

Plasma-Rich in Growth Factors Ameliorates Detrimental Effects of Cryopreservation on Human Sperm: A Prospective Study

Jafar Mirzaei, M.Sc., Mansoureh Movahedin, Ph.D., Iman Halvaei, Ph.D.*

Department of Anatomical Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

*Corresponding Address: Department of Anatomical Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
Email: ihalvaei@modares.ac.ir

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Abstract

Objective: Sperm cryopreservation results in damage to membrane integrity, sperm viability, sperm motility, and DNA structure. We aimed to evaluate the effect of plasma rich in growth factors (PRGF) on sperm parameters during the freeze-thaw process.

Materials and Methods: In the first phase of this prospective study, after sperm preparation, 10 normozoospermic specimens were cryopreserved by rapid freezing with different concentrations of PRGF including 0, 1, 5, and 10% to find the optimum dose. Sperm motility and viability were assessed in this phase. In the second phase of the study, based on the results of first phase, 25 normal sperm samples were frozen with 1% PRGF. All sperm parameters including motility, viability, acrosome reaction, and DNA integrity were assessed before freezing and after thawing.

Results: The rates of progressive and total sperm motility and viability were significantly higher in 1% PRGF compared to control, 5%, and 10% PRGF in the first phase ($P < 0.05$). Supplementation of freezing medium with 1% PRGF could significantly improve all sperm parameters including sperm motility, viability, normal morphology, acrosome integrity, chromatin structure, chromatin integrity, DNA denaturation, and DNA fragmentation in comparison with the control group.

Conclusion: It appears that the supplementation of freezing medium with 1% PRGF could protect human sperm parameters during cryopreservation.

Keywords: Freeze-Thawing, Growth Factor, Plasma Rich in Growth Factors, Platelet

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Introduction

Sperm cryopreservation is one of the main procedures routinely used in assisted reproduction technology. Several indications have been proposed for this technique including neoplastic and autoimmune disease, genetic disease affecting spermatogenesis, before vasectomy, gender reassignment, and sperm donors (1). It seems that oocyte and embryo cryopreservation have approximately found their optimum protocols but sperm freezing has remained to be modified to reach the optimum procedure. Cryopreservation could damage the cells via several mechanisms. Reactive oxygen species (ROS) is one of the most important factors that can damage the cells during cryopreservation (2). Sperm is a unique cell with very special features including a high level of polyunsaturated fatty acids in the plasma membrane, and a low level of antioxidants that make it vulnerable to the detrimental effects of cryopreservation. Spermatozoa also have a high number of mitochondria that could be a source of ROS production (3). Sperm cryopreservation can impair different sperm parameters. Different strategies have been recruited for ameliorating the adverse effects of sperm cryopreservation. Adding antioxidants to the cryopreservation medium has shown beneficial effects (4). Growth factors could act as antioxidants along with their roles in cell growth and differentiation (5).

In recent decades, platelets have been considered an important source of growth factors for regenerative medicine. Different methods using autologous platelet concentrates have been introduced including platelet-rich plasma (PRP) (6), plasma-rich in growth factors (PRGF) (7), and platelet-rich fibrin (8). PRGF was introduced in the late 90s for oral surgery (7). PRGF preparation is an easy method that needs simple centrifugation of fresh venous blood with adding anticoagulants and calcium chloride to release a pool of various growth factors (9). In comparison with other platelets concentrations, PRGF does not contain white blood cells (10). PRGF is a kind of plasma enriched by plasma proteins, coagulating agents, and circulating growth factors that later has an important role in tissue regeneration. The platelets contain α -granules that, after activation, release a pool of growth factors. PRGF has an antioxidant effect because it contains epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF β), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF-1) (11, 12). Beneficial effects of PRGF on neurobiology (13) and ophthalmology (14) have been previously described. The impacts of growth factors in the PRGF on the male reproductive system and spermatozoa have been solely evaluated in the previous studies. It was

shown that FGF, and VEGF have beneficial effects on sperm motility (15, 16). Beneficial effects of IGF-1 also have been reported in an animal study (17). Recently, it was shown that PRP has a partially protective effect on human sperm parameters during cryopreservation (18).

To the best of our knowledge, the impact of PRGF on sperm cryopreservation has not been assessed. The objective was to evaluate the role of PRGF on the detrimental effects of human sperm cryopreservation.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma (Sigma-Aldrich, Germany) unless otherwise stated. PRGF preparation.

