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The Effects of Autologous Platelet-rich Plasma Supplement during Sperm Cryopreservation on Post-cryopreserved Sperm Quality

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ABSTRACT

Objectives: This study aimed to evaluate human sperm vitality after cryopreservation by comparing a cryopreservation medium with and without the addition of autologous platelet-rich plasma (aPRP).

Materials and Methods: Semen samples were collected from normozoospermic men. Each sample was separated into two tubes: one without aPRP supplementation and one with 5% aPRP supplementation. Both tubes were cryopreserved for 14 days. Sperm parameters, including sperm concentration, motility, morphology, vitality, and deoxyribonucleic acid (DNA) fragmentation index (DFI), were measured and analyzed.

Results: Fifteen semen samples were included. After cryopreservation, sperm vitality was significantly higher in specimens with aPRP supplementation compared to those without (32.13% ± 12.02% vs 26.07% ± 9.30%, respectively, p = 0.009). There were no significant differences between the two groups in other sperm parameters, including sperm concentration (12.73 x 106/mL (interquartile range (IQR) 21.95 x 106/mL) vs 14.50 x 106/mL (IQR 25.31 x 106/mL), p = 0.053), morphology (4.40% ± standard deviation (SD) 0.98% vs 4.40% ± SD 1.18%, p > 0.999), total motility (8.73% (IQR 9.16%) vs 8.56% (IQR 11.24%), p = 0.410), progressive motility (5.67% (IQR 8.66%) vs 6.61% (IQR 6.35%), p = 0.887), and DFI (12.00% (IQR 15%) vs 17.00% (IQR 26%), p = 0.139).

Conclusion: The supplementation of cryopreservation media with aPRP significantly increased sperm survival rates after the freeze-thaw process. These findings suggest that aPRP may be an effective adjunct in cryopreservation protocols to improve sperm viability. The impact of combined additional high efficiency cryoprotectants on relevant reproductive outcomes, including fertilization, blastocyst formation, and pregnancy rates, needs further investigation.

Keywords: cryopreservation, platelet-rich plasma, semen analysis, sperm, sperm parameter.

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ผลของการแช่แข็งอสุจิแบบผสมส่วนประกอบเกล็ดเลือดเข้มข้นของตนเองต่อคุณภาพของอสุจิหลังละลาย

เฌอมินทร์ ฐิติภัทร์เลิศเดช, นิศานาถ บุญอิ้ง, กัญณภัทร เกสรสุคนธ์, ธัญชนก เจนจิตศิริ

บทคัดย่อ

วัตถุประสงค์: *เพื่อศึกษาความแตกต่างของอัตราการมีชีวิตของอสุจิที่ผ่านการแช่แข็งแบบปกติ และแช่แข็งแบบผสมส่วนประกอบเกล็ดเลือดเข้มข้น*

วัสดุและวิธีการ: *น้ำเชื้ออสุจิจากอาสาสมัครเข้าร่วมวิจัยที่มีคุณภาพอสุจิปกติ ถูกแบ่งเป็นสองกลุ่ม กลุ่มที่ไม่ผสมส่วนประกอบเกล็ดเลือดเข้มข้น และกลุ่มที่ผสมส่วนประกอบเกล็ดเลือดเข้มข้นร้อยละ 5 จากนั้นทั้งสองกลุ่ม ถูกนำไปเข้าสู่กระบวนการแช่แข็งเป็นระยะเวลา 14 วัน และเมื่อครบระยะเวลากำหนด ทั้งสองกลุ่มจะถูกนำมาวิเคราะห์คุณภาพอสุจิอีกครั้ง รวมถึงวิเคราะห์การแตกหัก deoxyribonucleic acid (DNA) ของอสุจิ (DNA fragmentation index)*

