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ORIGINAL

Investigation of the Effect of Biochemical Parameters of Platelet-Rich Plasma on Sperm and Expression Levels of BAX, BCL_2 and $Casp_9$ Genes under Freezing Conditions

Investigación del efecto de los parámetros bioquímicos del plasma rico en plaquetas en el esperma y los niveles de expresión de los genes BAX, BCL_2 y $Casp_9$ en condiciones de congelación de congelación

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Abstract

Objectives: Cryopreservation of spermatozoa is an important technique to help fertility. To improve semen freezing protocols, various compounds are used today to protect spermatozoa against damages. The purpose of this study is to examine the effect of platelet rich plasma on the quality of sperm parameters, oxidative stress level and chromatin heath and to assess the expression level of BAX, BCL2 and Caspase9 genes in the frozen sperm cells.

Methods: In this study, 40 normal semen samples were collected from patients who referred to the Fertility Research Center of Jahad Daneshgahi in Qom. These samples were frozen in different concentrations of platelet-rich plasma of 2.5, 5, 10, 20 and 25% by fast freezing and stored in liquid Nitrogen for 14 days. Sperm parameters were analyzed before and after freezing according to WHO 2010 protocol. To determine the optimal doze and study the effect of the intended platelet-rich plasma more accurately, complementary tests of DNA fragmentation using SDFA, and evaluation of the expression level of BAX, BCL2 and Caspas9 genes using Real-time PCR were conducted. The data was finally analyzed using one-way analysis of variance and post hoc-Tukey test.

Results: Study results show that concentrations of 5 and 10 %, as the best doses of platelet-rich plasma in the freezing process, significantly protect the parameters of motility, viability and morphology of the sperm; they also cause significant decrease in DNA fragmentation due to freezing ($p<0.05$). Gene expression results shows that optimal dose of platelet-rich plasma increases the expression level of BAX, BCL2 and Caspas9 genes ($p<0.05$) and the ratio of BAX/BCL2 shows a significant increase as well.

Conclusion: Results of this study show that appropriate dose of platelet-rich plasma can positively influence the frozen-thawed sperm and significantly protect the sperm against freezing damages.

Keywords: Infertility, Cryopreservation, Platelet-Rich Plasma, Apoptosis.

Resumen

Objetivos: La criopreservación de espermatozoides es una técnica importante para ayudar a la fertilidad. Para mejorar los protocolos de congelación de semen, hoy en día se utilizan varios compuestos para proteger a los espermatozoides contra daños. El propósito de este estudio es examinar el efecto del plasma rico en plaquetas sobre la calidad de los parámetros del esperma, el nivel de estrés oxidativo y la salud de la cromatina y evaluar el nivel de expresión de los genes BAX, BCL2 y Caspase9 en los espermatozoides congelados.

Métodos: En este estudio, se recolectaron 40 muestras de semen normales de pacientes que se derivaron al Centro de Investigación de Fertilidad de Jahad Daneshgahi en Qom. Estas muestras fueron congeladas en diferentes concentraciones de plasma rico en plaquetas al 2,5, 5, 10, 20 y 25% por congelación rápida y almacenadas en Nitrógeno líquido durante 14 días. Los parámetros espermáticos se analizaron antes y después de la congelación según el protocolo de la OMS de 2010. Para determinar la dosis óptima y estudiar con mayor precisión el efecto del plasma rico en plaquetas deseado, se realizaron pruebas complementarias de fragmentación de ADN mediante SDFA y evaluación del nivel de expresión de los genes BAX, BCL2 y Caspas9 mediante PCR en tiempo real. Finalmente, los datos se analizaron mediante el análisis de varianza de una vía y la prueba post hoc de Tukey.

Resultados: Los resultados del estudio muestran que concentraciones de 5 y 10%, como las mejores dosis de plasma rico en plaquetas en el proceso de congelación, protegen significativamente los parámetros de motilidad, viabilidad y morfología de los espermatozoides; también provocan una disminución significativa de la fragmentación del ADN por congelación ($p<0,05$). Los resultados de la expresión génica muestran que la dosis óptima de plasma rico en plaquetas aumenta el nivel de expresión de los genes BAX, BCL2 y Caspas9 ($p<0,05$) y la proporción de BAX/BCL2 también muestra un aumento significativo.

Conclusión: Los resultados de este estudio muestran que la dosis adecuada de plasma rico en plaquetas puede influir positivamente en el esperma congelado-descongelado y protegerlo significativamente contra daños por congelación.

Palabras clave: Infertilidad, Criopreservación, Plasma Rico en Plaquetas, Apoptosis.

Introduction

Despite a lot of efforts made to know the biology of freezing using various methods, it still causes overgeneration of Reactive Oxygen Species (ROS) and development of oxidative stress through exerting chemical and physical pressure on the sperm membrane. Under physiological conditions, spermatozoa produce a little ROS which is required for capacitation and acrosome reaction. When ROSs and RNSs are over-generated, biological deterioration called oxidative stress and nitrative stress occurs; these, in turn, influence the membrane structure and deteriorate the selective permeability of the sperm membrane and finally lead to cell death[1].

A platelet-rich extract consisting of various components is recently employed which has the following functions:

- Removing ROSs such as Hydroxyl radical and singlet oxygen and RNSs such as peroxy nitrite and Nitric oxide;
- Revitalizing external and internal oxidants such as Vitamin E, Vitamin C, catalase and glutathione reductase;
- Improving the capacitation of antioxidants such as superoxide dismutase, glutamate oxaloacetate transaminase, catalase and lactate dehydrogenase;
- Forming a strong shield in the sperm cell membrane against free radical attacks[2].