This step was based on a previous publication with a minor modification (10). Blood samples were taken from 5 healthy donors (all men, 27-40 years) who had not been infected with a viral infection during the last six months and screened for blood-borne viruses like human immunodeficiency virus, hepatitis B surface antigen, hepatitis C virus, cytomegalovirus and Epstein-Barr virus. Informed consent was obtained from all donors. The samples were mixed with 3.8% sodium citrate in a conical tube. After centrifugation (8 minutes, 580 g) at room temperature, the whole plasma layer was aspirated avoiding touching or aspirating the buffy coat layer containing leukocytes. 10% calcium chloride was then added to plasma and incubated at 37°C for 30 minutes. After that, the supernatant was collected and incubated at 56°C for 60 minutes. The plasma then was filtered, aliquoted, and stored at -80°C until use.

Semen collection and preparation

In the first phase of this prospective study, ten normozoospermic samples from patients who were referred to the *in vitro* fertilization (IVF) clinic of Gandhi Hospital were recruited. The abstinence period was 2-7 days. Only ejaculated samples were included in this study. Patients who took antioxidants in time of the study, heavy smokers, alcohol abuse, and with a history of varicocele were excluded from the study. This study was performed between June 2019 and October 2019. Informed consent was obtained from all patients. The ethics committee of Tarbiat Modares University has approved all parts of this study (IR.MODARES.REC.1397.055). The sperm preparation method was direct swim-up. The mean male age was 34.9 ± 4.38 years.

Sperm cryopreservation and thawing

In the first phase of study, the effect of different doses of PRGF on sperm motility and viability was evaluated. Beneficial effects of 0.31-10% PRGF on proliferation of human periosteal cells has been evaluated before (19). There was no data available on evaluating the effects of PRGF on sperm cryopreservation. To do this, ten sperm samples were included of which each sample was divided into four aliquots in the cryotubes containing 0 (control),

1, 5, and 10% (V/V) of PRGF. In the second phase of study, the best PRGF dose determined in the first phase was selected for further evaluations. In this phase, the effects of 1% PRGF (V/V) on sperm parameters were evaluated on twenty-five normozoospermic samples. After adjusting the dose of PRGF, 70 μ L Life Global sperm freezing medium (life global) was added for each 100 μ L sperm solution. After five minutes of equilibration at room temperature, the samples were cryopreserved by the rapid freezing method (20). For thawing, the cryotubes were placed in tap water for five minutes and then specimens were added to the pre-warmed human tubal fluid (HTF) solution supplemented with human serum albumin. The diluted spermatozoa were centrifuged for 15 minutes (300 g). The supernatant was then discarded and the pellet was re-suspended with the HTF medium for further analysis.

Analysis of sperm characteristics

Sperm motility, viability and morphology

Sperm motility was evaluated using a light microscope (400 x). According to WHO guidelines, sperm motility was divided into three categories of progressive motility, non-progressive motility (total motility: progressive+non-progressive), and immotile. At least 200 spermatozoa at 6 microscopic fields were evaluated each time and different categories of sperm motility were reported as percentage. Each sample was evaluated twice by two expert operators and the mean was reported after the calculation of error. Sperm viability was evaluated using the eosin-nigrosin staining method (21). The spermatozoa with red or dark pink heads were considered dead while spermatozoa with white heads were alive (light microscope 1,000x). At least 200 spermatozoa were checked and the rate of sperm viability was reported as percentage. Sperm morphology was assessed using the Papanicolaou staining method (21). Head abnormalities (shape, number, size, presence of vacuoles, and acrosome size), mid-piece abnormalities (insertion, thickness, and excess residual cytoplasm), and tail abnormalities (shape, number, length, and excess residual cytoplasm) were checked for each sample for at least 200 spermatozoa. The rate of normal sperm morphology was reported as percentage.

Aniline blue

Aniline blue (AB) can bind to the lysine amino acids in histone proteins and detects chromatin condensation. After the preparation of smears from the samples, they were fixed by 3% glutaraldehyde in 0.2 M phosphate buffer (pH=7.2, room temperature, 30 minutes). The slides were then stained with AB staining (in 4% acetic acid, pH=3.5, 10 minutes). Spermatozoa with highly condensed chromatin were light blue and abnormal cells were dark blue. At least 200 spermatozoa were evaluated under the light microscope (1,000X) and the results were reported as percentage (22).

Toluidine blue

Toluidine blue (TB) attaches to the phosphate group in

DNA and assesses the chromatin integrity. The air-dried slides were fixed and placed in 0.1 N HCl solution. The slides were then washed with distilled water and stained with 0.05% TB. The sperm heads with dark blue to purple was considered abnormal and light blue spermatozoa were considered normal (light microscope 1,000x) (22).