ผลการศึกษา: *อาสาสมัคร 15 คน ถูกนำมาเข้าร่วมกระบวนการวิจัย หลังผ่านกระบวนการแช่แข็ง พบว่า อัตราการมีชีวิตรอดของอสุจิ (sperm vitality) ในกลุ่มที่ผสมส่วนประกอบเกล็ดเลือดเข้มข้น สูงกว่าในกลุ่มกลุ่มที่ไม่ผสมส่วนประกอบเกล็ดเลือดเข้มข้น อย่างมีนัยสำคัญทางสถิติ, 32.13 ± 12.02 และ 26.07 ± 9.30, ตามลำดับ (p=0.009) ทั้งสองกลุ่ม ไม่มีความแตกต่างกันอย่างมีนัยสำคัญในอัตราความเข้มข้นของอสุจิ (sperm concentration) 12.73 x 106/mL (interquartile range (IQR) 21.95 x 106/mL) และ 14.50 x 106/mL (IQR 25.31 x 106/mL) (p=0.053); รูปร่างของอสุจิ (morphology), 4.40% ± standard deviation (SD) 0.98% และ 4.40% ± SD 1.18% (p>0.999); การเคลื่อนที่ของตัวอสุจิโดยรวม (total motility), 8.73% (IQR 9.16%) และ 8.56% (IQR 11.24%) (p=0.410) และการเคลื่อนที่ของอสุจิที่เคลื่อนที่เป็นเส้นตรงไปข้างหน้า (progressive motility), 5.67% (IQR 8.66%) และ 6.61% (IQR 6.35%) (p=0.887) รวมไปถึงอัตราการแตกหัก DNA ของอสุจิลดลง ในกลุ่มที่ผสมส่วนประกอบเกล็ดเลือดเข้มข้น เมื่อเทียบกับกลุ่มที่ไม่ผสมส่วนประกอบเกล็ดเลือดเข้มข้น 12.00% (IQR 15%) และ 17.00% (IQR 26%) ตามลำดับ (p=0.139).*

สรุป: *การผสมส่วนประกอบเกล็ดเลือดเข้มข้นของตนเอง ช่วยเพิ่มอัตราการมีชีวิตรอดของอสุจิหลังแช่แข็งและละลายอย่างมีนัยสำคัญทางสถิติ จากการศึกษาในวิจัยครั้งนี้ จึงแนะนำว่าการผสมส่วนประกอบเกล็ดเลือดเข้มข้นของตนเองอาจมีประโยชน์ในประบวนการแช่แข็งอสุจิเพื่อเพิ่มอัตราการมีชีวิตรอดหลังละลายแช่แข็ง การศึกษาเกี่ยวกับอัตราการปฏิสนธิ การเจริญเติบโตของตัวอ่อน และอัตราการตั้งครรภ์ยังต้องการการศึกษาต่อไปในอนาคต*

Introduction

Sperm cryopreservation is a procedure used to maintain male reproductive capacity by using several freezing techniques and special sperm freezing media. This ensures the availability of stored sperm for reproductive purposes at a later time, and may be used before cancer treatment[1], before undergoing a vasectomy[2] or other obstructive surgeries[3], in patients with autoimmune disorders or immunosuppressive therapies[4], hematopoietic stem cell disorder and transplantation[2], and even for donor sperm, which needs to be stored for at least 6 months to enable repeat blood testing for human immunodeficiency virus (HIV) before being used for insemination[2, 5]. It may also be used when a man is unable to provide a sufficient volume of semen on the day of egg retrieval in assisted reproductive technologies (ART)[6]. Osmotic alterations, freezing shock, intracellular ice crystal formation, and oxidative and mechanical stressors intrinsic to the freeze-thaw process are the main causes of decreased vitality, motility, and increased deoxyribonucleic acid (DNA) breakage of spermatozoa during semen cryopreservation[7]. The development of freezing procedures, freezing equipment design, vitrification techniques, and sperm freezing media supplementation have been explored to minimize cryoinjury[8]. Previous studies have shown that antioxidants, peptides, fatty acids, blood plasma, nanoscale material elements, biologically active equivalents, and botanical essential oils have significant cryoprotective effects and improve sperm quality following cryopreservation[5].