Cryopreservation of sperm is initial exposure of sperm to cryoprotectants, lowering the temperature below 0°C, storing, melting and, finally, removing the cryoprotectant and returning to the natural physiological state. Freezing human sperm is widely used in in vitro fertilization (IVF) plans under laboratory conditions to preserve the male gamete and provide a chance for subsequent fertilization. About 10-15% of couples are seeking infertility treatment and childbearing throughout the world; and 50% of these infertile couples have sperm-related problems. Sperm banking is used for several purposes: men whose fertility power is influenced by vasectomy, treatment with cytotoxic agents or by radiotherapy as well as men who are at the risk of sperm damage because of their working conditions[3]. Sperm freezing banks may also be used to store semen samples to be used in the following cases:

1. Infertility cases where a man cannot produce enough and adequate sperm to use ART.
2. Collecting semen is impossible on the day of obtaining the eggs for any reason.
3. Men with Azoospermia whose sperm is obtained through surgical techniques. However, sperm stored at low temperatures is less fertile than the fresh sperm. In the freezing process, the temperature of cells or the whole tissue is lowered to below 0°C and this temperature is usually 196°C below zero and the freezing occurs using Liquid Nitrogen (LN2)[4]. Long-term sperm storage is accomplished through controlling intracellular metabolism; in fact, no biochemical activity occurs below -196°C which results from lack of sufficient thermal energy required for chemical reactions at this temperature. Moreover, there is no fluid required for such a metabolic activity. However, the tissue of living cells might be damaged during the freeze-thawing process. Negative effects of freezing on sperm function are: decline in motility, viability, chromatin structure, the sperm plasma membrane, fertilization ability, preliminary fetal growth, implantation and, finally, reduced pregnancy chance[5].

Since sperm cells have inadequate antioxidants in their cytoplasm, they are more vulnerable to damages caused by freezing which may lead to impaired function due to molecular changes made in the membrane structure and sperm genome. Apoptosis is another mechanism that threatens the sperm health during the freezing. Survival of the cell after freezing is not only associated with potential damages resulting from the freezing process, but it also depends on the thawing process. Studies show that there is a negative correlation between ROS production and sperm morphology. Teratozoospermic samples with high percentage of Cytoplasmic droplets produce higher amounts of ROS which results from active cytoplasmic enzyme of Glucose 6 phosphate dehydrogenase (G6PD) that produces NADPH and increases ROS production. Lipid peroxidation is a well-known and harmful process because of making changes in lipid arrangement of the sperm membrane and decreasing the sperm motility. Lipid peroxidation control is applied on the reproductive process by antioxidant molecules and protective enzymes within the sperm and seminal plasma. Seminal plasma contains enzymes such as Superoxide dismutase (SOD), Glutathione peroxidase (GPX and Cat) which play a significant role in inhibiting the damaging effects of ROS. Lipid peroxidation reduces the sperm motility which may result from imbalance in the activities of SOD, GPX and CAT in the seminal plasma or lack of total Antioxidant Capacity of the cell. These enzymes are antioxidants that restrain Lipid peroxidation. Therefore, oxidative damage is not only associated with ROS production but it also depends on sperm antioxidant system and seminal plasma[6&7].

On the other hand, some studies show that there is no significant correlation between antioxidants and thawed semen fertility after freezing. Considering the damaging effect of freezing, we used the platelet-rich plasma to see its effects on protecting vital parameters and molecular structure of sperm under freezing conditions and evaluate the Bax, BCL_2 and $casp_9$ gene expression levels in the presence of the platelet-rich extract in the freezing process.

Sperm parameters

Sperm motility: In semen analysis, progressive motility of sperm is measured by calculating the rapid movement (a) plus slow movement (b). Total number

of the mobile sperm is measured by calculating both progressive and non-progressive motility. WHO and most laboratories assume 50% as the normal low sperm motility. However, Fertilizer Association considers 40% motility as a criterion for male factor infertility. Therefore, the normal low level indicates significant changes and depends on the regional laboratory experience. Generally, men who have greater than 10 million motile sperm per milliliter (ml) of semen enjoy a higher fertility chance than men who have 2-10 million sperm/ml. Sperm motility is induced by the wave motion of sperm flagellum which results from the conversion of ATP biochemical energy into Kinetic Energy and this causes microtubules in flagella slide along each other. Abnormalities in various components of flagellum in human spermatozoa can cause defects in sperm motility, which are classified based on defects in axoneme or preaxoneme structure. It seems that spermatozoa need to pass through cervical mucus and penetrate the transparent outer layer of the egg[8&9].

Materials and methods

Before the experiment, a vial containing Agarose was placed in a bain-marie boiling at 100°C for 5 minutes. The resulting solution was transferred to 0.5 ml microtubes with a volume of 100 µl and stored at 2-8°C.

