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Effect Intra-Testicular Injection of Autologous Platelet-Rich Plasma on Sperm Parameters and Regulation of the CFAP65 and SPEF2 genes in Heat-Stressed Rabbits

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Abstract

Autologous Platelet-Rich Plasma (PRP) has a potential effect on tissue repair through the proliferation and differentiation of tissue progenitor cells and is considered simple, safe, easy to prepare and use, easily applicable, and effective in use. Twenty New Zealand White rabbit were used and divided into two groups control (n=10), and the PRP group (n=10) which intratesticular injected with PRP (200 µl PRP per testes). In summer season, all animals were daily exposed to sunlight in the middle of the day for ten weeks of June, July, and August. The rectal temperature and temperature humidity index were obtained twice every week after exposure. The sperm parameters and gene expression of the target gene were analyzed before treatment and after 10 weeks. The Computer Assisted Semen Analysis (CASA) results showed that the sperm motility parameters were significantly ($P<0.05$) better for the summer heat-stressed in bucks injected with PRP than the control group. On the other hand, the gene expression of specific genes responsible for sperm motility (CFAP65 and SPEF2) results were noted that a high significant regulation ($p<0.05$) in the PRP group compared with the control. In conclusion, the PRP enhances the sperm parameters of rabbits that suffer from heat stress, as well as increases the regulation of CFAP65 and SPEF2 genes.

Keywords: ***Heat stress, Platelet-Rich Plasma, Sperm motility, Gene expression, Rabbit***

Introduction

Heat stress (HS) is defined as an environment that causes the body's temperature to rise over its set point. HS can impair reproductive processes by interfering with the activity of germ cells and perhaps other cells involved with reproduction (1, 2).During the spermatogenic cycle, exposure of the testes to an acute or chronic rise in temperature can lower the amount of sperm in the ejaculate and influence sperm characteristics such as motility, morphology, and plasma membrane integrity in various species. These occurrences can be followed by periods of partial or total infertility (3, 4).The loss in rabbit production of HS estimated from the percentages of decline in conception rate × pre-weaning mortality × litter weight at weaning was 73.0%. The provision of cool water restored 11/12 of heat loss. (5). Poor environmental conditions reduce farm animal fertility and semen quality, for example in rams, direct heat stress during the non-breeding season impairs

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testicular germ cells without having a significant influence on testicular endocrine function. (6). Testes are especially sensitive to HS due to the fact they are affected by physiologic abdomen temperature (7, 8). To achieve proper spermatogenesis, male animals' scrotal temperature should be 2 to 8 ℃ lower than their body temperature (4, 9). Acute or chronic high testicular temperature inhibits spermatogenesis and results in a decrease in spermatozoa number, which is connected with a transitory period of partial or total infertility in various species (1, 4). A type of autologous plasma called platelet-rich plasma (PRP) has a platelet concentration that is three to five times greater than average (10). PRP acquired the ability of therapeutic properties by activation of releasing several bioactive proteins, like Vascular Endothelial Growth Factor (VEGF), Insulin-like Growth Factor-1 (IGF-1), Fibroblast Growth Factor (FGF), and Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF), Superoxide Dismutase (SOD) and zinc, these factors are responsible for the continuous spermatogenesis and future fertility of a male (11). Furthermore, PRP is a source of growth factors that could contribute to tissue regeneration and may enhance both functional and structural disorders of the testis (12). Some studies supported the use of the PRP in several medical cases, while others countered its use (13). PRP has a potential effect on tissue repair through the proliferation and differentiation of tissue progenitor cells and it's simple, safe, easy to prepare and use, easily applicable, and effective in using (14). Little is known about PRP's use in testicular injection, and it is unknown how it increases sperm production in the testes (15). The process of spermatogenesis requires the control of a gene network to regulate gene expression in this network and many of these genes are specialized for sperm-generating cells (16). In this study, we employed rabbit models to investigate the temporal gene expression of the target genes CFAP65 and SPEF2 in spermatozoa cells by quantitative Polymerase chain reaction (PCR) using SYBR-green. Our objective was to ascertain whether PRP would result in gene expression patterns affecting spermatogenesis in rabbits which exposure to severe heat stress. We hypothesized that PRP would encourage the expression of a specific gene that controls sperm motility. By enhancing the expression of these genes, this research may contribute to the development of innovative therapeutic approaches to reduce the heat stress effect from which animals suffer, especially in hot summers. The cilia and flagella-associated protein 65 (CFAP65) is a protein-coding gene, involved in the development of the sperm flagellum that plays a role in sperm motility (17,18). The protein-coding gene sperm flagellar 2 (SPEF2) is a gene necessary for the proper development of the axoneme in spermatozoa, important for the morphology of the sperm head to mature normally, and necessary for fertility (19).

