Cellular and Molecular Biology

Journal homepage: www.cellmolbiol.org

Protective Effects of Autologous Platelet-Rich Plasma (PRP) on the Outcome of Cryopreservation in Rabbit Sperm

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ARTICLE INFO

Original paper

Article history:
Received: March 28, 2022
Accepted: April 28, 2022
Published: April 30, 2022

Keywords:
Platelet-Rich Plasma (PRP), Cryopreservation, Gene expression, Rabbit

ABSTRACT

Sperm cryopreservation is a cost-effective means of preserving gene resources and transporting sperm across distant locations. However, due to the general disadvantages of using freeze-thawed sperm, to prevent this irreversible damage, cryopreservation techniques must be improved by the addition of additional cryoprotection agents. This study aims to improve the freezability of buck semen using an intratesticular injection of Autologous Platelet-Rich Plasma **(**PRP) and confirm this theory by Casa laboratory analysis and gene expression detection of three genes (CATSPER1, SPAG5 and Hsp70). Twenty rabbits a New Zealand healthy male were randomly divided into two equal groups; the control and PRP group which enriched with PRP, the semen collection was applied after 10 weeks, then each sample was divided into two fractions; First fractions we apply the laboratory semen analysis directly, and the second fractions were cryopreservation, then thawed after one month to apply the same laboratory analysis. The results of CASA, DNA fragmentation, and real time-PCR analysis had statistically significant differences ($P<0.01$) when compartment of these values after and before freezing, yet we don't record any statistical differences between the C group and CR group. This study's findings are extremely significant, indicating that intratesticular injection of PRP is a good method for using in enhancing the rabbit sperm procedure after freeze-thawing.

DOI: http://dx.doi.org/10.14715/cmb/2022.68.4.14

Introduction

The use of sperm cryopreservation as a more economical and efficient means to maintain male gametes for assisted reproductive procedures has recently increased (ART). Sometimes, animal (ART) institutions may need to develop this procedure to retain the sperm's excellent quality and genetic potential (1). The freezing and thawing process can impact fertility because it reduces the motile degree and velocity of spermatozoa (2). Also, it causes increased DNA fragmentation which leads to damage sperm's plasma membrane, cytoplasm, and genomic structure (3,4). The cryopreservation-induced stress causes ultrastructural and functional damage in spermatozoa, like reduced motility, membrane integrity, and fertilizing activity (5). The detrimental effects of extreme freezing and thawing on sperm are still a major concern, as they reduce sperm fertility. The major causes of sperm cryodamage, may by resulting of osmotic alterations, cold shock, intracellular ice crystal formation, and oxidative stress (6).

As a result of the above, it was necessary to look for modern procedures that help improve the efficiency of cryopreservation. In this study, a new protocol was designed by intratesticular injection of autologous platelet-rich plasma (PRP) to reduce the spermatozoa effects by freezing and thawing process. Some recently published papers have suggested that it is possible to improve the sperm parameters after intra-testicular injection of PRP (7,8).

The PRP is a type of autologous platelet that has a concentration three to seven times higher than normal and is being used in several medical fields, with noteworthy results in dermatology, orthopedics, and sports medicine, stomatology, and reproductive medicine. (9). Many studies, like (10,11) have investigated the physiological and molecular mechanisms that lead to PRP therapy's success, and have discovered that PRP can help regulate the

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activity of Leydig and Sertoli cells, and growth factors have a beneficial impact on spermatogenesis and fertility, as well as a potential role on tissue repair via progenitor cell proliferation and differentiation.

Also, (12) were found that in the busulfan-treated mice, the PRP enhanced the number of spermatogenic stem cells, sperm motility, and testosterone levels. There are few studies on sperm motility evaluation using a computer-assisted sperm analyser (CASA) and gene expression variations of several critical regulatory transcripts involved in motility and fertility, as well as their relationship to sperm after cryopreservation, of genes associated with sperm fertility and motility like CATSPER1, SPAG5 and Hsp70

CATSPER1 (Cation Channel Sperm Associated 1) is a protein-coding gene that belongs to a family of putative cation channels that is found in the flagellum of the spermatozoa. The CATSPER has been shown to form a single channel, and disruption of one gene blocks the expression of the other in the flagellum. (13). CATSPER gene deficiency in Sperm leads to a high frequency of flagellar beats and a low bend amplitude, as well as an inability to hyperactivate during capacitation. (14)

The Specific sperm antigen5 (SPAG5) has 24 exons and is found on chromosome 17. It is found in large amounts in the testicles as well as other tissues such as the placenta, liver, and kidneys. These gene functional responsibilities regulated spermatogenesis. This gene's functional responsibilities regulated spermatogenesis and are critical for sperm activities including motility. The expression of the SPAG5 gene appears to be influenced by sperm cryopreservation (15).

