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Research Article

Autologous Platelet Rich Plasma Have Positive Effect on Ram Spermatozoa During Cryopreservation in Non-Breeding Season

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Abstract: The aim of this study was to evaluate the effects of autologous platelet-rich plasma (PRP) supplemented egg yolk based extender on ram semen after thawing. Semen samples were collected from nine Kıvırcık rams and each semen was divided into four equal aliquots and mixed with different concentrations of PRP supported extenders [no PRP (control), 12.5x10^6/mL PRP, 25x10^6/mL PRP, or 50x10^6/mL PRP)]. Motility, plasma membrane functional integrity, acrosome integrity, mitochondrial membrane potential, DNA integrity and malondialdehyde concentrations (MDA) were measured and analyzed after thawing. The results showed that 25x10^6/mL PRP group had positive effect on motility (61.67±3.81), membrane functional integrity (71.00±2.96) and MDA levels (5.13±0.64) at post-thawed (P<0.05). It was determined that 25x10^6/mL PRP and 50x10^6/mL PRP groups were more effective than other groups in terms of mitochondrial membrane potential (69.50±1.93), acrosome integrity (78.04±2.65) and DNA integrity (5.33±0.92). The results of the study showed that autologous PRP has a protective effect in the cryopreservation of ram spermatozoa.

Keywords: *Ram semen, Platelet-rich plasma, Cryopreservation, Post-thawed*

Otolog Trombositten Zengin Plazma Üreme Mevsimi Dışında Koç Spermatozoalarının Kriyoprezervasyonu Üzerinde Olumlu Etkiye Sahiptir

Öz: Bu çalışmanın amacı, otolog trombositten zengin plazma (PRP) ilave edilmiş yumurta sarılı sulandırıcılar eritme sonrası koç sperması üzerine etkilerini değerlendirmektir. Dokuz adet kıvırcık koçtan elde edilen sperma numuneleri dört eşit hacme bölündü ve farklı konsantrasyonlarda PRP içeren sulandırıcılar [PRP yok (Kontrol), 12.5x10^6/mL PRP, 25x10^6/mL PRP ve 50x10^6/mL PRP) ile sulandırıldı. Çözdürme sonrası motilite, plazma membranı fonsiyonel bütünlüğü, akrozomal bütünlük, mitokondriyal fonksiyon, DNA bütünlüğü ve malondialdehit konsantrasyonu (MDA) ölçüldü ve analiz edildi. Sonuç olarak, 25x10^6/mL PRP içeren sulandırcının çözdürme sonrası motilite (61.67±3.81), plazma membran fonksiyonel bütünlüğü (71.00±2.96), ve MDA seviyeleri (5.13±0.64) üzerine olumlu etkisi olduğunu gösterdi (P<0.05). 25x10^6/mL PRP ve 50x10^6/mL PRP gruplarının mitokondriyal fonksiyon (69.50±1.93), akrozomal bütünlüğü (78.04±2.65) ve DNA bütünlüğü (5.33±0.92) açısından diğer gruplara göre daha etkili olduğu belirlendi. Çalışmanın sonuçları, otolog PRP'nin koç spermasının dondurularak saklanması üzerine koruyucu bir etkiye sahip olduğu görüldü.

Anahtar sözcükler: *Koç sperması, Trombositten zengin plazma, Kriyoprezervasyon, Eritme sonrası*

Introduction

Cryopreservation that enables the maintenance of the biological function of spermatozoa for a long time has been widely used in various species (mammals, insects, avians, etc.). However, it has detrimental effects on spermatozoa because of the intracellular ice crystallization, osmotic changes, cold shock, oxidative stress and reactive oxygen species (ROS). Lipid peroxidation in the cytoplasm membrane of spermatozoon causes ROS production during cryopreservation. Free radicals lead to stress on the spermatozoa membranes therefore, viability, motility,

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and fertilizing ability of spermatozoon decrease during the freeze-thawed process. In recent years, various substances have been supplemented to the semen extenders against lipid peroxidation and to improve sperm quality at post-thaw [1-3].

Platelet-rich plasma (PRP) is a plasma component that contains 3-7 times a higher concentration of thrombocytes than the physiological concentration of whole blood [3-6]. Additionally, it contains hyperphysiological growth factors (GF) (insulin-like GF I, II, epidermal (GF), connective tissue (GF), platelet derived (GF), nerve (GF), vascular endothelial (GF), hepatocyte (GF), interleukin 8 (IL8), fibroblast (GF) and transforming (GF)], histamine, serotonin, calcium ions, zinc ions, superoxide dismutase (SOD) and adenosine triphosphate (ATP) [3,7,8]. It is used successfully in reproduction, dermatology, and orthopedics in mammals because of these important factors. In addition, many of these factors (VEGF, TGF, IGF-1, NGF, zinc ions, ATP, SOD and platelet-activating factor) have positively effect on sperm motility, viability, mitochondrial function, and DNA integrity at post thawed [3,9-12].

