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Effects of Date Palm Pollen Supplementations on The Expression of *PRDX1* and *PRDX6* Genes in Infertile Men: A Controlled Clinical Trial

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Abstract

Background: Accumulating evidences suggest that date palm pollen (DPP) induces antioxidant activity and improves semen parameters in male rats. However, there is a few scientific evidences in support of the DPP effects on human male fertility. Hence, the effect of oral consumption of DPP on sperm parameters and expression pattern of *Peroxiredoxin-1* (*PRDX1*) and *Peroxiredoxin-6* (*PRDX6*) genes was evaluated in men with infertility.

Materials and Methods: The current controlled clinical trial included 40 men with infertility (DPP group) and 10 normospermic fertile men as controls. The DPP group received gelatinous capsules of DPP (400 mg/kg) for 74 days. Semen sampling was done before and after treatment in the both groups. Semen analysis and 8-isoprostane concentration assessments were performed by computer-assisted sperm analysis and ELISA methods, respectively. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays were employed to explore expression of *PRDX1* and *PRDX6* genes.

Results: DPP consumption significantly improved semen volume (P=0.030), count (P<0.001) and morphology of sperm (P=0.023). Concentration of 8-isoprostane was significantly decreased after intervention in the DPP group (P<0.001). DPP consumption led to a significant elevation in the expression of *PRDX1* and *PRDX6* genes (P<0.001). Elevated gene expression of *PRDX6* and *PRDX1* was positively correlated with improved parameters of sperm including count, volume, motility and morphology.

Conclusion: Taken together, DPP seems to promote sperm quality through a decrease in reactive oxygen species (ROS) by increasing expression of antioxidant genes. Further large-scale studies are required to challenge this hypothesis (registration number: IRCT2015021221014N2)

Keywords: 8-Isoprostane, Male Infertility, *PRDX1*, *PRDX6*

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Introduction

Oxidative stress (OS) has been described as an important factor in the etiology of male infertility resulting from the excess generation and/or defective scavenging of reactive oxygen species (ROS) in the reproductive system (1). Spermatozoa have a finely balanced oxidant-antioxidant system to maintain ROS levels within the normal range (2). Under physiological conditions, an ROS generating enzymatic system provides low amounts of ROS required for sperm maturation and function (2, 3). It appears that the mitochondrial respiratory chain in mature and immature semen spermatozoa, leukocytes and environmental pro-oxidants are the primary sources of the excessive amount of ROS which in turn affects plasma membrane fluidity and DNA integrity through oxidation and peroxidation (4). An array of antioxidant enzymes and free radical scavengers neutralized the excessive ROS and enabled sperms to tolerate OS (5).

Peroxiredoxins are a family of thiol-specific enzymes that can function as antioxidants by eliminating harmful effect of excessive peroxynitrite (ONOO-) and hydrogen peroxide (H_20_2) (6). To date, six isoforms of peroxiredoxins have been identified in spermatozoa which are abundantly expressed in various subcellular compartments of these

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cells (5, 7). Peroxiredoxin-1 (PRDX1) enzyme contains two cysteine residues at the active site which are needed for its catalytic function (5). PRDX1 localized in the nucleus, the equatorial region and the flagellum of spermatozoa that highlights the antioxidant potential of PRDX1 in protecting the mitochondrial complex structures against OS (7). Peroxiredoxin-6 (PRDX6) is the most abundant isoform of peroxiredoxins found in all subcellular parts of the sperm and contains only one cysteine residue at its active site (5). Glutathione is the physiological electron donor to PRDX6 contributing to the neutralization of H2O2 and ONOO- in human sperms (8). Moreover, it has been documented that peroxiredoxins are involved in different cell signaling cascades (9). Owing to these critical roles, it is not surprising that dysregulation of *PRDX1* and *PRDX6* gene expressions and/or their function may alter normal sperm function and lead to male infertility (7, 10-13). In the past decades, accumulated evidences have demonstrated that date palm pollen (DPP) supplementation could positively impact sperm parameters and contribute to the improvement of fertility in male rats (14-17). Moreover, due to the presence of phenolic compounds, flavonoids and anthocyanins, DPP can induce expression and activity of antioxidant genes (18-20).