Material and solutions used in this experiment include:

- 0.5 ml microtube
- Denaturing solution A
- Lysing solution B
- Staining solution C
- Staining solution D
- Staining solution E
- 96° alcohol
- Treated slide

After microscopic evaluation, semen spermatozoa are washed twice in HamsF-10. To do this, 500 λ of semen sample is poured into the test tube; 1.5 ml HamsF-10 is added to it and centrifuged (200 g for 5 minutes). Finally, a suspension with 15-20 million spermatozoa is prepared. One of the 0.5 ml microtubes containing Agarose is placed in 100°C bain-marie for 5 minutes so that Agarose is thawed well. Then it is put in 37°C bain-marie. 30 µl of the sperm suspension is added to the microtube containing Agarose and mixed by turning it up-down. 30 λ of the above mixture is placed on the slide over the center of the hole S and a coverslip is placed on top; press it briefly to avoid the formation of air bubbles. All through the process, the slide should be placed horizontally. The slide should immediately be transferred on a cold surface (metal or glass surface) and stored in the fridge for 5 minutes. Then the slide is taken out from the fridge and the coverslip is slowly removed from the hole S. The slide is placed in a box horizontally and denaturing solution A is poured into the hole to fill up. After 7-minute incubation at room temperature in dark, the slide is tilted to remove the excess solution from the hole. Then the lysing solution B is poured on the hole and incubated for 15 minutes at room temperature in a biological safety cabinet. After 15 minutes, the solution is removed from the slide and the hole is washed with distilled water for 2 minutes. The slide is tilted again to fully remove the distilled water from the hole and immersed in increasing percentages of ethanol for dehydration (70, 90, 100% ethanol, each for 2 minutes). Ethanol is removed from the slide and the slide is placed on the staining dish. To stain the slide and view the aura around the sperm head, staining solution C is poured on the hole and the slide is incubated at room temperature for 75 seconds. Then it is tilted and the staining solution D is poured on the hole and the slide is incubated at room temperature for 3 minutes. Finally, the staining solution E is poured on the hole and the slide is incubated at room temperature for 2 minutes. Like previous stages, remove the solutions from the hole S and wash it in water. Then place the slide in tilted form to air dry at room temperature. View the slide with 100x lens. First, obtain the image with a 40x lens and then measure the aura diameter with 100x (oil immersion) magnification. In this stage, at least 200 perfect sperm (including both head and tail) should be counted and the percentage of the number of the sperm with and without aura should be reported.

Research Implementation Method

Location of experiments: This study was conducted in the Fertility Research Center of Jahad Daneshgahi and Azad University of Qom.

Table I: List of the required tools and instruments.

Instrument	Brand	Manufacturing country	Seller company
Optical microscope	Olympus	Japan	Tehran Jarah Novin
Fluorescence microscope	EURO Star	Germany	Nima Pouyesh Teb
37°C Incubator	Pars Azma	Iran	Pars Azma
Cell counter	Labtron	Iran	Behdad
Centrifuge	Hitech	Germany	Faradid Adak Gostaran
Laminar Flow Hood	Besat	Iran	Besat Iran
Thermal Cycler	----	Poland	ParaMed
Real-time PCR	Corbett	Australia	ParaMed
Microcentrifuge	Kiagene	Germany	Tajhizyar
Spectrophotometer	Photometer 5010	Germany	Farasamed

Research methodology

This is a case-control laboratory study where samples were selected among men aged 30-40 years old by an approved urologist between 2020-2021. Men with varicoceles, men who had a prior history of varicocele surgery, men who suffered from a systemic disease or were taking medication for such a disease, men with a prior history of Chemotherapy or Radiotherapy, anatomical problems in genital organs including testicular atrophy, low sperm count (oligospermia) or azoospermia, men on treatment with antiandrogen or androgen or testosterone, with aromatase inhibitors or anti estrogen drugs, or anti-depressants were excluded from the study.

Discussion

Seminal fluid collection: Subjects were asked to avoid intercourse for 3-4 days and then they were given a sterile and graduated container to collect their semen sample. The collected samples were stored in the 37°C Incubator for 20 minutes to get liquefied. After common evaluation based on WHO 2010 criteria, individuals' semen samples went through the laboratory processes.

Evaluation or analysis of sperm parameters: The analysis was conducted in two levels: macroscopic (appearance, liquefaction, volume, and pH) and microscopic level (sperm count, sperm motility, sperm morphology).

Microscopic tests: By microscope examination of semen, density, count, motility, morphological abnormalities, agglutination and viability of the sperm were studied. Although ordinary optical microscope is usually used to assess non-stained semen, Phase Contrast Microscope can be used for fresh and non-stained or washed semen sample for more accurate analysis. 10µl of the sample is first drawn on the slide by a sampler. Then it is covered by a 22 × 22 mm coverslip. Before viewing the sample, wait a minute until the sample is stabilized. This can be conducted at room temperature but the temperature should be 20-250°C. Very different number of spermatozoa in various fields indicates non-uniformity of the sample. Therefore, samples should be mixed again. Non-uniformity implies the presence of mucus, abnormal viscosity, abnormal dissolution and agglutination of the sperm which should be reported[10].

Sperm motility: Sperm motility is one of the most important factors to determine the potential fertility of individuals and the pregnancy rate in fertility centers is estimated based on the percentage of the progressive motility of an individual's specimen. While in andrology labs, the motility rate is calculated in two ways: by eye using the microscope or by the computer software (CASA); accurate assessment of sperm motility should be conducted at least 30 minutes to one hour after ejaculation and once the sample is liquefied. As time passes, the report would not be accurate owing to dehydration as well as PH and temperature changes. Sperm motility assessment should be conducted at the temperature of 37 °C or at room temperature[10]. First, a smear was made from sperm cell suspension. If the cell density is lower than 106×20 /ml of semen fluid, a drop of 10-20 µl is used; if it is greater than 106×20 /ml of semen fluid, a drop of 5-8 µl is used. Leave the drop in the middle of one end of the slide. Place the edge of the second slide at a 30-degree angle at the drop so that the sperm drop is spread in the width of the first slide. Hold the two slides firmly; draw the second slide slowly towards yourself; then, push the slide slowly forward along the first slide. This is known as the best technique (**Figure 1**).