Acridine orange

Acridine orange (AO) was used to detect DNA denaturation. The air-dried slides were stained by AO in a dark room for 10 minutes after fixing by Carnoy's solution (methanol-glacial acetic acid, 3:1). Green and yellow to red spermatozoa were considered normal and abnormal cells, respectively. At least 200 spermatozoa were evaluated each time and the rate of normal cells was reported as percentage (fluorescent microscope 1,000×, 460 nm filter) (20).

Sperm chromatin dispersion

Sperm chromatin dispersion (SCD) is an indirect method for the evaluation of DNA fragmentation. The sperm samples were mixed with agarose 1% (30 µL: 70 µL). Half of the sample (50 µL) was placed on a pre-coated slide with low melting agarose. After incubating at 4°C for 5 minutes, the slides were placed in a 0.08 N HCl solution and a lysis solution for 7 and 25 minutes, respectively. Then, the slides were washed with deionized water (5 minutes) and dehydration was performed by serially increased alcohol concentrations. Finally, the slides were stained by Wright color to see under the light microscope (1,000×). The spermatozoa with large or medium halo were considered normal while spermatozoa with small halo and no halo were considered abnormal. At least 200 spermatozoa were evaluated and normal cells were reported as percentage (23).

Acrosome reaction

The smears were prepared from samples after fixing with glutaraldehyde 3% for 30 minutes. Then, the slides were stained with Bismarck Brown and Bengal Rose for

10 and 25 minutes, respectively. Spermatozoa with light pink acrosome were considered intact acrosome and expressed as a percentage. At least 200 spermatozoa were evaluated under a light microscope (1,000×) (20).

Statistical analysis

SPSS software (version 15, Chicago, USA) was used for statistical analysis. The data are expressed as mean ± SD, median (min-max). To evaluate the normality of data, Shapiro-Wilk test was used. One-way ANOVA with Tukey and Kruskal-Wallis with Dunn were used for comparison between different groups for normal- and non-normal distributed data, respectively. The hypothesis was one-sided and the level of significance was set at $P \leq 0.05$.

Results

The freeze-thawing process decreased sperm total and progressive motility and adding PRGF (1%, 5%, and 10%) could increase these parameters. But only 1% PRGF showed a significant difference in comparison with 5% and 10%. Sperm viability was also decreased after thawing and it was only significantly increased in the 1% PRGF group. Our results showed that 1% PRGF had the best results to preserve the sperm motility and viability (Table 1). There was no obvious agglutination or aggregation in each experimental group.

The data of included semen in the second phase of study are shown in Table 2. Sperm motility decreased after thawing compared to before freezing. Adding 1% PRGF significantly increased both total and progressive sperm motility compared to the control group. Sperm viability also decreased after thawing in both groups and it was significantly increased in the 1% PRGF group compared to controls (73.25 ± 5.58 vs. 62.55 ± 4.5 , respectively). Sperm normal morphology and intact acrosome had also higher levels in the 1% PRGF group compared to the control group. Regarding the DNA integrity and chromatin packaging, our results showed that the rates of non-denatured DNA, non-fragmented DNA, and chromatin condensation were significantly higher in the 1% PRGF group compared to the control group (Table 3). Figure 1 shows AB and SCD tests.

Table 1: Sperm motility and viability after thawing in different doses of PRGF

Parameters	Before cryopreservation	PRGF concentration			
		0% (control)	1%	5%	10%
Total motility ^a	92.2 ± 2.19	45.4 ± 10.9	59.2 ± 6.4*	46 ± 16.3	48.2 ± 8.6
	93 (88-96)	45 (32-58)	61 (45-65)	50 (25-69)	49 (36-60)
Progressive motility	85 ± 3.08	35.4 ± 6.2	49 ± 5.8*	37.1 ± 17.6	36 ± 9.8
Viability ^a	94.1 ± 3.1	57.8 ± 7.3	67.3 ± 7.1*	58 ± 11.9	59.6 ± 7.8
	95 (89-97)	62 (44-62)	70 (52-75)	60 (40-73)	65 (50-66)

Data are presented as mean ± SD for data with normal distribution. ^a; Data are presented as mean ± SD, median (min-max) for data with non-normal distribution, and *; Significant difference versus control. All of groups had significant difference with before cryopreservation.