However, antioxidant effect of DPP on human sperm parameters and its mode of action to improve semen parameters have not been understood and well-documented. This hypothesis raised the point that perhaps DPP supplementation can reduce ROS levels, particularly through peroxiredoxins genes. Thus, deciphering this link likely provides insight into one of the molecular mechanisms of DPP on improvement of semen parameters in men with infertility. We designed the present attempt to explore influences of DPP on semen parameters and reduction of ROS level by quantifying expression of peroxiredoxins genes as well as 8-isoprostane, a marker for oxidative stress.

Materials and Methods

Ethics statement

We received approval for our study from the Institutional Review Board/Independent Ethics Committee (IRB/IEC) of Hormozgan University of Medical Sciences, Bandar Abbas, Iran (IR.HUMS.REC.1394.201). All clinical trial protocols were registered in Iranian Registry of Clinical Trials (IRCT2015021221014N2). According to the Declaration of Helsinki of World Medical Association, all participants signed an informed written consent after receiving full explanation on the study procedures and objectives.

Study design

This comparative clinical trial (before-after clinical trial study) consisted of infertile men admitted to the Omm-e-Leila Fertility and Infertility Center of Bandar Abbas (Iran) between January 21 and June 22, 2016. A total of 40 men with infertility were recruited by convenience sampling (the DPP group). Men with systemic or genetic conditions, abnormalities in reproductive tracts, alcohol and substance abuse, testicular trauma, and use of fertility drugs in the past six months were excluded from the study. The control group (n=10) were selected from fertile volunteers fathered a child during the past two years, with normal semen analysis to comparably evaluate rate of changes in expression of the studied genes, in order to reach this answer how much expression of the genes in DPP-treated infertile men averagely became near to normal men.

Details of participant enrollment, allocation and analysis are demonstrated in the CONSORT flow diagram (Fig.1). The DPP group were treated with 400 mg/kg DPP powder (21) in gelatinous capsules every day for 74 consecutive days and all participants completed the treatment course. Semen samples were obtained twice from participants in both groups: before and after the treatment period. Expression of the studied genes, ROS measurement and semen analysis were blindly accomplished by another one, who did not aware about the samples.

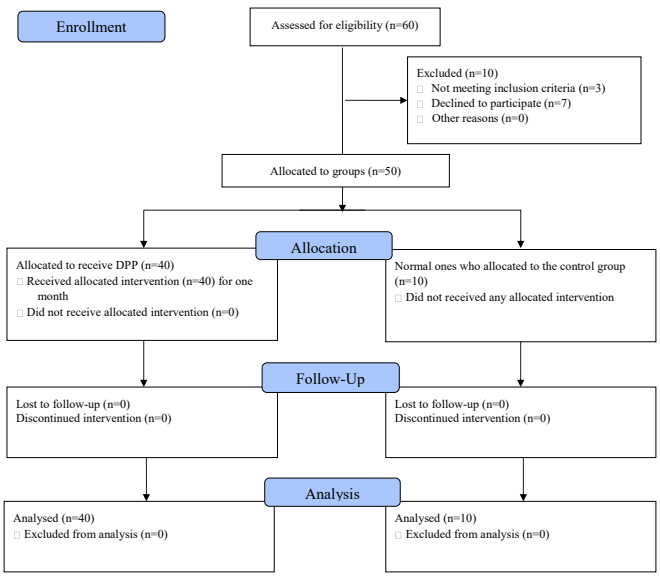

Fig.1: Consort flow chart.

