a 2% concentration of PRP increases the mean percentage of motile sperms[32]. Moreover, a study that was carried out in 2021 by Yan et al. investigated the effects of autologous PRP on post-thaw sperm progressive motility. Results revealed that a 5% concentration of PRP significantly preserves progressive movement [33]. Nabavinia et al.[21] also observed protective effects of PRP at 1×10^5/µL concentration similar to our study. In this study, all three concentrations (1×10^5/µL, 0.5×10^5/µL and 0.25×10^5/µL) exert protective effects on progressive motility and viability, while PRP did not change sperm count and morphology. Cryopreservation of stem cells which derived from umbilical cord at 40% concentration of PRP preserved proliferation and morphology of cells during cooling process. Results showed that the count of cryopreserved cells did not alter in experiment and control groups[34]. In this study also the count of sperm cells did not change in control group and at different concentrations of PRP may be due to the low count of OAT samples and their quality. Moreover, preparation of sperm samples can reduce sperm count.

According to the aforementioned studies, PRP at different concentrations showed protective effects on sperm parameters. These differences may be associated with techniques that are applied for PRP activation, various sperm freezing media that are used for sperm cryopreservation and also concentrations of PRP. For instance,

Bo Yan and colleagues activated PRP by cacl2 and thrombin, while we used freeze-thaw method to activate PRP[33]. Activation of PRP with different methods leads to a release of different amounts of PRP growth factors. Furthermore, in this study, PRP significantly decreased immotile sperms.

The stability and integrity of sperm plasma membrane and DNA play critical roles in preserving sperm fertilization capability and increasing embryo development. High levels of DNA fragmentation may decrease embryo formation and development and also increase miscarriage rate[35]. IGF-1 is another component of PRP with protective and positive effects. The addition of IGF-1 to the sperm medium and applying it in the cooling process has a considerable impact on sperm plasma membrane structure and mitochondria function. Treatment with IGF enhanced sperm fertilization potential and count compared to the control group. Using PRP did not affect DNA fragmentation which was caused by freezing-thawing technique, while decreased the percentage of sperm with fragmented DNA as a result of centrifugation induced stress in the entire process [18,36,37]. Moreover, NGF, one of the PRP contents, can reduce the adverse effects of cryopreservation technique on DNA integrity[19]. In this study, although OAT samples have a high percentage of DNA damage, PRP at concentration of 1×105/µL significantly decreased DNA fragmentation as a result of IGF and NGF releasing from platelets. This result is in line with a recent study which investigated the effects of PRP on normospermic samples during freezing-thawing method[21]. It was reported that PRP with a concentration of 1×10^5/µL can attenuate DNA damage of post-thaw sperm. Yan et al. showed that DNA fragmentation did not significantly decrease by different concentrations of PRP. The method that they used to evaluate DNA damage was terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay which is for double-strand DNA fragmentation detection[33]. In other cells, high amount of histidine is presented, while in spermatozoa cell protamine is the most abundant amino acid. When histones replaced by protamine abnormally, DNA damage elevates. Histones replacement can be detected by CMA3 staining[38]. The results of this method showed that high concentration of PRP which used in this study (1×10^5/µL) decreased protamine deficiency as a result of freezing side effects. As mentioned above, DNA integrity was also preserved by high concentration of PRP which confirmed the results of CMA3 test. Our results demonstrated that the addition of PRP as a cryoprotectant to the freezing medium did not change the percentage of sperms with intact acrosome compared to the samples that froze without PRP. If sperm carry out acrosome reaction before sperm-oocyte fusion they become incapable of fertilizing oocyte. Therefore, PRP based on it protective agents such as calcium, zinc and ATP which play critical roles in sperm capacitation and acrosome reaction did not maintain acrosome integrity [14,15]. For acrosome integrity, it would be better to use PRP with another antioxidant to benefit from protective effects of PRP and preserve integrity of acrosome.

Oxidative stress is one of the main causes of the cryo-damage and is the important purpose of making cryopreservation more ideal. Various substances with antioxidant properties are added to the sperm freezing medium to overcome the adverse impacts of freeze-thaw method on sperm quality[7]. SOD, one of the PRP ingredients, can decline free radicals of oxygen. It was reported that SOD can preserve DNA integrity in the sperms which stored at 5 °C for 24 h by decreasing lipid peroxidation[39]. Therefore, SOD may be contributing factor in reducing DNA damage which was assessed by SCD test in present study. In this study, MDA level was measured to assess the level of lipid peroxidation and the results revealed that a high level of MDA which produced by OAT samples are decreased by high concentration of PRP (1×10^5/µL). It was demonstrated that oxidative stress which induced in rat spleen improved by PRP injection due to different features of PRP such as anti-inflammatory, anti-apoptotic, and anti-oxidative effects[39]. In addition, PRP ameliorated negative effects of oxidative stress induced by hydrogen peroxide (H_2O_2) in sperm cells and sperm parameters expect morphology[32].

PRP attracts all the attention in clinical regenerative medicine field due to various properties. In the reproductive field, PRP exerts positive effects in rejuvenating and repairing of endometrial tissue, intrauterine adhesions, increasing ovarian reserve, improving ischemia-reperfusion injury of testicular tissue and sexual disturbance in men[40-42]. Moreover, PRP as cryoprotectant use to reduce possible complications of freezing-thawing procedure of normal semen samples[21,33]. In the present study, OAT samples which are low in quality were selected to search the effects of PRP. Similar to normal samples, PRP preserves all sperm biological characteristics except morphology. PRP decreases MDA as an oxidative stress indicator and protects DNA integrity against cryo-damge. Although this study same as other research confirmed cryoprotective role of PRP, more studies are required to evaluate all aspects of PRP application as a promising strategy for maintaining male gametes in liquid nitrogen for a long time.

CONCLUSIONS

Taken together, this study showed that PRP can preserve sperm parameters and improve DNA fragmentation, protamine deficiency and MDA level in OAT samples which possess low quality. Moreover, among three different concentrations of PRP, PRP at concentration1×105/µL (about 25%) exerts more cryoprotective effects. It should be considered that PRP application as cryoprotectant agent in freezing method can prevent further deterioration of OAT sample quality.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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