Table 2: Semen characteristics included in the second phase of this study

Parameters	Mean	Minimum	Maximum	Standard deviation
Male age (Y)	34.9	24	42	4.38
Count (10 ⁶ /mL)	112.6	50	200	26.74
Progressive motility (%)	64.6	50	81	9.66
Non-progressive motility (%)	9.95	2	20	3.78
Total motility (%)	74.55	59	87	9.16
Immotile (%)	25.45	13	41	9.16
Normal morphology (%)	8.46	5	12	1.43
Round cell (10 ³ /mL)	435	200	700	159.85

Table 3: Sperm parameters before cryopreservation, and after thawing in control and 1% PRGF groups

Parameters	Groups		
	Before cryopreservation	Control	1% PRGF
Progressive motility*	81.10 ± 5.87 ^{ab}	33.85 ± 5.95 ^{ac}	47.20 ± 9.31 ^{bc}
	82 (65-88)	34 (25-44)	46 (30-71)
Total motility	88.65 ± 4.13 ^{ab}	55.55 ± 6.63 ^{ac}	64.45 ± 7.06 ^{bc}
Viability	92.05 ± 3.25 ^{ab}	62.55 ± 4.5 ^{ac}	73.25 ± 5.58 ^{bc}
Normal morphology*	14.4 ± 3.33 ^{ab}	6.2 ± 1.82 ^{ac}	9.85 ± 2.66 ^{bc}
	14 (8-21)	6 (4-10)	10 (6-16)
Intact acrosome*	93.60 ± 2.7 ^{ab}	88.5 ± 2.6 ^{ac}	90.35 ± 2.39 ^{bc}
	93.5 (89-98)	89.5 (84-92)	90.5 (86-95)
Aniline blue (sperm chromatin structure)	87.2 ± 3.65 ^{ab}	62.55 ± 4.77 ^{ac}	76.15 ± 4.1 ^{bc}
Toluidine blue (sperm chromatin integrity)*	87.7 ± 4.61 ^{ab}	63.3 ± 5.89 ^{ac}	77.6 ± 5.03 ^{bc}
	88.5 (74-92)	63.5 (51-76)	79 (65-85)
Non-denaturated DNA (AO)	91.15 ± 3.26 ^{ab}	71 ± 5.95 ^{ac}	81.3 ± 3.88 ^{bc}
Non-fragmented DNA (SCD)*	90.25 ± 4.63 ^{ab}	63.70 ± 6.19 ^{ac}	78.25 ± 6.63 ^{bc}
	90 (75-96)	63 (55-79)	79 (62-87)

Data are presented as mean ± SD for data with normal distribution. AO; Acridine orange, SCD; Sperm chromatin dispersion test, and *; Data are presented as mean ± SD, median (min-max) for data with non-normal distribution. Similar letters have significant difference.

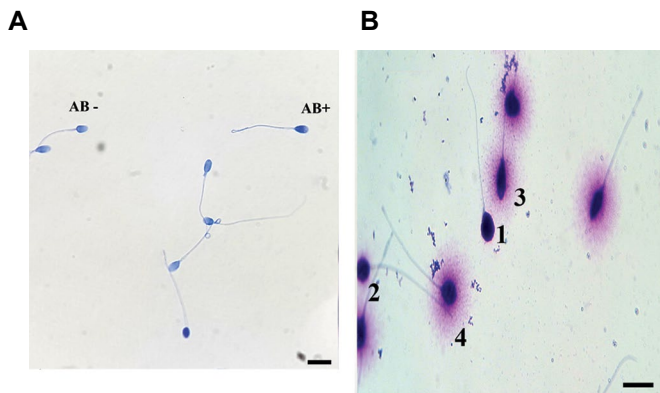


Fig.1: Aniline blue and sperm chromatin dispersion tests. **A.** Aniline blue shows normal light blue spermatozoa with highly condensed chromatin (AB-) and abnormal dark blue spermatozoa (AB+) (scale bar: 10 μ m). **B.** Sperm chromatin dispersion test shows DNA fragmentation with four patterns of halos. 1; Abnormal cell with no halo, 2; Abnormal cell with small halo, 3; Normal cell with medium halo, and 4; Normal cell with large halo (1000 \times) (scale bar: 10 μ m).

Discussion

PRGF could be considered as a suitable example of endogenous regenerative medicine in which the patient's own blood plasma and growth factors are used for treatment. PRGF has been introduced as the first 100% autologous PRP. White blood cells are considered as a source of ROS generation in culture media and they are omitted during PRGF preparation. It has several other advantages including the low volume of venous blood needed, it is not time-consuming, easy, inexpensive and safe. PRGF has more beneficial effects on tissue regeneration compared to PRP (19).