Figure 1: Making smear for staining.

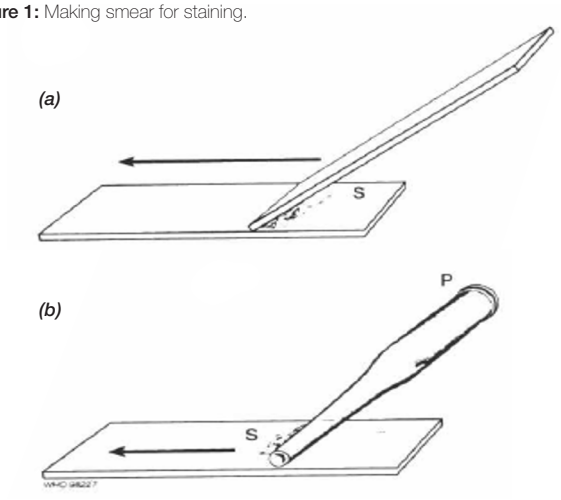
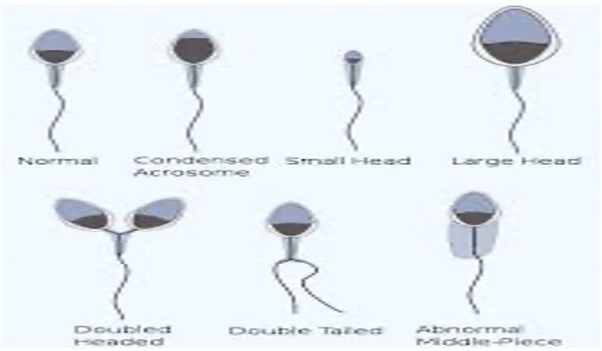


Figure 2: Normal sperm and various related abnormalities.



Sperm vitality assessment: Sperm vitality in semen sample is determined by the number of the live sperm. This assessment is recommended for semen fluid sample with the sperm having progressive motility less than 40%. This technique is based on differentiating the live from dead sperm by Eosin-nigrosin staining. Live cells become red by applying Eosin and Nigrosin provides a dark background in slide for easier assessment. This test was performed using Vitality Assay Kit (Dayan Zist Azma-Iran) which contains solution A and B.

Figure 3: Eosin-nigrosin staining to test sperm viability.

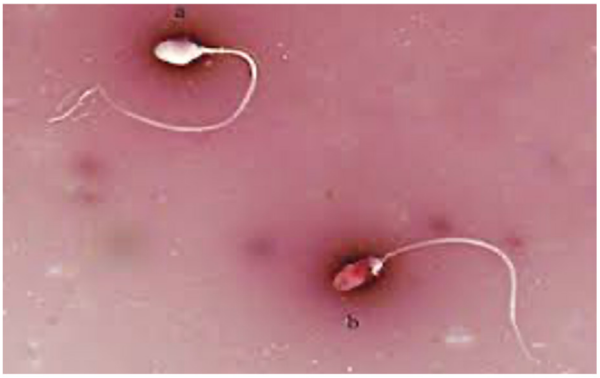
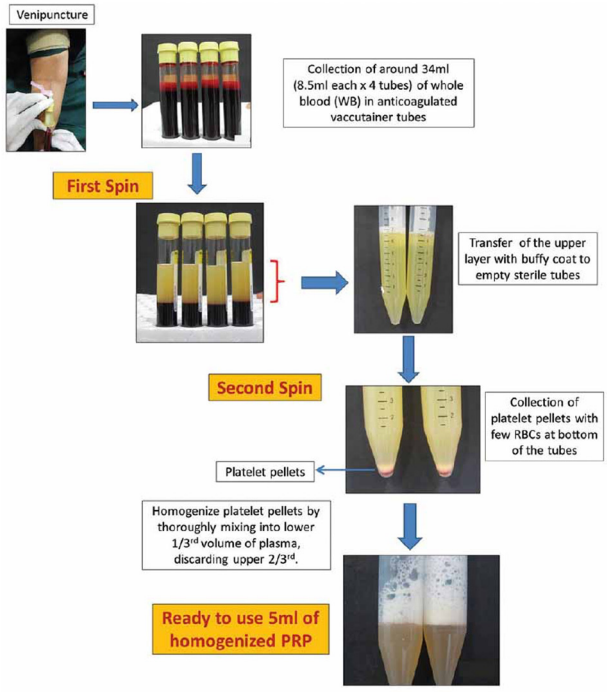


Figure 4: Stages of preparing PRP.



After sample analysis, the sperm is centrifuged and a volume of 2 ml is prepared; it is then divided into four equal parts. One part is frozen only by the freezing solution and the remaining three parts are frozen with different concentrations of PRP plus freezing solution with 1:1 proportion (semen to solution). Sperm freezing solution is added slowly by drops to the semen, immersed in Nitrogen vapor and transferred to the liquid Nitrogen.

Real Time PCR studies to measure gene expression

Preparation of primers

1. Add sterile distilled water in the amounts determined by the company to each tube containing primer, and centrifuge. A concentration of 100 pmol is obtained.
2. Store this solution as the main stock at -20°C.
3. Take 10 µL of each primer R, F; mix it with 90 µL of water to obtain a volume of 100 µL. The concentration will be 10 pmol (**Table II**).

Real-time PCR technique

To determine the concentration of genes under study, fluorescent dyes or fluorescent oligonucleotide indicators are used. During PCR reaction, as the concentration of the given genes increases, the amount of fluorescence in solution increases as well. By measuring the fluorescent light intensity at the end of each cycle, a curve is finally obtained. Then the concentration of target genes in the sample under study is measured using standard charts.