HSPA4 (Heat Shock Protein Family A (Hsp70) member 4) is one of the chaperone molecules that is generally produced when the cells are subjected to all-encompassing stressors. Yet, it has been shown that the expression of the HSP70 gene/protein decreases throughout the cryopreservation procedure (16). A reduction in HSP70 levels throughout duration sensitizes spermatozoa leading to cell apoptosis and alterations of the capacitation (17). As a result, it appears that maintaining a greater antioxidant defense in spermatozoa throughout freezing and thawing is critical. The purpose of this study was to determine the role of PRP in antioxidant defense as well as its impact on HSP expression and, as a result, rabbit semen post-thaw quality. The aim of the present study was, therefore, evaluation of benefit intratesticular injection of autologous PRP on the ability of sperm to tolerate the cryopreservation and throwing processes in male rabbits and find a relation between sperm parameters and gene expression patterns of a few selected genes related to motility and fertility of sperm, like CATSPER1, SPAG5 and Hsp70

Materials and methods

Animals and Study design

The period of this study was from October 2021 to February 2022. Twenty healthy New- Zealand rabbit males aged 11-13 months and weighing 1.90-2.70 kg were selected. All animals were confirmed to be free of any visible abnormalities of the reproductive organs, all bucks were kept under standard environmental conditions, fed with nourishing fodder, hay, and root crops. During the adaptation period of two weeks, the rabbits were taught to service an artificial vaginal delivery system. These animals were randomly divided into two equal groups: the control (C) which was kept without any injection, and the PRP (P) which was treated with autologous PRP.

Platelet-Rich Plasma (PRP) preparation

From each animal of the PRP group, 10 ml of whole blood was drawn, then mixed with a 3.2% sodium citrate tube with a whole blood/anticoagulant ratio of 9/1, this mixture was added to the PRP gel tube with the Activator. (Biozek Medical®, Laan van de Ram, Bulgaria), and centrifugation as per the manufacturer's recommendations. The PRP collected (2ml) were drawn into the Eppendorf tube and stored at −80°C for later use. The PRP was used for intra-testicular injection as described by (7). The concentration of the PRP in this study was (4000 × 10^9 platelets/1 microliter) 200 µl single-dose injection into each testis parenchyma of PRP group animals, by inserting a sterile needle gauge 21 along the longitudinal axis and pushing the fluid with the withdrawal process (12)

Semen collection

The semen samples were collected after 10 weeks of PRP injection (18) by disposable artificial vagina device pre-filled with warm water (40°C) and maintained in a water bath (38ºC). The sperm

concentration (10^6/ml semen) was calculated by direct cell count using the Hemocytometer (MARIENFELD®, Neubauer-Improved Platelet Counting Chamber, USA) according to the company guide.

The semen samples of both groups (Control & PRP) were divided into two fractions: the first fraction protective without frizzing (C & P) and the second fraction kept by cryopreservation (CR & PR) by diluted (50×10^6 sperm/ml), using Tris egg-yolk medium (Rabbit semen extender Lepus series) according to performer as a description by (19). After one month; Thawing was achieved by immersing the straws for six sc. in the water bath set at 65°C (20), then processing the laboratory analysis and gene expression.

DNA fragmentation

The DNA fragmentation evaluation of Halo sperm kit (Halotech DNA, Spain) as an improved Sperm Chromatin Dispersion (SCD) test. A microscope was used to examine 500 spermatozoa per sample. We relied on the (21) study, were referred to the spermatozoa with intact DNA having a medium or large halo. Yet, the spermatozoa with absent, degraded, or small halos were thought to have DNA fragments.