Platelet-rich plasma (PRP) is a concentrated serum of platelets that has a concentration around seven times higher than the normal platelet concentration. Enriched with growth factors, cell-activating substances, cytokines, cell adhesion molecules (CAMs), and angiogenic factors, this substance enhances the quality of sperm parameters. An example of one of these factors is the hormone insulin-like growth factor-I (IGF-I), which is an important regulator of spermatogenesis and functions as an effective polypeptide that promotes cell division, metabolism, and differentiation. Furthermore, it has been shown that IGF-I and vascular endothelial growth factor (VEGF) enhance sperm motility, vitality, and the structural integrity of mitochondria and plasma membranes after cryopreservation[9, 10]. Moreover, studies have demonstrated that fibroblast growth factor 2 (FGF-2) stimulates the process of phosphorylating fibroblast growth factor receptors (FGFRs), triggering activation of the flagella. This leads to an increase in both total and progressive sperm motility[11]. The superoxide dismutase (SOD) in PRP neutralizes reactive oxygen species (ROS), which induce oxidation of the sperm plasma membrane to protect against cryoinjury[12, 13]. PRP derives its cellular activity and potential therapeutic benefits mostly from its extensive variety of cytokines, various growth factors, and peptide hormones[14, 15].

We assessed the impact of adding an autologous platelet-rich plasma (aPRP) to sperm cryopreservation medium on improving sperm motility and vitality and minimizing DNA fragmentation after vitrification, in comparison to traditional cryopreservation media. In a previous study conducted in China[6], the addition of PRP during sperm cryopreservation significantly promoted sperm motility and viability, while providing minimal protection against excessive formation of ROS and intracellular freezing damage. Furthermore, a study published in Iran[16] found that PRP had an advantageous effect on both sperm motility and survival rates or vitality.

Therefore, we hypothesized that aPRP may act as a cryoprotectant, protecting sperm from damage caused by the freeze-thaw process. This study aimed

to analyze the impact of aPRP supplementation on human sperm by evaluating indicators such as sperm viability, concentration, motility, and DNA fragmentation after the cryopreservation and thawing process.

Materials and Methods

The participants in this study were 20 men, aged between 20 and 40 years old, who were receiving care or were medical staff at Rajavithi Hospital in Bangkok, Thailand and who volunteered to participate. Prior to their participation in the clinical trial, all participants provided informed consent. Once consent was obtained, semen analysis was performed on each sample.

All 20 semen samples were obtained by masturbating into sterile plastic containers after a period of sexual abstinence for 2 to 7 days. The samples were immediately analyzed for sperm parameters within one hour of collection, utilizing a computer-assisted semen analysis (CASA) system according to the 6th edition of the World Health Organization (WHO) guidelines[17].

Samples that did not satisfy the minimal requirements established by the WHO guidelines or had a sperm volume below 2 mL were excluded from this study. Only samples that met the WHO criteria were included, and each semen sample was divided into two specimens, each containing 1 mL each (Fig. 1).

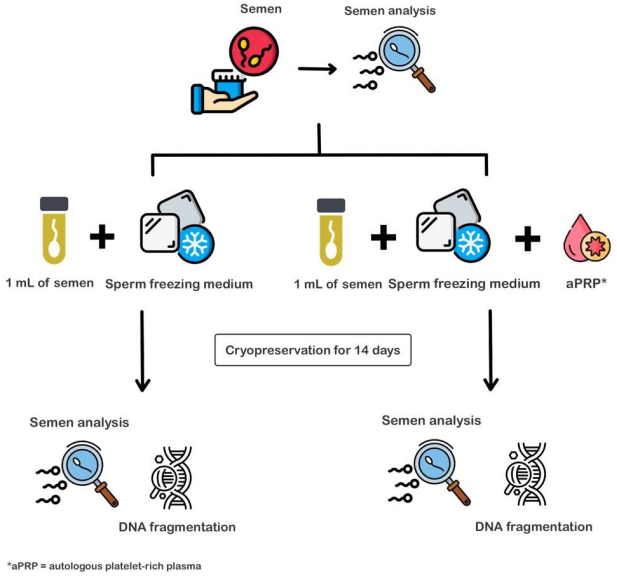

Fig. 1. Study flow diagram shows the methods applied to the control group (left side) and the autologous platelet rich plasma (aPRP) supplementation group (right side).

Assessment of sperm concentration and motility was conducted utilizing a CASA system. Sperm motility was categorized into three distinct groups: progressive motility, characterized by active movement of sperm, either at a fast or slow speed, in a linear or circular motion; non-progressive motility, encompassing all other patterns of active tail movements without any obvious progression; and immotile, indicating the absence of active tail movements. This study considered both progressive motility and total motility, which can be further classified as progressive motility and non-progressive motility, according to the 6th edition of the WHO guidelines[17].