To the best of our knowledge, this is the first study that evaluates the protective effects of PRGF on human sperm cryopreservation. Our findings showed that PRGF 1% protects human sperm during cryopreservation. The beneficial effects of PRGF in infertility treatment and assisted reproduction have been previously described. It was shown that PRGF is useful for proliferation and migration of endometrial fibroblast and thickness of endometrium in IVF cycles (24). Also, Chang et al. found that PRP affects endometrial growth and increases pregnancy rate in implantation failure cases (25). An excess amount of ROS is the main candidate for the adverse effects of cryopreservation on spermatozoa. Antioxidant effect of PRGF has been shown on retinal pigment epithelium (5). Besides, PRGF could protect the function of mitochondria. It can increase the level of glutathione, as a major antioxidant molecule, against increasing ROS. PRGF also reduced nuclear factor erythroid 2-related factor 2 (Nrf2) gene expression in the Keap1-Nrf2 pathway (an important antioxidant pathway) (5).

The protective effects of PRGF on cryo-damage are related to its ingredients and growth factors that each of them has been shown to have beneficial effects on sperm parameters. It was found that IGF-I has a receptor on sperm cells (26) and a lower level of IGF-I in serum

was associated with abnormal sperm parameters (27). Also, a significant positive correlation was shown between seminal IGF-I and sperm count (28). Miao et al. (29) found that the incubation of normozoospermic samples with IGF-I increases sperm motility. It was also reported that IGF-I can preserve canine sperm motility during hypostorage (4°C) via acting on mitochondrial membrane potential (30). The protective effect of IGF-I on ovine sperm motility and plasma membrane integrity during cryopreservation has been approved as well (17). Li et al. (31) showed that sperm incubation with IGF-1 decreases sperm DNA damage may be due to its effects on mitochondrial cytochrome c/caspase pathway. EGF, another component of PRGF, also has receptors on sperm cells (32). It was shown that EGF can modulate acrosome reaction via actin polymerization (33). Animal studies showed that EGF improves ram sperm motility and viability during hypostorage at 4°C (34) and its effect on bovine sperm acrosome reaction has been described (35). The receptor of VEGF has been found in the male reproductive system and this protein was found in spermatids, seminal plasma, Sertoli cells, and Leydig cells. The positive effects of VEGF on sperm motility were shown in the previous study (16). Tohidnezhad et al. (36) found that VEGF ameliorates oxidative stress damage by activating the Nrf2 pathway. TGF β has anti-inflammatory effects and it was shown that seminal TGF β has a correlation with sperm motility and affects sperm function (37). FGF2 is the most important member of the FGF family. It was shown that sperm incubation with FGF2 could result in increasing sperm motility (15). FGF receptors have been found on sperm cells and it was proposed that FGF2 can increase FGFR phosphorylation in the sperm tail and modulate the sperm motility through the ERK1/2, PI3K and AKT pathway (38). Using another platelet concentrate, PRP containing similar growth factors, was shown to have positive effects on human sperm parameters. Recently, Bader *et al.* showed that incubation of 2% PRP with sperm increases sperm motility and viability and decreases sperm DNA fragmentation (39). They found that 2% PRP significantly increases sperm motility compared to controls and 5% PRP did not increase the sperm motility while 10% PRP reduced total sperm motility in the non-stressed group and in the stress group (treated with H₂O₂). They concluded that 2% PRP was the best dose for the preservation of sperm motility and viability. In a very recent study with low sample size, Yan *et al.* evaluated the possible effects of PRP on human sperm cryopreservation (18). They showed that 5% PRP preserved sperm motility, viability and integrity of plasma membrane but sperm DNA fragmentation, intracellular ROS and mitochondrial membrane potential were the same between different groups. Osmotic stress that has occurred during cryopreservation may affect sperm morphology. Spermatozoa during cryopreservation are exposed to change in osmolality which may change sperm morphology. Different proteins in the PRGF may have protective effects on osmotic shock and cell membrane during different stages of cryopreservation

(40). All of these factors together may have additive and synergistic effects to ameliorate the detrimental effects of cryopreservation on spermatozoa. It is suggested to evaluate the possible effects on PRGF on poor quality samples in the next step. This study was designed for research purposes that shows PRGF can be used for sperm cryopreservation and would provide new insights for use of autologous PRGF in assisted reproduction in the next studies in the concept of personalized medicine.

Conclusion

It seems PRGF can preserve sperm parameters including motility, viability, morphology, and the integrity of acrosome and DNA during cryopreservation. More studies with larger sample size are required to confirm our results. PRGF as one of the most important sources of growth factors could be a good candidate for adding to the freezing medium to ameliorate the detrimental effects of the freeze-thaw process on sperm parameters.

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Authors' Contributions

I.H., M.M.; Conceived and designed the study and revised the manuscript. J.M.; Conducted the study and wrote the manuscript. J.M., M.M., I.H.; Analysed the data. All authors read and approved the final manuscript.

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