Material and Method

The experiment was conducted in the Dewaniya region; it is one of the governorates of the Middle Euphrates region which is included in the sedimentary floodplain of Iraq, located between latitudes 31º 57 18 to 32º 01 30) north, and longitudes (44º 52 13.70 to 44º 58 29.16) east (20). The study was conducted according to the Ethical Committee of the Faculty of Veterinary Medicine, University of Al-Qadisiyah- Iraq, and conformed to the Guidelines for the ethical use of animals. (21)

Animals and experiment design

During the period from June to August ,(2022) twenty bucks (New Zealand Whites have pink eyes) rabbits, aged 15-20 months, and weighing range of 2.25-2.75 kg were housed in battery cages (two rabbits per cage) in a semi-close rabbit house with adequate ventilation, it was determined that none of any discernible

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reproductive organ problems, the bucks were kept in a regular setting and fed nutritious, hay, and root vegetables, the bucks were instructed to maintain an artificial vaginal delivery system for the two-week adaption period. We divided these animals into two groups, ten bucks for each: the 1st group served as control (C1) and receives either no treatment, while the 2nd group (P1) was used for PRP inra-testecular injection.

Rectal temperature and Temperature Humidity Index

Throughout the experiment (10 weeks) from June to August. All animals were exposed every day to 1 h of sunlight between 02:00 and 03:00 pm (≥ 45°C). The rectal temperature (RT) and temperature humidity index (THI) were obtained twice every week after exposure to sunlight. We measured the mean of RT by using a conventional clinical thermometer (Vet Digital Flexible Thermometer) by inserted into the rectum for two minutes to read the temperature there. The THI method was suggested as a way of evaluating the severity of heat stress using both ambient temperature and relative humidity (22). The THI values obtained were then classified as follows: <27.8= absence of heat stress, 27.8 - < 28.9= moderate heat stress, 28.9 - <30.0 = severe heat stress, and 30.0 or more = very severe heat stress, calculated using the equation modified by (5).

PRP preparation and intra-testicular injection

On the first day, 10 ml of whole blood was drawn from P1 group animals (ear vein method), then added to the PRP gel tube with activator (Biozek Medical®, Laan van de Ram, Bulgaria), after mixing with a 3.2% sodium citrate tube, and centrifugation under the manufacturer's instructions. A sample of PRP (2ml) was drawn into an Eppendorf tube and kept at -80°C for future use. Intra- testicular injection of autologous PRP after animals sedative of the P1 group with xylazine (1.5 mg/kg body weight) (23). Then, using a sterile needle gauge 21, a single dose of 200 µl PRP into each testis parenchyma along the longitudinal axis while forcing the fluid out of the needle during the withdrawal process (17,24).