Evaluation of sperm viability was conducted using the eosin-nigrosine staining technique. Equal volumes of 10 µl of each sample were combined with a dye solution including 1% eosin Y and 10%

nigrosine and then rapidly smeared onto a glass slide and allowed to dry spontaneously at room temperature prior to being analyzed using 1,000x light microscopy. Spermatozoa exhibiting red or dark pink heads were classified as non-viable cells, while unstained spermatozoa were considered live or viable[17]. The ratio of dead to living spermatozoa was established by observing a minimum of 200 sperm cells[6].

Spermatozoa are composed of a head and tail. The midpiece refers to the segment of the tail that is linked to the head and includes a thicker section housing mitochondria. The remaining section of the tail comprises the main sperm component, which is an axoneme or ciliary structure encased by thick outer fibers, together with a fibrous covering including longitudinal columns and an endpiece. Given the limited visibility of the endpiece under a light microscope, it is reasonable to regard the cell as consisting of a head (and neck) and tail (midpiece and major piece). In order to classify a spermatozoon as normal, the head, midpiece, tail, and cytoplasmic residue must all be normal. All borderline forms must be regarded as abnormal[17].

To evaluate sperm morphology, ejaculate smears were prepared by air-drying, followed by fixation and staining to enhance the visualization of spermatozoa. In this study, the slides were fixed using a fixative for 15 minutes before undergoing Papanicolaou staining, in accordance with WHO recommendations[17]. Subsequently, the slides were analyzed under 1,000x light microscopy. A minimum of 200 spermatozoa were examined to assess their morphological characteristics and determine the proportion of those with normal forms.

DNA fragmentation was assessed using the sperm chromatin dispersion (SCD) assay kit (HaloSperm®, Halotech, Madrid, Spain). In summary, a small amount of undamaged or post-thaw semen was added to molten agarose, cooled to 37°C, thoroughly mixed, and then set on a Super-Coated Slides (SDS) in the SCD assay kit. In accordance with the manufacturer's instructions, the solidified gel slides were placed in denaturation and lysis solutions, fixed, stained, and examined using bright-field microscopy[18]. Three hundred sperm were randomly selected for each sample and examined using bright-field microscopy with a green filter. The percentage of sperm exhibiting green fluorescence was used as the DNA fragmentation index (DFI), as determined by a CASA system.

Spermatozoa with a substantial or moderately sized halo were deemed normal, suggesting the absence of DNA fragmentation, whereas spermatozoa with a small or no halo were categorized as having fragmented DNA. The DFI was determined by calculating the proportion of spermatozoa with fragmented DNA, encompassing those with a small halo, no halo, and halo-degraded (resulting in fragments) (Fig. 2).

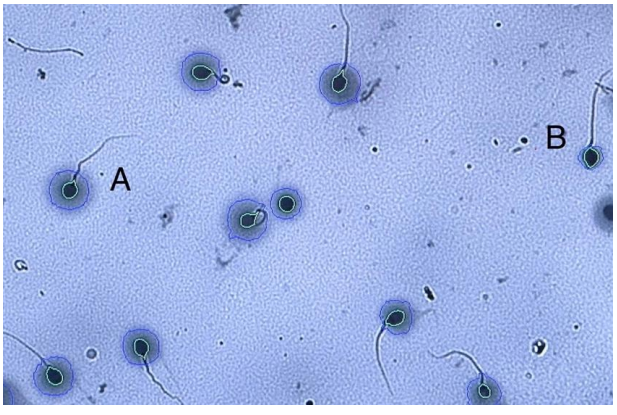

Fig. 2. A: Sperm with a large-sized halo (big halo) means a normal sperm or no deoxyribonucleic acid (DNA) fragmentation, B: Sperm with a small-sized halo (small halo) means a sperm with fragmented DNA, as seen using a computer-assisted semen analysis system.

To prepare aPRP, a blood sample was collected from a donor. The blood was collected into four 3 mL tubes filled with sodium citrate as an anticoagulant solution. After mixing the anticoagulant and blood by inverting the tubes several times, the samples were moved into centrifuge tubes and centrifuged at 1,000 revolutions per minute for 10 minutes.