Semen analysis and isolation of spermatozoa from seminal fluid

After a 3-5 days abstinence cycle, fresh semen was sampled via masturbation in sterile plastic vessels, followed by liquefying for half an hour. Initial analysis of semen was done immediately according to the sperm quality analyzer IIC (United Medical Systems Inc., USA) according to the world health organization (WHO) protocols (22, 23). The spermatozoa were purified by Goodrich protocol (24), and then the samples were washed twice with BSA-free Ham's-F10 medium and stored in Qiagen RNA later solution (Germany) at -80°C awaiting extraction of RNA.

Purification and measurement of free 8-Isoprostane

Isoprostane is a free radical-catalyzed peroxidation of essential fatty acids. It was measured as marker for OS in sperms. Free 8-isoprostane was purified in duplicate with the aid of Affinity Column (Cayman Chemical, USA). For precipitate isolation, specimens were all initially centrifuged with 15000 g, followed by diluting the supernatant in the presence of column buffer (at 1:5); the next steps were performed on the basis of manufacturer's instructions. Fifty mili-liters of specimens were applied to assess concentration of 8-isoprostane via an enzyme-linked immunosorbent assay reader (STAT FAX 2100; Awareness Technology Inc, USA) at a wavelength of 405 nm.

cDNA synthesis and quantitative reverse transcription polymerase chain reaction

The manufacturer's protocol was followed to extract total RNA from the specimens through the NucleoSpin® RNA Midi kit (Macherey-Nagel, Germany) and RNA yield was exposed to Thermo Scientific RNase-free DNase I (USA). The extracted RNA quantity and quality were evaluated by electrophoresis on agarose gel and NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, USA), respectively. Subsequently, based on the manufacturer's protocol, 1 µg of total RNA was reverse transcribed to cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Quantitative reverse transcription PCR (qRT-PCR) was run in duplicate with the aid of gene-specific primers and Tli RNaseH Plus SYBR® Premix Ex Taq™ II kit (Takara Bio Inc., Japan) through the Rotor-GeneTM 6000 system (Corbett Research, Australia) based on the guidelines of manufacturer. The utilized primers for qRT-PCR assays were:

PRDX1-
F: 5'-CAAAGCCACAGCTGTTATGC-3'
R: 5'-GAGAATCCACAGAAGCACC-3′
PRDX6-
F: 5'-CTTTGAGGCCAATACCACCG-3'
R: 5'-AGATGGTCCTCAACACTGTC-3′
and *β-actin*-
F: 5'-ATGGAATCCTGTGGCATCCA-3'
R: 5'-CGCTCAGGAGGAGCAATGAT-3′.

Primary and secondary endpoints

Our primary endpoint was the impact of DPP on the gene expression of *PRDX1* and *PRDX6*, while our secondary endpoint was the impact of DPP on the parameters of sperm.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences version 22.0 (SPSS Inc., USA) software and $P<0.05$ was considered statistically significance level. Chi-square and paired t test were performed to compare sperm parameters before and after treatment. mRNA expression levels of *PRDX1* and *PRDX6* genes were compared in the DPP group before and after treatment with healthy individuals using the qRT-PCR. Gene expression ratio was determined via the method of $2^{-\Delta\Delta ct}$. Kolmogorov Smirnov normality test showed the abnormal distribution of *PRDX1* and *PRDX6* gene expression levels and thus, non-parametric tests were used for comparison. The Wilcoxon test was run to compare expression levels of *PRDX1* and *PRDX6* before and after treatment within the groups. Expression level of the genes was compared after treatment between groups, by a two-tailed Mann-Whitney U-test. Association of variable sperm indices and free 8-isoprostane levels with expression levels of *PRDX1* and *PRDX6* genes were clarified via chi-square and one-way ANOVA tests. Spearman's correlation test was used to determine correlations of *PRDX1* and *PRDX6* expressions with the variable sperm parameters and free 8-isoprostane levels.

Results

Clinical and demographic profiles of the DPP group and healthy controls are shown in Table 1.