The effects of PRP based extenders have been examined on the outcomes of routine spermatological evaluations (motility, viability, acrosomal integrity etc.) [3]. But previous reports have not focused on the effects of PRP on ram semen cryopreservation. Therefore, the aim of the current study was to evaluate the effect of various concentrations of autologous PRP on the quality of ram spermatozoa at post-thawed.

Material and Methods

Ethical Approval

The study was approved by Scientific Ethical Committee (Bursa Uludag University, Türkiye) (No: 2021-04/03).

Chemicals

Chemicals were purchased from Merck (Darmstadt Germany) and Sigma (St. Louis, MO, USA) in the study.

Experimental Design

This study was designed to determine the effectiveness of PRP supplemented to the semen extender on ram semen cryopreservation. For this intention, diverse concentrations of autologous PRP [no PRP (control), 12.5x10^6/mL PRP, 25x10^6/mL PRP, or 50x10^6/mL PRP)] were used in the extenders during non-breeding season. Each experiment was replicated five times throughout the study.

Semen Extender Preparation

Extenders contained 223.7 mmol/L Tris, 66.6 mmol/L citric acid, 55.5 mmol/L fructose, 4.03 mmol/L EDTA, 4 g/L penicillin G, 100.4 mmol/L trehalose, 3 g/L dihydrostreptomycin, 20% egg yolk in distilled water. Autologous PRP was supplemented to each group according to the experimental design [13].

For PRP preparation, commercial PRP kits (S&M PRP Unique STR Kits) were used. Briefly, PRP was obtained from each ram's blood sample 1 h before blood draw. Ram semen was collected five times every other day and PRP was prepared again in each application. 10 mL blood were collected from each ram and centrifugation (15 min at 3200 rpm) was made using a tabletop cold centrifuge device. The extraction of PRP was completed based on the method outlined in the commercially available separation system. From each ram, 800x10^6 - 920x10^6/mL autologous PRP platelet were obtained. The obtained PRP was activated by 10% calcium chloride. PRP concentrations were detected by a fully automatic five-type blood cell analyzer (Sysmex XT-1800i).

Semen Collection and Dilution

Nine Kıvırcık rams maintained with the same state of affairs were used for semen collection. Semen was collected with electrically stimulated ejaculation (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand). Collected ejaculates were transferred to the laboratory in a water bath (37°C). Only ejaculates which have rapid wave (> +3 on 0-5 scale), >75% motility and >1.0x10^9 spermatozoa/mL were used.

Briefly, each ram ejaculate was split into four equal aliquots and diluted (37°C) to a final concentration of almost 150x10^6 (spermatozoon/mL) with PRP supplemented extenders and control extender. Diluted semen was gradually cooled to 4°C and then equilibrated for 2-h at 4°C.

Semen Freezing and Thawing

After the equilibration, each diluted semen was loaded into 0.25 mL straws. Cryopreservation and thawing procedures were performed by the method of Alcay et al.[14]. According to this method, straws were frozen at 3°C/min from +5°C to -8°C and at 15°C/min from -8°C to -120°C using the Nicool Plus PC freezing machine (Air Liquide, France). Then, the sperm-filled pipettes were dipped in liquid nitrogen and left in the liquid nitrogen container until examined.

Semen Evaluation

After thawing, sperm motility, plasma membrane integrity (hyposmotic swelling test (HOST)), capacitation status (Chlortetracyclin (CTC) staining), mitochondrial activity (R123; Invitrogen TM, Eugene, OR, USA) and DNA integrity (terminal deoxynucleotidyl transferase) using -mediated dUTP pseudo-end labeling (TUNEL)) was evaluated. All measurements and evaluations made from the beginning to the end of the study were made by the same person.

- Motility

Ram sperm motility assessment was performed using a phase-contrast microscope at 400x magnification (Olympus BX51-TF - Olympus Optical Co., Ltd., Japan) with the slide heated to 37°C.

- Membrane Functionality

For the assessment of the plasma membrane functional integrity, the hypo-osmotic swelling test method is used which was previously applied by Alcay et al.[14]. Following this method, the membrane integrity of the ram sperm was evaluated by observing the frizzled tails.

- Acrosome Integrity

For this evaluation method, a sample of 10 µL spermatozoa was added in 100 mL of PBS and then centrifuged for 5 min. The sperm pellet obtained after centrifugation was resuspended again in 100 mL PBS and smeared on the slide. Smeared-slides were left in acetone fixation at 4°C for 15 min. After fixation, smears were stained with FITC-PSA solution for 1 h at 37°C. After the staining process was completed, at least 200 ram spermatozoa were evaluated under a fluorescent attachment microscope [15].