Semen collection

We collected the semen from all animals by using an artificial vagina (25), these samples were transferred to the laboratory at room temperature, and each sample was divided into two sections to apply the computer-assisted sperm analysis (CASA) and gene expression analysis. The semen collection was repeated after 10 weeks (depending on the results obtained in our previous study by Abdulla et al. (26) that were showed that the best time for improving the semen parameters after PRP intra-testicular injection in rabbits was between 8 and 10 weeks. For the comparison between before and after treatment, these animal groups were renamed to the C2 group and P2 group respectively, to apply these laboratory analyses (CASA and Gene expression).

CASA analysis

The CASA technique was used to monitor the sperm motility of rabbits and specifically set up the parameters of total sperm numbers (million/ejaculation), total motility (PM+NP) %, progressive motility (grade A + grade B) % and Immotility % by use CASA system "CEROS II®, Zeiss, IMV-Technologies Co. France" with Advanced microscopy, simplified (Olympus, Europa), a Makler counting chamber (20 µm) was used for each sample (27).

Gene expression

Initially, we separated the seminal plasma from the spermatozoa to obtain a population of spermatozoa from the surplus ejaculates. The seminal samples were thoroughly washed by centrifugation with HEPES Buffer Solution (Capricorn Scientific GmbH, Germany), and the pelts were kept at -80°C until needed (28).

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We applied to extract the total Ribonucleic acid (RNA) from purified spermatozoa according to the instructions of RNA Purification Kit "Thermo Scientific ® Gene JET RNA Purification Kit, California, USA", then All samples were treated with Deoxyribonucleic (DNase) "DNase I, Thermo Scientific ® RNase-free kit, California, USA", the concentration and purity of total RNA were assessed using a Nano-drop spectrophotometer (OPTIMA®, SP-3000 Nano, UV/Vis Spectrophotometer. Tokyo, JAPAN), and a ratio reading at A260/280 nm, with only samples having a ratio of 1.8 to 2.0. The specific primer sequences were designed by the GenBank database, using the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) (Table 1). The Sequence Manipulation Suite, PCR Primer Stats http:// www. Bioinformatics. org/ sms2/ pcr_primer_stats. html) was used to check each designed primer pair for the possibility of dimer formation. We utilized the Reverse Transcription PRP (RT-PCR) technique to synthesis the complementary Deoxyribonucleic acid (cDNA) by (Thermo Scientific ® Revert Aid First Strand cDNA Synthesis Kit, California, USA) with random primers, According to manufacturer recommendations to convert this total RNA to cDNA.

Real-Time PCR (RT-PCR)

Quantitative real-time PCR was used to analyze the total RNA of target genes to perform the thermal reaction by (Excecycler 96® Thermal cycler for Real-time PCR, Bionner, Korea), with master mix contend cDNAs, total RNA, reverse primer and forward primer, then add to SYBR Green Master Mix (AccuPower® Greenstar™ qPCR PreMix, Bionner, Korea), then complete this mixture according to the manufacturer instructions, to verify the previously chosen genes' expression in spermatozoa RNA by relative quantitation PCR reactions using the $2^{-\Delta\Delta Ct}$ calculation (Livack method), with a reference gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as follows: The PCR amplification comprised according to (29), each sample class was tested run in triplicate, the standard deviations for each mean CT value were determined.

Table (1): The Gene ID, primer sequence and product size of target genes were used for quantitative real-time-PCR analysis.

Gene	Gene ID	Primer sequence	Product size (bp)
CFAP65	100349492	F: 5′- GGTCCTTCCTCTTCCCAAGC-3′ R: 5′- GTTGTCCTGCACACGCATTT-3′	104 bp
SPEF2	100353102	F: 5′-ATGAGCCAAGGATGGTGGTG-3′ R: 5′-ACGGGATACCCCCATCAGAA-3′	174 bp
GABDH	100009074	F: 5′-TGAGCGAGCTTACAACCAAC-3′ R: 5′-ATCACAAACATGGGGGCATC-3′	104 bp

Ethical approval:

The researchers obtained ethical approval from the research Ethical Approval Committee of the College of Veterinary Medicine, University of Al-Qadisiyah.