Steps of cDNA synthesis: After RNA extraction using HyperScript™ RT premix (with Random hexamer) kit by Gene All Company, cDNA was synthesized. The extracted RNA with appropriate concentration and volume of 20µl of DEPC water is added to 0.2 ml microtube of the intended company (**Table III**).

cDNA synthesis temperature is as follows in **table IV**. After cDNA synthesis, they are stored at -70°C.

Table II: Sequence of primers used.

Accession No.	Product size(bp)	Primer		Gene
NM_001357943.2	92	TGGCTACAGCAACAGGGTG CTCTTGTGCTCTTGCTGGG	Forward Reverse	GAPDH
NM_001291429.2	149	CGGCAACTTCAACTGGGG TCCAGCCCAACAGCCG	Forward Reverse	BAX
NM_000657.3	114	GGTGCCGGTTCAGGTACTCA TTGTGGCCTTCTTTGAGTTCG	Forward Reverse	BCL2
NM_032996.3	120	CAGTCCCGAAGTTCTCAAGG GATGTTCTTCACTGTGGGGC	Forward Reverse	Cas9

Table III: Components of cDNA synthesis.

Amount	Compounds
10µl	HyperScript™ RT premix (with Random hexamer)
1pg - 2µg	Extracted RNA
Final volume should be 20µl	DEPC water

Table IV: Temperature process of cDNA synthesis.

Time	Temperature	Primer
5 min	25	Random hexamer
55 min	55	
5 min	95	

Optimization

To analyze interpretation of the intended gene expression value, an internal control gene or a housekeeping gene is required. Main genes are BCL_2, BAX and $CASP_9$ and the internal control gene is GAPDH[11]. For optimization and achieving the best results, all components required for the experiment were tested in different concentrations and different temperatures which finally the most optimal concentration and temperature were selected (**Table V** to **XII**).

Note: The optimal temperature and concentration is the one that displays a single pick thawing curve in real time PCR test.

Note: Housekeeping genes are genes that are expressed consistently in all tissues of the body, such as GAPDH.

Table V: Optimal concentration of Real Time PCR reaction for GAPDH.

Concentration	The amount required for total volume of 20 µl	Compounds
____	10 µl	real Q-plus 2x Master mix Green
5µm	0.5 µl	PrimerF
5µm	0.5 µl	PrimerR
____	1µl	cDNA
____	Up to 20µl volume	ddH2O(DW)

Table VI: Real Time PCR plan for GAPDH.

Cycle	Time	Temperature(°c)	Step
1	10 min	95	Initial Denaturation
40	10 sec	95	Denaturation
	40 sec	60	Annealing
	20 sec	72	Extension
1	10 sec	55-95	Melting Curve

Table VII: Optimal concentration of Real Time PCR reaction material for BCL_2.

Concentration	The amount required for total volume of 20 µl	Compounds
____	10µl	real Q-plus 2x Master mix Green
5µm	0.5 µl	PrimerF
5µm	0.5 µl	PrimeR
____	1 µl	cDNA
____	Up to 20µl volume	ddH2O(DW)

Table VIII: Real Time PCR plan for BCL_2.

Cycle	Time	Temperature(°c)	Step
1	10 min	95	Initial Denaturation
40	10 sec	93	Denaturation
	40 sec	59	Annealing
	25 sec	72	Extension
1	10 sec	55-95	Melting Curve

Table IX: Optimal concentration of Real Time PCR reaction material for BAX.

Concentration	The amount required for total volume of 20 µl	Compounds
____	10µl	real Q-plus 2x Master mix Green
5µm	0.5 µl	PrimerF
5µm	0.5 µl	PrimerR
____	1 µl	cDNA
____	Up to 20µl volume	ddH2O(DW)

Table X: Real Time PCR plan for BAX.

Cycle	Time	Temperature(°c)	Step
1	10min	95	Initial Denaturation
40	10sec	93	Denaturation
	40sec	58	Annealing
	25sec	72	Extension
1	10sec	55-95	Melting Curve

Table XI: Optimal concentration of Real Time PCR reaction material for $casp_9$.

Concentration	The amount required for total volume of 20 µl	Compounds
____	10µl	real Q-plus 2x Master mix Green
5µm	0.5 µl	PrimerF
5µm	0.5 µl	PrimeR
____	1 µl	cDNA
____	Up to 20µl volume	ddH2O(DW)

Table XII: Real Time PCR plan for $casp_9$.

Cycle	Time	Temperature(°c)	Step
1	10 min	95	Initial Denaturation
40	10 sec	93	Denaturation
	40 sec	59	Annealing
	25 sec	72	Extension
1	10 sec	55-95	Melting Curve

Calculating gene expression value

After the test, once CTs for the reference gene and the intended gene was obtained, ΔΔCT method was used to calculate the gene expression value.

Groups ΔCT = CT Target – CT GAPDH

Control ΔCT = CT Target – CT GAPDH

ΔΔCT = ΔCT sample – ΔCT Refrence $\rightarrow$ $2^{-(\Delta\Delta CT)}$

Gene duplication curve

Duplication curve graph was drawn for BCL_2, $Casp_9$, BAX and GAPDH, as the internal control gene, by measuring the Fluorescence intensity changes. In this graph, Y-axes represent intensity of Fluorescence signal and X-axes display the reaction number. In the graph, the area where Fluorescence signal is first detected is called the threshold. In other words, this area indicates the amount of product which is duplicated exponentially in all samples so that the Fluorescence leaves the background and begins to rise above it. The cycle where the curve intersects the threshold line for generated Fluorescence is called CT. The lower this point, the greater the number of the intended gene version.