CASA analysis

The sperm motility was evaluated by the CASA method (CEROS II®, Animal Semen Analysis, Zeiss, IMV-Technologies Co., France), with a sperm chamber (Leja products BV ® 4 Chamber Slides depth 20 µm, IMV-Technologies Co., France), These sperm parameters that were chosen: Total motility %; Progressive motility (% STR>85%); Sperm hyperactivity* (VCL >35 µm/s, ALH >2.5 µm/s; vitality (% live sperm) (22); Spermatozoa morphology by using the staining kit for spermatozoa morphology (SoermFunc®, Diff-Quik, China), this slide was fixed and stained with the manufacturer guidelines, a minimum of 200 motile spermatozoa analysed per sample,

RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

For the C & P groups before freezing, samples are directly handled by the laboratory analysis. As for the groups CR & PR; each treatment's straws were taken from the liquid nitrogen tank and allowed to thaw at ambient temperature. The contents were placed in a 1.5 ml Eppendorf tube, then centrifuged at 700 rpm (12 min. /4 ˚C). All samples' pellets were stored at -80°C until RNA was extracted.

The RNA isolation using (Thermo Scientific ® GeneJET RNA Purification Kit, California, USA), with adding DNase I (Thermo Scientific ® RNase-free kit, California, USA). The integrity of total RNA was verified by agarose gel electrophoresis by Cleaver®, NANOPAC-300, Scientific Ltd., UK. The concentration and purity of total RNA were determined by the ratio reading at A260/280 nm, only samples have a ratio of 1.8 to 2.0, by Nano-drop spectrophotometer (OPTIMA®, SP-3000 Nano, UV/Vis Spectrophotometer. Tokyo, JAPAN). The cDNA synthesis by reverse transcription (RT-PCR) was used (Thermo Scientific ® RevertAid First Strand cDNA Synthesis Kit, California, USA) with random primers.

The Gene-specific primers were designed from sequences available in the GenBank database https://www.ncbi.nlm.nih.gov/ using Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) as shown in table1.

Table1. The primer sequence and annealing temperature were used for quantitative real time-PCR analysis.

Gene	Gene ID	Primer sequence	Product size (bp)
CATSPER1	100357804	F: 5′- GTGTCGTTCCACTGTCCACTCC-3′ R: 5′- CCACACGGGCAAAGACAGGC-3′	163
SPEG5	100346395	F: 5′- GCTCACTTCGGCGTGCAGAG-3′ R: 5′- ACCCAACTGAGCTCCCCACTG-3′	171
Hsp70	100338049	F: 5′- CCCCAATCTGAAAACAGCAGCTA-3′ R: 5′- ACACTGTAGTGCCCACCAAGAA-3′	185
GABDH	100009074	F: 5′-TGAGCGAGCTTACAACCAAC-3′ R: 5′-ATCACAAACATGGGGGCATC-3′	104

The real-time PCR reaction was done using (Excecycler 96® Thermal cycler for Real-time PCR, Bionner, Korea), with first-strand cDNAs (2.0µl) from

total sperm cells RNA using 0.2 µl from both reverse and forward primers, 10ul of SYBR Green Master Mix (AccuPower® Greenstar™ qPCR PreMix, Bionner, Korea), 7.6µl nuclease-free water.

The settings of relative quantitation PCR reactions using the $2^{-\Delta\Delta Ct}$ calculation, with the reference gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). As follows: The PCR amplification comprised of a 2 min initial step at 95 ºC, followed by 40 cycles of 30-sec denaturation at 95 ºC and 30 seconds of annealing at 58 ºC, and 30-sec extension at 72 ºC. The last extension took 5 minutes at 72 ºC, according to (23).

Statically Analysis

IBM® SPSS® statistical software 27.0, 2020; for Windows (SPSS Inc., Chicago, IL, USA) was applied. All the data were normally analysing used Duncan's post-hoc test by one-way analysis of variance (ANOVA). The P values of less than 0.05, 0.01, and 0.001 were used to signify statistical significance (24)

Results and discussion

The data of spermatic parameters results in statistically significant differences (P<0.01) in concentration (Figure 1), total sperm motility, progressive motility, hyperactivity and vitality in the P group were a high compared with C, CR and PR groups, also don't record statistical differences between PR and C. yet, these parameters were decreased after cryopreservation in -196℃ for one mouth and thawing in CR group, but it was less effective in the PR group. (Table 2).