Statistical analysis

The results (mean + standard error of the mean) of each parameter, and the variance evaluated by Paired Samples t-test - SPSS. The statistical significance was determined as follows: $p<0.05$. IBM® SPSS® statistical software 27.0, 2020; for Windows was used to

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investigate all of the variables (30).

Results

Rectal Temperature and Temperature–humidity index

According to data from table (2) that recorded statistically significant differences (P < 0.05) in the 2nd, 3ed, 7th and 10th weeks compared to other times, it turned out that the experiment rabbits were overheating at this time. On the other hand, the heat stress indicator (THI) shows that there was a high-intensity effect in this period because of the higher value than 30, according to the global measurements of this indicator, as shown in this table, this value was high throughout the experiment period, reaching the highest value in the seventh week (41.06), but the lower value in the first week (36.48). Therefore, in any case, it is very influential.

Table (2): Mean of the Rectal Temperature and Temperature–humidity index for all animals during the experiment period.) June, July, and August)

Exp. period	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10
RT	39.75 ±0.12	40.13* ±0.06	40.15* ±0.09	39.85 ±0.15	40.01 ±0.11	39.90 ±0.19	40.10 ±0.06	39.93 ±0.15	39.69 ±0.10	40.15* ±0.05
THI	36.48	37.41	39.12	40.20	39.98	39.88	41.06	39.02	37.89	36.62

RT: Rectal Temperature, THI: Temperature–humidity index, W: week, the star marker in the horizontal row referred to statistical significance (P<0.05).

Sperm motility

The results of CASA analysis of sperm parameters percent (total sperm count, total motility, progressive motility) decreased significantly (P < 0.05) after 10 weeks of exposure to heat stress in the C2 (274.7, 49% and 2%) respectively compared to before heat exposure in the C1 group (311.5, 87% and 63%) respectively. Yet, the sperm immotility percent was high in C2 (51%) vs C1 (13%). In contrast, these semen parameters were increased significantly (P < 0.05) after exposure in the P2 group vs the P1 group, so the sperm immotility percentage decreased (30%) in this group after the 10th week of PRP injection compared with the P1 group (14%). However, the percent of sperm parameters when compared between the P2 with C2 groups showed a significant increase (P < 0.05). These results referred to that intra-testicular injection PRP have improved sperm parameters in heat-stressed rabbits (Table 3).

Table (3): sperm parameters (Mean± SE) of bucks and their improvement after PRP intra-testicular injection after 10 weeks.

Sperm Parameters	Control group		Treated group with PRP		*p*-value
	C1 (n=10) before heat expousure	C1 after 10 w heat exposure	P1 (n=10) before heat expousure	P1 after 10 w of heat exposure	
Total sperm numbers	311.5^{a}±15.89	274.7^{c}±14.42	340.9^{a}±20.88	304^{b}±21.72	0.000

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(million/ejaculation)					
Total motility% (PM+NP)	87^{a}±1.5	49^{c}±2.8	86^{a}±2.1	70^{b}±3.8	0.000
Progressive motility%	63^{a}±2.8	22^{c}±1.3	64^{a}±2.9	52^{b}±3.9	0.000
Immotility%	13^{c}±0.85	51^{a}±3.4	14^{c}±0.9	30^{b}±3.1	0.000

a,b,c,d: Means within a row with different superscripts differ significantly (p<0.05).

Gene expression

The relative quantitative expressions of messenger RNA (mRNA) for CFAP65 and SPEF2 in spermatozoa cells of rabbit by Real-time PCR assay was operationalized during summer are presented in table (4) and figure (1). The mRNA expression during the summer season was found significant downregulation of these target genes in the control group (C2) after heat stress. Yet, in rabbits enriched with an intra-testicular injection of PRP, the CFAP65 and SPEF2 were increased ($p < 0.01$) in the P2 group (4.35 and 4.94) fold change respectively, whine compared with the other groups.