Melting curve

Due to the non-specificity of the fluorescent dye of SYBR Green and for exclusivity of primers and ensuring the duplication of specific pieces as well making sure that non-specific pieces such as primer dimers are not present in the product, melting curve is drawn. This dye binds all double- stranded DNA. Once bound, fluorescent light is detected by the detector. As the quantity of cDNA copies increases during PCR reaction, fluorescence generation increases as well; and then it is stabilized. These changes

are displayed in the fluorescent curve. At the end of PCR reaction, products are melted by increasing temperature and they become two-stranded as the temperature decreases. Fluorescent changes of the specimen in this stage are displayed in the melting curve and it indicates the specificity or non-specificity of the product.

Examining the gene expression value and results of ΔΔCT

$$\text{Fold} = \frac{E^{-(Ct_{Treat} - CT_{control})_{target}}}{E^{-(Ct_{Treat} - CT_{control})_{ref.}}} \quad (1)$$

Statistical analysis

For data analysis, SPSS version 16 (SPSS, Chicago, IL, USA) was used. Normal distribution of data was determined by Kolmogorov-Smirnov test. Results were reported as Mean ± standard deviation. Means of the groups were analyzed using one-way analysis of variance (ANOVA) and Post- hoc Tukey test. Values p<0/05 are considered as significant.

Results

Statistical analysis of data in this study indicates that the data are normally distributed. In addition, the effect of platelet-rich plasma on sperm quality, oxidative stress value, and chromatin health was studied; the level of apoptotic BAX expression, anti-apoptotic BCL2 and CASP9 in sperm cells under freezing were examined using sperm analysis techniques, viability, chromatin emission evaluation and Real time to assess changes in sperm molecular and vital parameters under freezing conditions in the presence of platelet-rich plasma and without its presence. In the first phase, samples were obtained from 30-40-year-old patients who referred to Rooya Fertility Center of Jahad Daneshgahi in Qom. Those who met the requirements joined the study by consent. Participants had abstained from sexual intercourse for 2-3 days and met the criteria to join the study. Then macroscopic and microscopic sperm analysis was performed according to WHO 2010 protocol.

Sperm Parameter Means before the freezing process

Sperm parameters were studied in participants before the freezing process. Mean, standard deviation, minimum and maximum values for each parameter is displayed in **table XIII**.

Table XIII: Sperm parameters before freezing.

Sperm parameters before freezing	Min	Max	Mean±SD
Specimen volume (ml)	5/1	5/4	324/0 ± 2/3
Sperm concentration (106/ml)	25	95	754/29 ±6/52
Total motility value (%)	45	60	325/10 ± 2/51
Progressive movement (%)	35	55	875/7 ± 7/42
Vitality (%)	45	90	215/20 ± 7/69
Normal morphology (%)	4	7	315/1 ± 1/5

Determining optimal concentration of platelet-rich plasma

To determine the optimal concentration of platelet-rich plasma in freezing process, parameters of sperm motility (%), progressive movement (%), concentration (million/milliliter), morphology (%), vitality (%) and coiled-tail (%) were evaluated and the right concentration was selected accordingly.

Examining sperm motility percentage

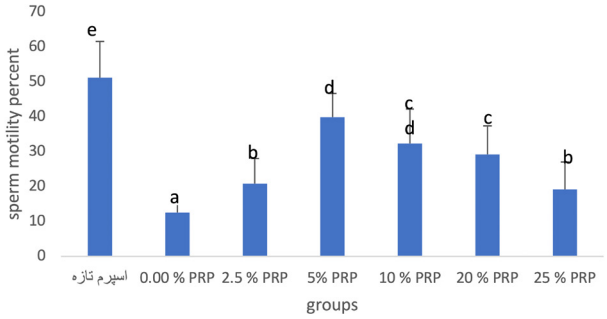
The results show that the fresh sperm group had the highest motility rate and the frozen and melted sperm with concentration of 0.00% PRP had the lowest motility compared to other groups. ANOVA test showed that the freeze-thawing process causes significant reduction in the percentage of sperm motility (%) in groups (p<0.0001).

Tukey post- hoc test showed that:

- The mean difference between fresh sperm group and other groups is significant.
- The mean difference between 0.00% PRP group and other groups is significant.
- The mean difference between 2.5%PRP group and 25%PRP group is not significant but it is significantly different from other groups.
- The mean difference between 5%PRP group and 10%PRP group is not significant but it is significantly different from other groups.
- The mean difference between 10%PRP group and groups of 5%PRP and 20%PRP is not significant but it is significantly different from other groups.
- The mean difference between 20%PRP group and 10%PRP group is not significant but it is significantly different from other groups.
- The mean difference between 25%PRP group and 2.5%PRP group is not significant but it is significantly different from other groups.

Comparison of the means of sperm motility percentage is displayed in the **figure 5**.

Figure 5: Comparison of the means of sperm motility percentage



Calculating the percentage of sperm progressive movement

Results of the **figure 6** show that 25% PRP group has the lowest and 25% PRP has the greatest percentage

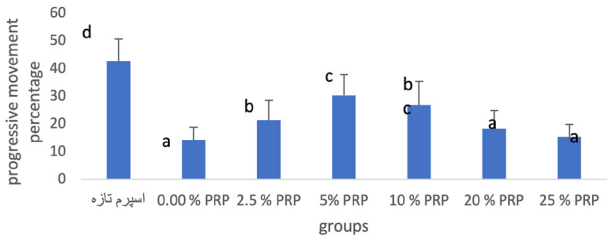
of sperm progressive movement. One-way analysis of variance (ANOVA) test showed a significant mean difference in sperm progressive movement percentage (%) between the groups (p<0.0001).