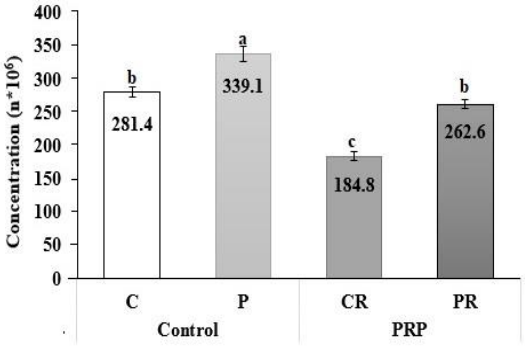

Figure 1. The percentages of spermatozoa concentration for samples of fresh (C) group (n = 10), and PRP therapy (P) group (n = 10) compared with cryopreservation of control (CR) group (n = 10), and PRP therapy (PR) group (n = 10). The different letters in each row values were provided a different significantly (P<0.01), one-way ANOVA with Dunkin (*post-hoc* test).

Table 2. Spermatic parameters and DNA fragmentation (Mean ± SEM) of buck semen as affected by autologous PRP intra-testicular injection.

Sperm parameter	Fresh		Frozen	
	C	P	CR	PR
Total sperm motility (%)	60.8^{b}±2.14	78.6^{a}±1.59	46.8^{c}±1.33	55.01^{c}±1.38
Progressively sperm (%)	39.8^{b}±0.88	48.4^{a}±0.98	30.1^{d}±1.06	34.6^{c}±1.07
Hyperactivity (%)	8.6^{b}±0.72	14.5^{a}±1.0	4.8^{c}±0.53	9.5^{b}±0.72
Vitality (%)	60.61^{b}±3.04	77.5^{a}±1.91	48.0^{c}±3.18	59.4^{b}±2.07

C; control group, P; PRP group, CR; control freezing group, PR; PRP freezing group. a,b,c,d: Means within a row with different superscripts differ significantly (p<0.01); S.E.M- Standard error of the mean. The (*) in each row values were provided a different (P<0.01), one-way ANOVA with Dunkin (post-hoc test).

The data was obtained after analyzing the DNA fragmentation of freezing rabbit sperm treated with PRP which was stored for one month at -196 °C and thawing. The DNA fragmentation was downed in the P group compared with the C group. Yet, the PR group showed a lower percentage of DNA fragmented than the CR group. (Figures 2 and 3)

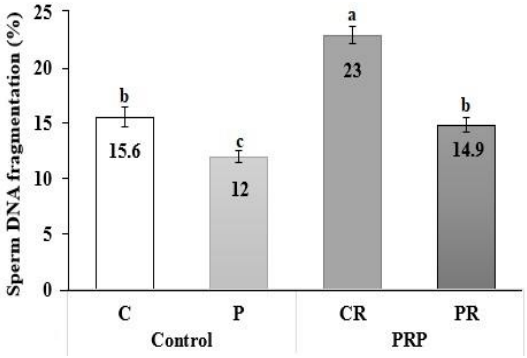

Figure 2. Role of the positive effect of the autologous PRP intratesticular injection anti-impact of sperm cryopreservation stress on sperm DNA fragmentation. The results are means ± S.E.M. The different superscript letters in each bar represent significant differences (P<0.01), one-way ANOVA with Dunkin (*post-hoc* test).

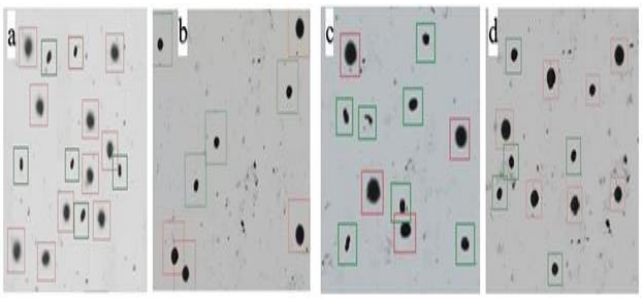

Figure 3. Microscopic field visualized after staining with SCD technique. Those sperm cells with fragmented DNA are indicated by a green square, and sperm with a big halo of DNA dispersion is indicated by a red square. (100x).

The preparation of normalized cDNA libraries was done in the Biotechnology faculty, University of Al-Qadisiyah, Iraq , from 1.8 µg pooled total RNA. First-strand cDNA synthesis was carried out with an oligo (dT) primer. The obtained cDNA population. The Normalization was achieved by one cycle of denaturation. (Figure 4).