After centrifugation, the samples had divided into three distinct layers: the supernatant (uppermost layer), the intermediate buffy coat (middle layer), and the red blood cells (bottom layer). Subsequently, the upper layer was discarded, and the middle layer was transferred to another centrifuge tube then centrifuged again at 1,000 revolutions per minute for 10 minutes.

Following the second centrifugation, the sample was roughly divided into three, with the upper two-thirds of the supernatant being discarded. The layer of liquid remaining at the bottom was identified as PRP[19]. Following this, the PRP in the specimen was measured via a cell analyzer; 1×106 platelets per µL were used as autologous aPRP in this study[6, 20].

After completion of the semen analysis, each semen sample was separated into two 1 mL samples: one for aPRP supplementation and the other without, according to the procedure outlined in a previous study[6]. Each specimen was carefully titrated at room temperature with an equivalent volume (v/v; 1:1) of sperm-freezing medium (glycerol, sucrose, and egg yolk-free solution (Origio®)). This was done by adding the medium drop by drop into the semen and carefully mixing the solution after each addition. The mixture requiring aPRP supplementation was supplemented with 5% aPRP and properly mixed. After 10 minutes, the mixtures were transferred into a sterile cryotube and positioned for a 30-minute upward exposure to liquid nitrogen vapor at a height of 3–5 cm above the liquid nitrogen surface. After that, the samples were cryopreserved in liquid nitrogen for 14 days. After 14 days, the samples were thawed at a temperature of 37°C for 10 minutes. Immediately following centrifugation at 2,000 revolutions per minute for 5 minutes, spermatozoa analysis was performed. The supernatant was discarded, and the spermatozoa resuspended with sperm washing medium[16].

Statistical analysis of the data collected in this study was conducted using SPSS software, version 25 (IBM Corp., Armonk, NY, USA). The quantitative findings were reported as the mean ± standard deviation, and median ± interquartile range (IQR) were used to represent the quantitative results for non-normally distributed data. The statistical analyses included the use of the paired student's t test, McNemar test, and Wilcoxon signed-rank test. A p value of less than 0.05 was considered as statistically significant.

Results

Twenty participants were enrolled between August 1, 2023, and August 1, 2024. Two participants were excluded due to abnormal semen analysis, according to the 6th edition of the WHO guidelines[17]. The specimens of 18 participants were divided into two prior to the freeze-thaw process. After 14 days of freezing and thawing, three pairs of specimens had a laboratory error: two of them were not analyzed for sperm motility, and one of them was not analyzed for DNA fragmentation. A total of 15 pairs of specimens were included in the final analysis (Fig. 3). The baseline characteristics of these 15 participants are displayed in Table 1.

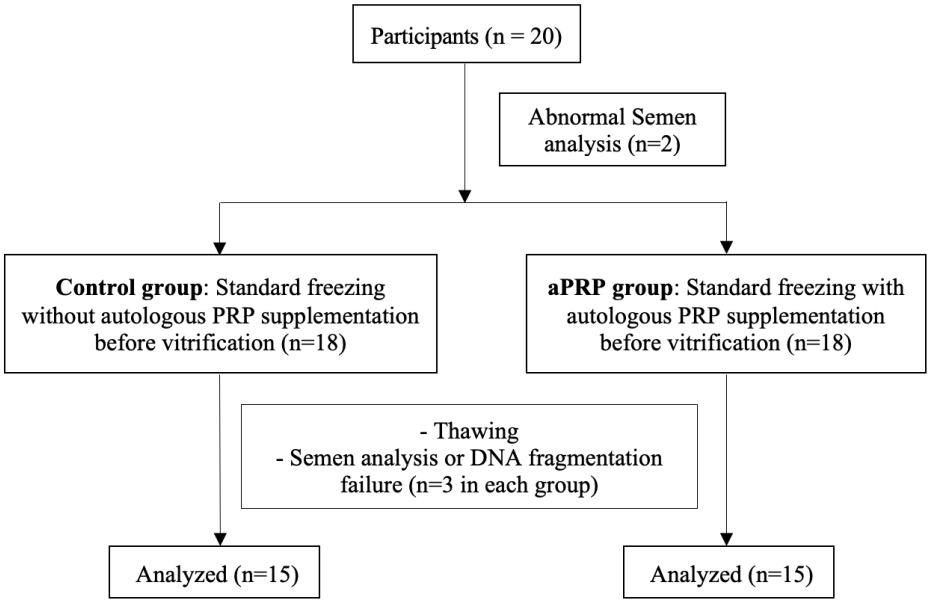

Fig. 3. Consort Flow Diagram.