Table 1: Demographic and clinical characteristics of the cases and healthy controls

Demographic variable	Cases		Controls	
	n (%)	Mean ± SD	n (%)	Mean ± SD
Age (Y)				
≤30	30 (75)	29.3 ± 3.21	4 (40)	27.25 ± 1.70
>30	10 (25)	33.25 ± 3.65	6 (60)	32.83 ± 2.04
Weight (Kg)	40 (100)	88.1 ± 11.47	10 (100)	86.40 ± 10.80
Height (M)	40 (100)	1.66 ± 0.08	10 (100)	1.67 ± 0.08
BMI (kg/m^2)				
Normal (<25)	4 (10)	23.45 ± 0.86	2 (20)	23.22 ± 0.43
Overweight (25≤ - <30)	8 (20)	27.65 ± 1.19	2 (20)	27.50 ± 0.73
Moderately obese (30≤ - <35)	19 (75.5)	32.57 ± 1.57	4 (40)	32.30 ± 1.58
Severely obese (35≤ - <40)	4 (10)	37.09 ± 1.31	2 (20)	37.64 ± 1.85
Very severely obese (≥40)	5 (12.5)	43.75 ± 2.50	-	-

BMI; Body mass index.

DPP supplementation significantly improved count and morphology of sperm, as well as semen volume

The findings of this study demonstrated a significant enhancement in semen indices like semen volume, sperm count and sperm morphology after treatment with DPP (Table 2), while differences of the other parameters including viscosity, liquefaction, pH, appearance and motility of sperm before and after DPP treatment were not statistically significant. Comparison of sperm parameters of the DPP group with healthy controls after treatment revealed significant differences in semen parameters including count, volume, motility and morphology (Table 3).

Table 2: The comparison of sperm parameters and free 8-Isoprostane of the infertile participants before and after treatment

Variable	Before treatment		After treatment		P value†
Sperm parameters	**n (%)**	**Mean ± SD**	**n (%)**	**Mean ± SD**	
Count					**<0.001**
Normal (≥15 million/ml)	10 (25)	16.75 ± 1.35	31 (77.5)	45.90 ± 28.79	
Abnormal (<15 million/ml)	30 (75)	5.29 ± 3.95	9 (22.5)	5.98 ± 3.59	
Volume					**0.030**
Normal (≥1.5ml)	35 (87.5)	3.32 ± 1.27	40 (100)	3.72 ± 1.30	
Abnormal (<1.5ml)	5 (12.5)	1.18 ± 0.16	-	-	
Appearance					0.320
Normal	39 (97.5)	-	40 (100)	-	
Abnormal	1 (2.5)	-	-	-	
Viscosity					0.320
Normal	39 (97.5)	-	40 (100)	-	
Abnormal	1 (2.5)	-	-	-	
Liquefaction					0.562
Normal	39 (97.5)	-	39 (97.5)	-	
Abnormal	1 (2.5)	-	1 (2.5)	-	
pH					0.166
Normal	39 (97.5)	7.72 ± 3.22	40 (100)	7.64 ± 0.16	
Abnormal	1 (2.5)	7.00	-	-	
Motility					0.154
Normal (≥40%)	13 (32.5)	46.69 ± 12.01	13 (32.5)	53.15 ± 11.67	
Abnormal (<40%)	27 (67.5)	23.55 ± 8.42	27 (67.5)	27.51 ± 7.48	
Morphology					**0.023**
Normal (≥30%)	5 (12.5)	42.40 ± 4.27	8 (20)	46.25 ± 5.82	
Abnormal (<30%)	35 (87.5)	23.40 ± 7.75	32 (80)	27.09 ± 6.71	
Free 8-Isoprostane (ηg/ml)	40 (100)	3.84 ± 3.90	40 (100)	1.76 ± 1.64	**<0.001‡**

Bold values indicate statistically significant differences (P<0.05). †; P value were calculated by chi-square test and ‡; P value were calculated by t test.