- Mitochondrial Activity

Mitochondrial functions were assessed with fluorescent stains, PI, and Rhodamine (R123) combination [16]. Semen samples were first washed with phosphate-buffered saline and then centrifuged at 4000 g, 30 s. PBS was added to dilute the sperm sample (1/10 mL). Rh123 was then added to a final concentration of 5 µg/mL, and the sample incubated for 5 min at 37°C in the dark. At least 200 spermatozoa were used and the results were expressed as a percentage (%).

- DNA fragmentation

DNA fragmentation rates were evaluated by the TUNEL technique using *In Situ* Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim Germany) according to the manufacturer's protocol with slight modifications [17]. In brief, one drop of resuspended spermatozoa was smeared on a glass slide and fixed with 10% formaldehyde for 20 min. The slides were washed in PBS and stored at 4°C until use. The permeabilized slides were incubated in the dark chamber at 37°C for 1 h with the TUNEL reaction mixture which contained terminal deoxynucleotidyl transferase (TdT) plus dUTP label. TUNEL positive sperm cells. Each microscopic field was evaluated first under fluorescence microscopy (400× magnification) to determine the number of reactive sperm and then under phase-contrast microscope to determine the total number of sperm per field.

- Malondialdehyde (MDA) concentrations

To assess the MDA concentrations the method of Sharafi et al.[18] was performed. Briefly, 0.25 mL of diluted semen sample was treated with 0.25 mL of cold 20% (w/v) trichloroacetic acid to precipitate the protein. During the centrifugation, the precipitated protein was pelleted and the supernatant was incubated with (w/v) thiobarbituric acid for 10 min in a 100°C boiling water bath. After the incubation in the hot water bath, the sample was allowed to cool. Absorbance was determined using the Spectrophotometer (Mannheim Boehringer Photometer 4010). MDA concentrations were expressed as nmol/mL.

Statistical Analysis

Data obtained from this study are presented as mean ± standard deviation. Shapiro Wilk test was used for the normality test. The mean values of the obtained parameters were analyzed using the Kruskal Wallis test, which is an ordered one-way analysis of variance test. Statistical differences between the means of the treatment groups with differences were determined by the Mann Whitney U test. Differences with P values less than 0.05 were considered statistically significant. All analyzes were computerized using SPSS (SPSS 23.0 for Windows; SPSS, Chicago, IL, USA).

Results

In the study, the mean percentages of motility, plasma membrane functionality, acrosome integrity, mitochondrial function, and DNA fragmentation rates of nine fresh semen samples were 81.25±2.31, 88.87±2.85, 93.00±2.44, 89.75±3.49, and 4.25±0.46 respectively. Sperm quality was significantly reduced by the cryopreservation process compared with the fresh samples (P<0.05).

Table 1 shows the effects of different concentrations of PRP on ram sperm parameters at frozen-thawed. PRP25 and PRP50 groups significantly improved the motility, in comparison to the control and 12.5 groups without PRP addition at post-thawed (P<0.05). In addition, the highest percentage of motility rates were obtained from the PRP25 group (P<0.05). The percentages of plasma membrane functional integrity were higher in PRP groups compared to the control group (P<0.05). Also, the highest membrane integrity rate was obtained in the PRP25 group (P<0.05).

The higher acrosome integrity and mitochondrial function rates were obtained in the PRP groups compared to the control group (P<0.05). The highest DNA damaged spermatozoa were obtained control group compared to the PRP groups at post-thawed (P<0.05). As shown in *Table 2*, it was found that the MDA levels were lower in PRP25 group compared to the other groups (P<0.05).

Discussion

Platelet-rich plasma which has a source of growth factor has been widely used in regenerative medicine because of

Table 1. *The mean of studied ram sperm post-thawing parameters on different extender groups*

Variable	PRP Concentrations (x10⁶/mL)			
	0	**12.5**	**25**	**50**
Motility (%)	50.00±0.52[a]	52.60±0.82[a]	61.67±0.78[b]	56.88±1.20[c]
HOST (%)	60.46±0.68[a]	63.80±0.66[b]	71.00±0.60[c]	66.88±0.76[d]
Acrosomal Integrity (%)	73.00±0.42[a]	75.60±0.47[b]	78.04±0.54[c]	76.58±0.45[bc]
Mitochondrial function (%)	60.58±0.72[a]	64.64±0.61[b]	69.50±0.39[c]	66.42±0.83[bc]
DNA fragmentation (%)	9.75±0.20[a]	8.92±0.16[b]	5.33±0.19[c]	5.96±0.20[c]

Data is presented in Mean± S.E.M. Different letters within the same rows show significant differences among the groups (P<0.05)

Table 2. *Malondialdehiyde (MDA) levels in frozen-thawed drone sperm*

Groups	Control	PRP12.5	PRP25	PRP50
MDA (nmol/mL)	7.75±0.41[a]	7.00±0.27[a]	5.13±0.23[b]	5.63±0.18[c]

Data is presented in Mean± S.E.M. Different superscripts (a,b,c,b) in the same line indicate significant differences (P<0.05)

its cytoprotective properties. In the current study, we have investigated the effect of autologous PRP supplemented extenders on the quality of ram spermatozoa at post-thawed.