The gene expression level of target genes (CATSPER1, SPEG5 and Hsp70) of the P group (3.64±0.17, 5.37±0.53 and 3.97±0.39 (respectively were upregulation (P < 0.01) than C group (2.60±0.21,3.01±0.24 and 2.39±0.28 (respectively . Although, these expressions were upregulation (P < 0.01) in the PR group (2.40±0.19 ,2.87±0.18 and 2.89±0.22 (respectively, vs. the CR group (1.40±0.07 , 2.02±0.17 and 2.89±0.22 (respectively. Yet, there was no significant difference between C and PR (P < 0.01). (Figure 5).

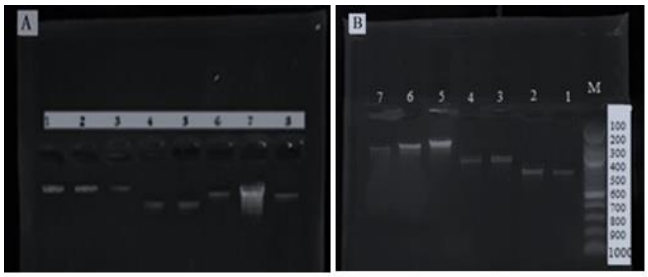

Figure 4. Detection of Complementary DNA (cDNA) template agarose 1% stained Ethidium bromide, synthesized using sperm cell's total RNA as the template by reverse-transcriptase RT-PCR. Lane 1-3, CATSPER1 (lane 4-5), SPEG5 (lane 6-8), Hsp70 (A). Lane 1-2 (CATSPER1 length of 390 bp), lane 3-4 (SPEG5 length of 320 bp), lane 5-7 (Hsp70 length of 240 bp) (B). Marker type 1000 bp of DNA Ladders (TrackIt™ 100 bp DNA Ladder, Invitrogen, Thermo Fisher Scientific, USA) (M).

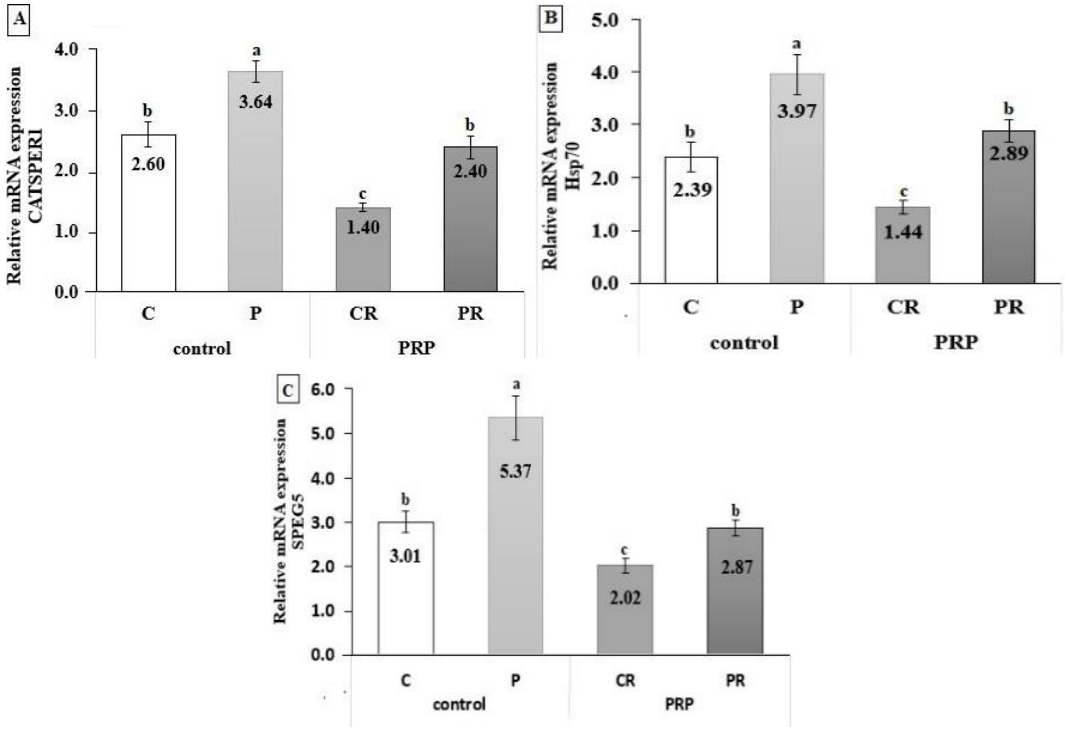

Figure 5. Comparison of relative mRNA expression for target genes. CATSPER1 (A), SPEG5 (B) ,Hsp70 (C) by real-time RT-PCR of control and PRP group before freezing (C &P) and after freezing (CR &CR) respectively. The Values are the means ±SE (n=10). The different letters in each row values were provided a different significantly (P<0.001), one-way ANOVA with Dunkin (*post-hoc* test).