DNA: deoxyribonucleic acid, aPRP: autologous platelet-rich plasma, PRP: Platelet-rich plasma

Table 1. Baseline characteristics of 15 participants.

Characteristic	
Age (yrs.), mean ± SD	27.3 ± 3.8
BMI (kg/m^2), mean ± SD	23.7 ± 3.5
Education level, n (%)	
Bachelor's degree	14.0 (93.3)
Master's degree or above	1.0 (6.7)
Occupation, n (%)	
Medical staff	6.0 (40.0)
College student	5.0 (33.3)
Other	4.0 (26.7)
Underlying disease, n (%)	
None	11.0 (78.6)
Allergic rhinitis	3.0 (21.4)
Current medication, n (%)	
None	11.0 (73.3)
Yes	4.0 (26.7)
Smoking status, n (%)	
None	14.0 (93.3)
Yes	1.0 (6.7)
Alcohol consumption, n (%)	
None	13.0 (86.7)
Yes	2.0 (13.3)
History of reproductive surgery, n (%)	
Never	15.0 (100.0)

BMI: body mass index, SD: standard deviation

The baseline sperm parameters of these 15 participants before cryopreservation are shown in Table 2. The analysis of the effects of sperm parameters after cryopreservation (freeze–thaw) without and with the addition of aPRP on sperm concentration, motility, morphology and vitality is presented in Table 3.

Table 2. Analysis of baseline sperm parameters for 15 participants prior to cryopreservation.

Semen parameters	Before cryopreservation
Sperm vitality (%), mean ± SD	81.07 ± 13.04
Sperm concentration (x106/mL), median (IQR)	40.46 (40.94)
Motility	
- Total motility (%), median (IQR)	72.49 (37.65)
- Progressive motility (%), median (IQR)	58.87 (35.63)
Normal morphology (%), mean ± SD	4.53 ± 1.30

IQR: interquartile range, SD: standard deviation

Table 3. The effects of cryopreservation on sperm parameters and DNA fragmentation without and with the addition of autologous platelet-rich plasma (aPRP).

Semen parameters	Without aPRP supplementation	With aPRP supplementation	p value
Sperm vitality (%), mean ± SD	26.07 ± 9.30	32.13 ± 12.02	0.009
Sperm concentration (x106/mL), median (IQR)	14.50 (25.31)	12.73 (21.95)	0.053
Motility			
- Total motility (%), median (IQR)	8.73 (9.16)	8.56 (11.24)	0.410
- Progressive motility (%), median (IQR)	5.67 (8.66)	6.61 (6.35)	0.887
Normal morphology (%), mean ± SD	4.40 ± 1.18	4.40 ± 0.98	> 0.999
DNA fragmentation index (%), median (IQR)	17.00 (26.00)	12.00 (15.00)	0.139

IQR: interquartile range, SD: standard deviation, aPRP: autologous platelet-rich plasma

Post-cryopreservation, the viability of sperm was markedly diminished in comparison to the fresh samples in their initial state. Supplementation with aPRP significantly improved the vitality of sperm in comparison to the control group that lacked aPRP (mean difference 6.07, 95% CI 1.81-10.32, p = 0.009). No statistically significant variations were seen in sperm concentration, motility (both total and progressive), or morphology between the two groups.

This study examined the impact of cryopreservation on sperm DNA fragmentation, both with and without the addition of aPRP. The results are presented in Table 3. The experimental group receiving aPRP supplementation had a lower DNA fragmentation index than the control group without aPRP. Nevertheless, there were no statistically significant variations in DNA fragmentation index, regardless of the addition of aPRP (p = 0.139).