Tukey post- hoc test showed that:

- The mean difference between fresh sperm group and other groups is significant.
- The mean difference between 0.00% PRP group and groups of 20%PRP and 25%PRP is not significant but it is significantly different from other groups.
- The mean difference between 2.5%PRP group and groups of 20%PRP and 25%PRP is not significant but it is significantly different from other groups.
- The mean difference between 5%PRP group and 10%PRP group is not significant but it is significantly different from other groups.
- The mean difference between 10%PRP group and groups of 2.5%PRP and 5%PRP is not significant but it is significantly different from other groups.
- The mean difference between 20%PRP group and groups of 0.00%PRP and 25%PRP is not significant but it is significantly different from other groups.
- The mean difference between 25%PRP group and groups of 0.00%PRP and 20%PRP is not significant but it is significantly different from other groups.

Different letters represent a significant difference between groups and similar letters represent that there is no significant difference. Comparison of the means of the percentage of sperm progressive movement is displayed in the **figure 6**.

Figure 6: The mean of sperm progressive movement percentage.



Different letters represent a significant difference between groups and similar letters represent that there is no significant difference (p<0.05).

Assessing the percentage of sperm with coiled-tail

Papanicolaou method was used to assess the percentage of sperm with coiled-tail which is displayed in **figure 7**. The highest percentage of the sperm with coiled-tail is found in 0.00%PRP group and the lowest percentage is found in the fresh sperm group.

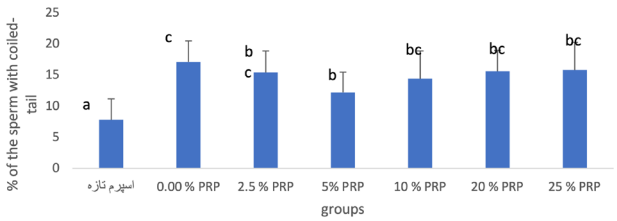
One-way analysis of variance (ANOVA) test showed a significant mean difference in the percentage of the sperm with coiled-tail (%) between the groups (p<0.027).

Tukey post- hoc test showed that:

- The mean difference between fresh sperm group and other groups is significant.
- The mean difference between 0.00%PRP group and groups of 5%PRP and fresh sperm is significant but it is not significantly different from other groups.
- The mean difference between 2.5%PRP group and fresh sperm group is significant but it is not significantly different from other groups.
- The mean difference between 5%PRP group and groups of fresh sperm and 0.00%PRP is significant but it is not significantly different from other groups.
- The mean difference between 10%PRP group and fresh sperm group is significant but it is not significantly different from other groups.
- The mean difference between 20%PRP group and fresh sperm group is significant but it is not significantly different from other groups.
- The mean difference between 25%PRP group and fresh sperm group is significant but it is not significantly different from other groups.

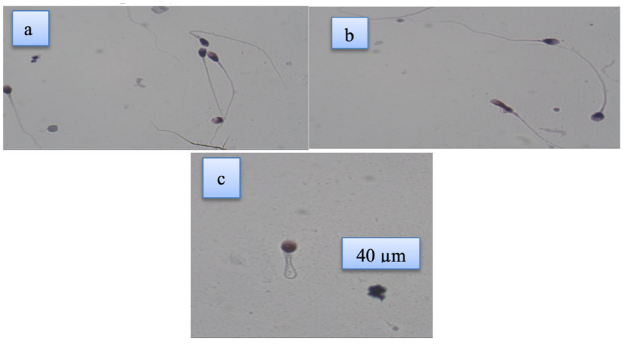
Comparison of the means of the percentage of the sperm with coiled-tail is reported in the **figure 7**.

Figure 7: The mean of the percentage of the sperm with coiled-tail



Different letters represent a significant difference between groups and similar letters represent that there is no significant difference (p<0.05).

Figure 7[11]: Sperm morphology illustrated in Papanicolaou way before freezing (a), after freeze-thaw (b) and the sperm with coiled-tail due to freezing process (c)



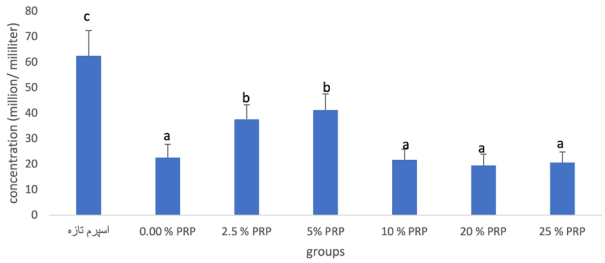
Assessing sperm concentration (million/milliliter)

The lowest sperm concentration is found in 20%PRP group and the highest concentration is found in the fresh sperm group. One-way analysis of variance (ANOVA) test showed a significant mean difference in sperm concentration between the groups (p= 0.004).

Tukey post- hoc test showed that:

- The mean difference between fresh sperm group and other groups is significant.
- The mean difference between 0.00%PRP group and groups of 10%PRP, 20%PRP and 25%PRP is not significant but it is significantly different from other groups.
- The mean difference between 2.5%PRP group and 5%PRP group is not significant but it is significantly different from other groups.
- The mean difference between 5%PRP group and 2.5%PRP group is not significant but it is significantly different from other groups.
- The mean difference between 10%PRP group and groups of 0.00%PRP, 20%PRP and 25%PRP is not significant but it is significantly different from other groups.
- The mean difference between 20%PRP group and groups of 0.00%PRP, 10%PRP and 25%PRP is not significant but it is significantly different from other groups.
- The mean difference between 25%PRP group and groups of 0.00%PRP, 10%PRP and 20%PRP is not significant but it is significantly different from other groups.