Table (4): Fold change expression of target genes, calculated by ***ΔΔ***Ct method.

target genes	Groups	Ct $_{target}$ Mean±SD	Ct $_{GABDH}$ Mean±SD	***Δ***Ct Mean±SD	***ΔΔ***Ct	$2^{-\Delta\Delta Ct}$ mean (max-min)
CFAP65	C1	26.38± 0.60	22.99±0.56	3.36±0.82	3.79±0.82	0.09 (0.099-0.083
	C2	30.17± 0.61	23.02±0.46	7.15±0.78		
	P1	24.94±0.49	23.02±0.463	1.99±0.674	-1.93±0.674	4.35 (4.652-4.083)
	P2	23.21±0.49	23.02±0.45	0.06±0.66		
SPEF2	C1	25.02±0.58	22.99±0.55	2.04±0.8	2.85±0.8	0.18 (0.223-0.123)
	C2	27.91±0.50	24.67±2.0.5	4.89±0.707		
	P1	29.89±0.43	22.99±0.56	6.90±0.7	-1.96±0.7	4.94 (5.497-4.373)
	P2	27.96±0.44	23.02±0.36	4.94±0.57		

C1: control group before exposure to sunlight (untreated), C2: control group after exposure (treatment), P1: PRP group before exposure to sunlight (untreated), P2: PRP group after exposure (treatment).

According to (31), the variance of the ΔCt is calculated from the standard deviations of the target and reference values using the formula: $S = (S1^2 + S2^2)^{1/2}$ (S1= standard deviation of Ct $_{target}$ =S2 ,standard deviation of Ct $_{GABDH}$ The .(*Δ*Ct= Ct $_{target}$- Ct $_{GABDH}$ The .*ΔΔ*Ct= *Δ*Ct $_{treated}$- *Δ*Ct $_{untreated}$, the standard deviation same standard deviation of ΔCt.

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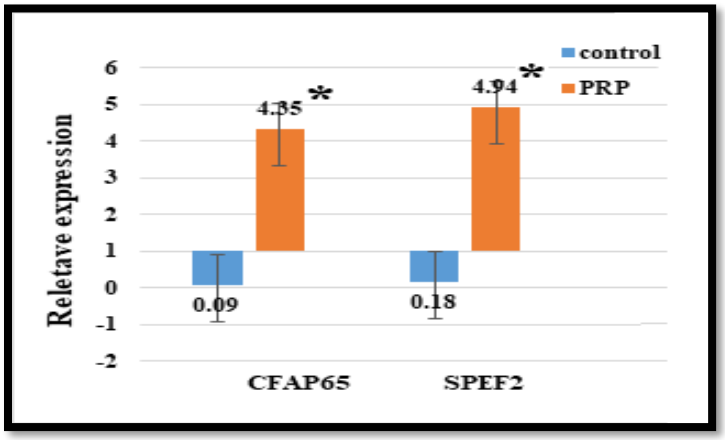

Figure (1): The gene expression rabbit spermatozoa cells of target genes (CFAP65 and SPEF2), to compare the $2^{-\Delta\Delta Ct}$ value of the control and PRP groups before and after heat stress. The statistic represents significant differences ($P<0.05$).