Discussion

Sperm cryopreservation is becoming more prevalent for reproductive purposes at a later time, as mentioned earlier. Previous studies have shown that the motility and viability of sperm decrease according to thawing conditions compared to the pre-freeze state[21]. The reduced metabolic rate of spermatozoa during cryopreservation leads to substantial functional and structural alterations due to exposure to cryoprotectants and freeze-thaw processes[21].

The formation of ROS during the cryopreservation process has the capacity to cause oxidative stress and damage the lipid peroxidation of sperm membrane

proteins and DNA[22]. Moreover, the freeze-thaw process can interfere with the glycolysis pathway, leading to a decrease in adenosine triphosphate (ATP) synthesis and thus reducing sperm viability and motility[21, 23]. Oxidative stress is common after freezing; therefore, studies have been conducted in an effort to minimize the production of ROS after the freezing process[24].

Several studies have found that PRP performs effectively in several medical fields including orthopedics, dermatology, stomatology, sports medicine, and reproduction. This is because it is highly safe and effective, uncomplicated to prepare, and does not trigger immunity or other adverse effects[15, 25]. One study revealed that the administration of PRP injections might significantly reduce oxidative stress in the spleen of mice. These advantageous effects can be related to the elimination of the inflammatory, oxidative stress, and anti-apoptotic action of PRP[26]. Nabavinia et al conducted a study using non-autologous frozen PRP and found that the percentage of sperm progressive motility and viability in samples treated with a 1×105/μL concentration of PRP were significantly higher than in the control group[16]. Bo Yan's study used autologous non-frozen PRP to study the effect of several concentrations of PRP on sperm parameters, and found that 5% PRP significantly enhanced sperm motility and viability while also providing protection against oxidative stress and intracellular cryopreservation damage[6]. Buffalo oocytes inseminated with sperm cryofrozen in 5% PRP demonstrated higher fertilization, cleavage and blastocyst rates and lower polyspermy compared to the control. It was concluded that cryofreezing buffalo spermatozoa in autologous PRP-enhanced semen extender increased cryotolerance and fertilizing capability[27]. Therefore, 5% of non-frozen aPRP was used in the current study.

This study found that 5% aPRP supplementation significantly increased sperm vitality post-cryopreservation, consistent with findings from previous studies[6, 16]. However, previous studies found that it not only increased sperm vitality, but also significantly increased sperm progressive motility. There was no statistically significant difference between samples with and without aPRP supplementation in terms of sperm concentration, morphology, total motility, or progressive motility in this study.

Some studies have found that PRP may induce head-to-head sperm agglutination[28]. In this study, we found that head-to-head sperm agglutination increased after thawing, with the majority of agglutination occurring near the slide's border, which may have led to inaccuracies in the CASA system. This may be the reason for the decreasing sperm concentration. Head-to-head motility, shown as sperm agglutination, may lead to failure to detect motility in the CASA system. If sperm motility is inadequate, the vitality test is important in order to distinguish between immotile dead sperm and immotile living sperm[17]. The Bo Yan study showed that the addition of a coagulant to activated aPRP had no impact on sperm agglutination[6]. In our study, we noticed an elevated rate of sperm agglutination, possibly because aPRP was not activated before its administration.

During this study, the sperm morphology after thawing of both aPRP-treated and untreated samples was similar to the morphology prior to freezing, indicating that the aPRP and cryopreservation processes did not damage sperm morphology.

The limitations of this study included the relatively small sample size, which was calculated using the two-dependent-means method, as referenced in Bo Yan's study[6] Similarly, prior studies have also utilized small sample sizes. Another limitation lied in the methodology for preparing aPRP before freezing and aPRP activation as discussed previously, the sperm-washing techniques, and the process of centrifugation after thawing, which may have resulted in a decrease in the concentration of sperm, resulting in inaccurate analysis. The further development of methods to reduce sperm agglutination and other combined additional high efficiency cryoprotectants may prove advantageous in the future. Moreover, a well-designed randomized controlled trial

would enhance the reliability of the findings. Finally, the fertilization rate, rate of blastocyst formation, and pregnancy rate also need further investigation.

Conclusion

Supplementation with aPRP in a standard sperm cryopreservation protocol had a significant positive effect on increasing sperm survival rates and sperm vitality. There were no adverse effects on other sperm parameters, including sperm concentration, motility, or morphology.

Potential conflicts of interest

The authors declare no conflicts of interest.

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