Cryopreservation ensures that genetic materials are preserved for a long time. However, it is known that the freezing-thawing process has undesirable effects on the fertilization ability of spermatozoa. These negative effects decrease motility, viability, plasma membrane, and acrosome integrities of spermatozoa. In the study, ram sperm have been negatively affected by the cryopreservation process compared to fresh spermatozoa (P<0.05).

Motility provides useful information concerning spermatozoon quality and oocyte penetration ability [19]. The cryopreservation process damages the cell membrane structure and inhibits the production of ATP, which ultimately leads to a reduction in sperm motility. PRP contains multiple biologically active ingredients which responsible for sperm motility and viability. In this study, we have shown that the presence of autologous PRP concentrations in the extenders increased ram spermatozoon motility compared to the control group at post-thawed (P<0.05). In the studies, the motility rates of ram spermatozoon cryopreserved with various commercial or non-commercial extenders ranged between 25% - 62% [13,20-24] at post-thawed. Our study shows that post-thawed motility values in high dose PRP groups (PRP25 and PRP50) were in good agreement with the findings of these studies. In our study, although rainbow trout seminal plasma (RTS) supplementation caused a clear increase in motility, the PRP12.5 group had not sufficient effect to make a statistical difference compared with the control group at post-thawed. When the PRP doses were compared among each other, the PRP25 group caused an increase in motility.

The functionality of the plasma membrane that is essential for spermatozoon metabolism plays a crucial role in the oocyte fusion of spermatozoon [25]. However, coldshock and lipid peroxidation have negatively affect membrane permeability and integrity during cryopreservation [26]. Therefore, it is crucial to keep integrity during the cryopreservation process to avoid cellular damage. HOST is the optimized test for detecting the subtle changes of spermatozoon membrane functionality [14]. In the study, the plasma membrane functional integrity in the PRP25 group was higher than in the other groups (P<0.05). The HOST values are in agreement with the earlier researches [13,14,20].

Acrosome examination is an important method that determines the fertilization ability of spermatozoa [27]. During the cryopreservation process, acrosome integrity and fertilization ability deteriorate. However, PRP groups successfully protected acrosome integrity during the cryopreservation process. These results are in agreement with the previous researches [23,28,29].

Sperm needs the energy to carry out its functions and it can mostly obtain ATP through the glycolytic and oxidative phosphorylation pathways [30]. Mitochondrial membrane potential is evaluated as a parameter related to the production of ATP by the spermatozoon mitochondria through oxidative phosphorylation and capacitation. Low mitochondrial membrane potential may occur due to spermatozoa anomaly or cryo damage during freeze-thawed process [31]. Therefore, it is important to determine the potential of the mitochondrial membrane for sperm quality [32,33]. In the study, mitochondrial function was better preserved in PRP groups compared to the control group (P<0.05). Similar results were obtained in previous research [30,34-36].

Sperm DNA integrity is important not only for the successful transfer of genetic material to future generations but

also for proper fertilization, quality embryo development and pregnancy. Protecting the integrity of DNA during cryopreservation also has great importance not to disrupt the early development of the embryo [27]. IGF-1 and NGF which were ingredients in PRP have been proven to improve DNA integrity. In this study, it was observed that PRP groups were resistant to the cryopreservation process. A similar result was obtained in previous research [3].

Oxidative damage is one of the most common cryopreservation damages and it may be evaluated by MDA levels which is a key product of polyunsaturated fatty acid's peroxidation in the cells [37,38]. PRP has an antioxidant effect in mammalian cells therefore it has also a positive effect on sperm during cryopreservation. In our study, the lower MDA levels were obtained PRP25 and PRP50 groups (P>0.05).

Considering all sperm parameters; autologous PRP has a protective effect on cryopreservation of ram spermatozoa. The PRP25 group was the optimum for semen preservation. Future studies might be focusing on PRP supplementation to evaluate reproductive success when used to fertilize the sheep.

Availability of Data and Materials

The authors declare that the data supporting the study findings were obtained from the corresponding author (S. Alçay).

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Conflict of Interest

The authors declare that there were no conflicts of interest in the realisation of this research.

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Author Contributions

SA, MA, HS designed the experiment. SA, AA, DK, performed the experiment. AA, MY, MK analyzed the data. SA, AA made tables, and wrote the paper. All authors reviewed and approved the final manuscript.

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