The goal of cryopreservation is to maintain as many live, normal spermatozoa as possible after they have been thawed while preserving the sperm quality criterion that was before freezing, even though spermatozoa cryopreservation is an often-used procedure for preserving their biological function, roughly half of spermatozoa do not survive the freezing-thawing operation (25). Inappropriate freezing or thawing rates are inversely correlated with spermatozoa survivability since the spermatozoa experience a substantial alteration in their chemical and physical features. (26)

Spermatozoa are susceptible to harmful chemical and physical consequences of the cryopreservation process, including the production of intracellular ice crystals and disintegration, increased permeability of membranes, cellular dehydration and osmotic damage (27). As a result, it harms the spermatozoa fertility by causing damage to their cell membranes and DNA which leads to limiting the sperm motility (28)

The present study investigated intratesticular injection of autologous PRP less effect of the cryopreservation and thaw sperm stress on sperm and regulating of genes expression. The data of concentration and CASA analysis of a total sperm motility, progressive motility, hyperactivity and vitality that statistically significant differences ($P<0.01$) in group P compartment with group C, and the group PR compared with group CR (Figure 1 & Table 2). Furthermore, the data of DNA damage in the PR group were lower than in the CR group. Yet, the sperm samples of group P showed a lower percentage, compared to C group sperm samples ($p < .001$). (Figure 2)

By monitoring caspase-3 and IGF-1 mRNA expression, the (29) study was able to determine the efficiency of supplementary PRP for testicular injury repair in diabetic experimental rats. Many research studies indicated that low mRNA expression in cryopreserved spermatozoa samples results in decreased motility. (30) ,Therefore our current study has highlighted some genes that are responsible for sperm fertility like CATSPER1, SPAG5 and Hsp70. Quantitative PCR is a valuable technique for evaluating the quality of sperm after they have been thawed. The HSP (Hsp70 and Hsp90) mRNA expression levels based on qPCR have been suggested as molecular markers to determine the fertilization ability of post-thaw sperm. (31). Previous studies stated that Hsp70 plays an important role as an indicator of thermo tolerance that were measurable by performance indicators of quantifying the expression of the Hsp70 gene. (32). The quality of bull spermatozoa may also be accessible by the quantification of Hsp70 gene expression rather than motility alone, according to the findings of the (33) study.

The results of figure (5) appearance the statistically significant difference ($P\leq0.01$) of CATSPER1 mRNA expression, after freezing were up-regulation of these target genes in PR group, vs. CR group, these findings agree with those of a previous study, which demonstrated that cryopreservation alters the mRNA–protein relationship and makes mRNA molecules highly vulnerable to degradation (34). Besides that, other studies found significantly higher levels of CATSPER2 and CATSPER3 mRNAs in high motility spermatozoa than in the low-motility fraction (35). This downregulation of CATSPER1 in sperm cells of the CR group by freezing and thawing process after one month leads to the low synthesis of the CATSPER1 protein which is the responsible formation of the CATSPER channel which plays a vital role in regulating sperm motility, hyperactivity and male fertility. It was also explained that by (36). Thereto, the sperm lacking CATSPER show abnormally high flagellar beat frequency and low bend amplitude, as well as a failure to hyperactivity during capacitation. (37)

According to these results of our study, the down-regulation of mRNA- SPAG5 in sperm cells in the CR group, then goes back to normal or slightly higher in the PR group (Figure 5), whereby agreements with (38) that were noted in the sperm-specific antigens possibly, if defective, cause infertility through creation sperm cell dysfunction. This clearly shows that sperm cryopreservation causes challenges and adverse effects can affect the expression of this gene (39).