Figure 8: The mean of sperm concentration in the fresh sperm group and frozen-thawed sperm groups with PRP concentrations.



Different letters represent a significant difference between groups and similar letters represent that there is no significant difference (p<0.05).

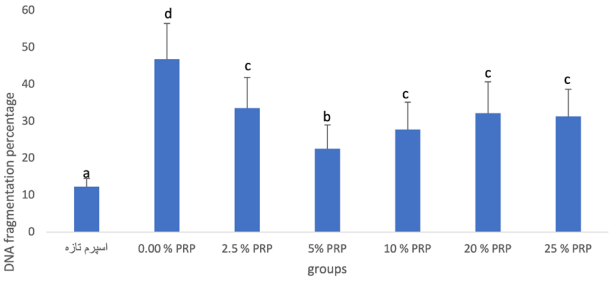
Comparing the effect of different concentrations of platelet-rich plasma on DNA fragmentation in the fresh sperm and the frozen-thawed sperm

Chromatin emission evaluation test was used to assess DNA fragmentation which is indicated in **figure 9**. The lowest rate of DNA fragmentation is found in the fresh sperm group and the greatest amount is found in 0.00%PRP. One-way analysis of variance (ANOVA) test showed a significant mean difference in ROS between the groups (p= 0.012).

Tukey post- hoc test showed that:

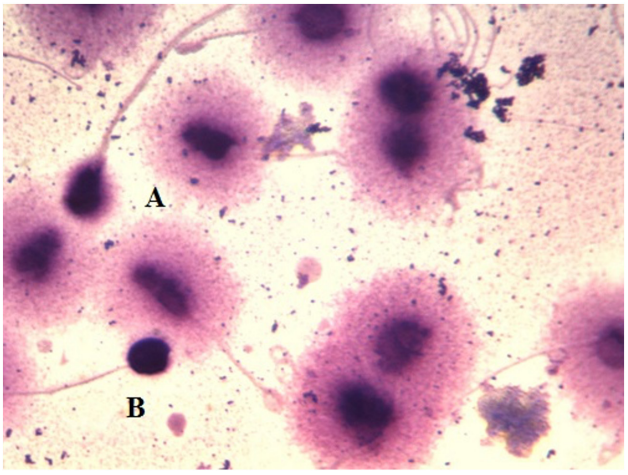
- The mean difference between fresh sperm group and other groups is significant.
- The mean difference between 0.00%PRP group and other groups is significant.
- The mean difference between 2.5%PRP group and groups of 10%PRP, 20%PRP and 25%PRP is not significant but it is significantly different from other groups.
- The mean difference between 5%PRP group and 10%PRP group is not significant but it is significantly different from other groups.
- The mean difference between 10%PRP group and 5%PRP group is not significant but it is significantly different from other groups.
- The mean difference between 20%PRP group and groups of 2.5%PRP, 10%PRP and 25%PRP is not significant but it is significantly different from other groups.
- The mean difference between 25%PRP group and groups of 2.5%PRP, 10%PRP and 20%PRP is not significant but it is significantly different from other groups.

Figure 9: Comparison of DNA fragmentation percentage.



Different letters represent a significant difference between groups and similar letters represent that there is no significant difference (p<0.05) (**Figure 10**).

Figure 10: DNA fragmentation by staining Chromatin emission evaluation.



The effect of different concentrations of platelet-rich plasma on expression of CASPASE9 Apoptosis in the frozen-thawed sperm

Expression level of CASPASE9 Apoptosis in samples was calculated using Real Time PCR and the values are reported in the **figure 11**.

- The ratio of expression level of CASPASE9 Apoptosis in the frozen-thawed sperm with 0%PRP to the fresh sperm increased by 9.91 times which was significant (p<0.0001).
- The ratio of expression level of CASPASE9 Apoptosis in the frozen-thawed sperm with 2.5%PRP to the fresh sperm increased by 6.03 times which was significant (p<0.0001).
- The ratio of expression level of CASPASE9 Apoptosis in the frozen-thawed sperm with 5%PRP to the fresh sperm increased by 2.22 times which was significant (p<0.039).
- The ratio of expression level of CASPASE9 Apoptosis in the frozen-thawed sperm with 10%PRP to the fresh sperm increased by 5.52 times which was significant (p<0.0001).
- The ratio of expression level of CASPASE9 Apoptosis in the frozen-thawed sperm with 20%PRP to the fresh sperm increased by 6.55 times which was significant (p<0.0001).
- The ratio of expression level of CASPASE9 Apoptosis in the frozen-thawed sperm with 25%PRP to the fresh sperm increased by 8.38 times which was significant (p= 0001).

Figure 11: Changes of caspase9 expression level.

Conclusion

Study results show that concentrations of 5 and 10 %, as the best doses of platelet-rich plasma in the freezing process, significantly protect the parameters of motility, viability and morphology of the sperm; they also cause significant decrease in DNA fragmentation due to freezing (p<0.05). Gene expression results shows that optimal dose of platelet-rich plasma increases the expression level of BAX, BCL_2 and Caspas9 genes (p<0.05) and the ratio of BAX/BCL_2 shows a significant increase as well.

Conflict of interest

Authors do not have any conflict of interest to declare.

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