DPP supplementation significantly reduced OS marker concentration

Mean free 8-isoprostane concentration before treatment in the DPP group and healthy controls were 3.84 ± 3.89 and 1.62 ± 0.65, respectively. Seminal plasma concentrations of free 8-isoprostane were significantly higher in the DPP group before treatment, by comparing with the control group (P<0.001, Tables 2, 3). Free 8-isoprostane concentration was significantly decreased in the DPP group after treatment with DPP (Table 2). Moreover, after treatment, concentration of this marker was higher in the DPP group, compared to the control group, while it was not statistically significant (P=0.087, Table 3). The probable correlation was explored between 8-isoprostane concentration and seminal parameters. The reduced concentration of 8-isoprostane displayed a negative correlation with sperm count after treatment with DPP (r=-0.360, P=0.001), while it indicated no correlation with the other parameters of semen.

Table 3: The comparison of sperm parameters and free 8-Isoprostane levels of the date palm pollen (DPP)-treated patient group (after treatment DPP-group) with healthy controls

Variable	DPP-treated patient	Normal Controls	P value†
Sperm parameters			
Count (million/ml)	36.92 ± 30.41	81.90 ± 22.18	**<0.001**
Volume (ml)	3.72 ± 1.30	2.75 ± 0.95	**0.032**
pH	7.64 ± 0.16	7.67 ± 0.27	0.685
Motility	35.85 ± 15.07	61.50 ± 12.86	**<0.001**
Morphology	30.92 ± 10.10	61.60 ± 10.41	**<0.001**
Free 8-Isoprostane (ηg/ml)	1.76 ± 1.64	1.62 ±0.65	0.087

Data presented as mean ± SD. Bold values indicate statistically significant differences (P<0.05). †; P value were calculated by independent t test.

DPP supplementation significantly elevated the antioxidant gene expression

Comparison of the gene expression levels demonstrated that *PRDX1* and *PRDX6* mRNA was significantly

overexpressed in infertile individuals compared to the healthy controls (P<0.001, Fig.2A, B). By comparing the expression level of *PRDX1* in the DPP group before and after treatment, a significant increase was seen after treatment (P<0.001, Fig.2A); nevertheless, the difference between the study groups after treatment was not statistically significant, regarding mean PRDX1 expression levels (P=0.188, Fig.2A). The mRNA expression level of *PRDX6* was increased significantly in the DPP group after treatment (P<0.001, Fig.2B). Comparison of *PRDX6* gene expression between the DPP group and healthy individuals after treatment indicated a lower (but not statistically significant) mRNA expression level in the healthy controls, compared to DPP group (P=0.577, Fig.2B).

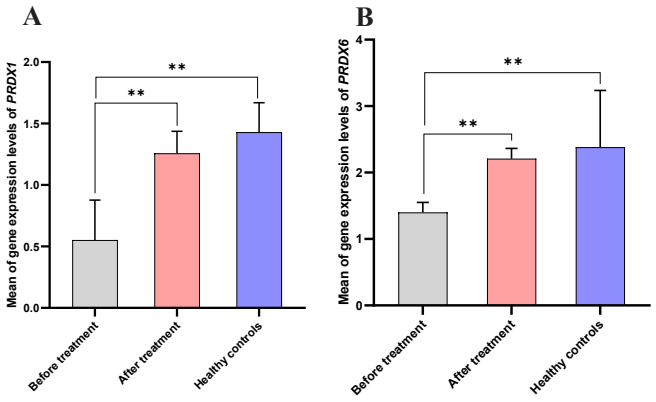

Fig.2: Mean of gene expression levels of *PRDX1* and *PRDX6* in cases before and after treatment, and the healthy controls. **A.** Upregulation of *PRDX1* expression in cases before treatment compared with cases after treatment and healthy controls. **B.** Upregulation of *PRDX6* expression in cases before treatment compared with cases after treatment and healthy controls. **; Indicate statistically significant differences(P<0.05).