In our study results; the cryopreservation significantly reduced the mRNA expression levels of heat shock protein (HSP70) in rabbit sperm, these findings (Figure 5) agree with many previous studies such as (17) which indicated these effect in bull sperm. Based on those results, we suggest the intratesticular injection of PRP may have an important molecular role to enhance the testicular cells to the regulation of CATSPER, SPAG5 and Hsp70, this result was confirmed by the researcher (40) were suggested the PRP contains the highest concentration of growth factors with known anabolic enhances tendon matrix gene expression patterns. However, there was no difference in CASPASE-3 and IGF-1 mRNA expression of the diabetic rat testis given insulin and PRP compared to those without PRP (41). Furthermore, the freeze-thaw process of cryopreservation can modulate mRNA stability,

protein and gene expression, and epigenetic content of sperm (42).

The numerous cytokines such as platelet-derived growth factor (PDGF), transforming growth factors β (TGF-b) and vascular endothelial growth factor (VEGF) included in PRP contribute to its biological activity and possible therapeutic effect on sperm quality and motility (43) Zinc and calcium ions are closely related to sperm capacitation and acrosome reaction (44). Furthermore, it has been found that the platelet-activating factor protects sperm from cryodamage and improves sperm quality following cryopreservation (45). Additionally, many cell signaling molecules directly engaged in tissue healing, including serotonin, sphingosine-1-phosphate, and adenosine diphosphate, is found in PRP granules, and these chemicals can boost survival signals (46). All of the above operations were referred to the PRP may be implicated with sperm parameter enhancement. The study revealed that as compared to the controls group the PRP group had the same effect on sperm quality following the freezing-thawing technique. Specifically, improving sperm total motility increases progressive motility and acts on inhibition of the DND fragmentation (Figure 2). These findings confirmed that intra-testicular injection of PRP may enhance spermatozoa production. This hypothesis was in agreement with the results of (21) which referred to the PRP molecular role which may strengthen the cells' natural defences. Also, the PRP has a favourable impact on histological analysis according to a study by (47) that showed the successful use of PRP in restoring the gonads of male mice that were in a toxic state. The beneficial effect of PRP is mainly related to its numerous bioactive components, like IGF-1 which has a major role in tissue repair (48), furthermore, IGF-1 can inhibit apoptosis in germinal cells, according to studies done in testicular cells (49). This interaction allows inter-and intracellular signalling pathways to control cell growth, proliferation, and differentiation through. These PRP local effects include releasing of other growth factors, chemotaxis, Mitogenesis, and extracellular matrix creation (50).

Conclusions

This study's findings are extremely significant, indicating that intratesticular injection of autologous PRP is a good method for using in enhancing the rabbit sperm procedure after freeze-thawing. Our findings suggest that cryopreservation is a viable option for rabbit sperm when injection of PRP, seems more effective. The reproductive effects of the sperm appeared to give a rise to that of fresh sperm after using the improved freezing process. In further studies, the beneficial effects of PRP reported here must be validated by intensive rabbit rearing systems, to increase the safety of preserving sperms from freezing on the one hand, and to improve the movement of weak sperms on the other hand.

Acknowledgments

The authors are much thankful to the staff of the Sciences Faculty and Medicine Faculty, University of Sfax, Tunisia, and we would like to warmly thank the Faculty of Biotechnology and Veterinary Medicine, University of Al-Qadisiyah, Al Diwaniyah, Iraq to provide all necessary assistance for this work. We appreciate, assistant Prof. Mohammed A Al-Askari of the Department of Medical Biotechnology, University of Al-Qadisiyah, for the advice and support on association analysis, and Husam Raheem of the Ph.D. student at the Rutgers Cancer Institute of New Jersey, to help him with the language editing.

Interest conflict

The authors declare that they have no conflict of interest.

Author contributions

Project supervisor: Tarek Rebai; Co-advisor: Al-Delemi, D.H.J.; Research concept and design: Abdulla, A.K.; Capturing and housing of the animals: Abdulla, A.K. and Al-Delemi, D.H.J; laboratory experiments: Abdulla, A.K. and Delemi, D.H.J.; Data and statistical analysed: Abdulla, A.K. and Tarek Rebai; Writing the article: Abdulla, A.K. and Tarek Rebai. The final manuscript has been read and accepted by all contributors.

Abbreviations

STR = Straightness of the path velocity (VSL/VAP); VSL = Straight line velocity (μm/s; VAP= Average path velocity; VCL = Curvilinear velocity; ALH = Amplitude of Lateral Head displacement.

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