Discussion

The average body temperature of a rabbit is between 38.5 and 39.5 ◦C, with individual variations between 0.5 and 1.2 ◦C. Heat stress occurs when the ambient temperature is more than 30 ◦C, heat stress occurs in rabbits when the temperature rises above 35°C because they are unable to control their body temperatures (32, 33). The current study's findings in the table (2) that the rectal temperature of summer heat-stressed rabbits were rather high (39.69±0.10 - 40.15±0.09), this explains the effect of the hot summer season on the rabbit's temperature, depending on (5) that were explained the THI indicated in rabbit bucks which exposure to severe heat during the summer season lead to severe heat stress which is described in the above material and method, So our results can be confirmed in a table (2) which showed that all THI values were more than 30.0, yet these results agreement with (34) study that was referred to the THI value which was>30.0 indicates to that bucks were exposed to heat stress throughout June-August and moderate in September. The data in Table (3) explained that total sperm numbers showed no significant differences were detected between all experimental groups. While sperm motility was significantly higher ($p<0.01$) in the control sample compared to experimental groups (P1 and P2). The increase of total motility (48 %) was observed in group P2 after PRP injection compared with the P1 group, this significant difference explains the important role of the PRP intra-testicular injection in enhancing spermatogenesis after 10 weeks from injection. The positive effect of PRP has been suggested to be caused by the presence of many biological compounds in PRP like cytokines “IGF-1, PDGF, EGF, VEGF and growth factors which reduce oxidative stress and raises testosterone levels (35, 12). Furthermore, numerous studies have demonstrated that the effect of PRP has developed into a desirable biologic tool in regenerative medicine (36). Our findings suggest that PRP has a role in enhancing sperm motility, these results were in agreement with our previous study (37). On the other hand, the study of (38) found the possible effect of PRP on ischemic injury in testicular torsion in rats, in which PRP was injected into the testicular tissue upon de-torsion. Additionally, PRP has no negative effects on male fertility (39). The PRP is being employed for tissue regeneration by including several growth factors, VGEF, which promote

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germ cell multiplication, sustain their life cycle, and prevent germ cell death (40).

In this study, we investigated the PRP effect on expression levels of target genes (CFAP65, and SPEF2) and their relationship to enhancing sperm motility, which was decreased by heat stress. In recent years, significant progress has been made in our understanding of the pathophysiology of flagella-related disorders, although the pathogenic genes and mechanisms of flagellum production remain unclear (41). The results of our study point to the expression of CFAP65in spermatozoa cells was down-regulation ($p \leq 0.01$) in the control group (C2) after heat stress which has low sperm motility after heat exposure of rabbits for 10 weeks. Although, after intra-testicular PRP injection, the relative expression of these genes was upregulation ($p < 0.01$) when calculating the relative expression in the PRP group (Table 4 and Figure 1). These results were aggregative with previse investigation which referred to the down-regulation of CFAP65 protein expression in the sperm flagellum led to sperm flagellum dysplasia and abnormal sperm ultrastructure in mouse testes (18). This gene may be encoding a transmembrane protein with putative coiled-coil domains. This gene's mutation may result in significant chromosomal inversion, abnormal comb morphology, and sperm motility problems (17). Furthermore, this study indicated the down expression of the SPEF3 gene in spermatozoa cells of the C2 group, yet it was increased after 10 weeks from PRP injection, these results explained the molecular PRP effect by regenerating testicular cells to enhance regulation of this gene, these results referred to stimulation the spermatogenesis and sperm motility. This observation agrees with previous investigations, as a study of Li *et al.* (42) that were referred to the SPEF2 gene is essential for the development of the sperm tail and ciliary which is necessary for an activity for sperm movement. Further, the SPEF2 is a determinant of sperm motility during bull spermatogenesis and its expressed in testes and sperm, yet the molecular processes that control SPEF2 gene expression are yet unclear (43).

Conclusion

To sum up, our study has offered additional insights into the biological role of PRP intra-testicular injection for enhancing sperm parameters in animals suffering from heat stress. Yet, On the other hand, regulation of the gene expression in spermatozoa cells provides evidence that uses of the PRP may be enhancing the regulation of CFAP65 and SPEF2 genes, which are responsible for sperm motility. This upregulation may have a direct correlation between these genes and the progressive motility rate in rabbit spermatozoa. Further research is necessary to confirm these findings and identify how these genes have direct control of sperm motility, particularly in the case of the genes CRHR1 and SPEF2, about which little is known about spermatozoa.

Conflict of Interest: there is no conflict of interest.

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