The increased expression of PRDX1 and PRDX6 genes were correlated with improvement of semen parameters

To find a possible relationship between the altered gene expression and semen parameters in the participants after treatment, we evaluated correlation of *PRDX1* and *PRDX6* fold changes with sperm parameters and seminal plasma levels of free 8-isoprostane. We observed a significant association between the increased *PRDX1* expressions and improvement of semen parameters including count, motility, volume and morphology. Similarly, the increased expression of PRDX6 was significantly associated with the increased count, motility, volume and improved morphology of sperm (Table 4). Our findings showed a significant correlation of the altered *PRDX1* expression level with sperm motility and morphology. In addition, altered expression of the PRDX6 gene was significantly related to count, appearance, pH and morphology. Nonetheless, we did not find any significant correlation of the altered expression of *PRDX1* and *PRDX6* genes with decreased concentration of 8-isoprostane levels (Table S1, See Supplementary Online Information at www.ijfs.ir).

Table 4: Association of *PRDX1* and *PRDX6* gene expressions fold change with seminal fluid parameters

Variable	Expression of the genes		P value†
Sperm parameters	Before treatment	After treatment	
Mean expression level of *PRDX1*			
Count (million/ml)	0.5403	1.1813	**<0.001**
Volume (ml)	0.5600	2.4780	**0.036**
Motility	0.4504	1.0550	**0.001**
Morphology	0.5334	1.3189	**0.001**
Mean expression level of *PRDX6*			
Count (million/ml)	1.3627	2.2410	**0.003**
Volume (ml)	0.9600	2.5180	**0.028**
Motility	1.5370	2.2145	**0.047**
Morphology	1.3623	2.1518	**0.002**

Bold values indicate statistically significant differences (P<0.05). †; P value were calculated by Wilcoxon test.

Discussion

In the current study, we found that DPP supplementation led to significant improvement of sperm morphology, semen volume and sperm count in infertile men. We also found that expression of *PRDX1* and *PRDX6* genes was significantly upregulated in the DPP group compared to the healthy controls. Significant improvement in the sperm parameters of our study was consistent with several previously published studies (14, 15, 17, 25). In a study conducted by Rasekh et al. (25), a significant improvement in count, morphology and forward progressive motility of sperm was identified after consumption of DPP. Bahmanpour et al. (15) reported that DPP consumption led to a significant improvement of count, motility, morphology and DNA quality of sperm in male rate. Similar results were obtained from studies performed by Mehraban et al. (14) and Iftikhar et al. (17); they demonstrated a beneficial impact of DPP on sperm indices in male rats.

The observed positive effects of DPP on the parameters of sperm in our work and the other reports can be attributed to gonad-stimulating compounds such as estradiol, estrone and estriol present in DPP (26). Accordingly, it has been demonstrated that DPP consumption can improve testosterone, estradiol, follicle-stimulating hormone and luteinizing hormone levels, which led to increased weight of testis, epididymis, and sexual behaviors (15, 16, 27). Therefore, it seems that efficacy of DPP with respect to sperm parameters is at least in part due to the presence of its estrogenic components.

Since OS is a major contributor to male infertility, an antioxidant-based treatment strategy can be beneficial to restore fertility. In the past decades, there has been great interest in the use of herbal remedies, as natural antioxidant sources with minimum side-effects, to treat male infertility in developing countries (14, 28). From ancient times, DPP was used in traditional medicine of Egypt for enhancement of male fertility (28). Phytochemical screening revealed that DPP was a natural source of

minerals such as zinc, calcium and selenium, vitamins including A, B, C and niacin, phenolic components such as gallic, coumaric, ferulic, and protocatechuic acids, and flavonoids like luteolin, quercetin and apigenin (28-30). Having these components altogether, DPP can be considered as an antioxidant agent. Hence, we examined effect of DPP supplementation on sperm parameters. We also assessed its efficacy on 8-isoprostane concentrations and expression pattern of *PRDX1* and *PRDX6* genes in infertile men.

We found that concentration of 8-isoprostane (a marker for lipid peroxidation) was significantly higher in the infertile individuals compared to the normospermic healthy controls. Our finding were in agreement with those of Khosrowbeygi and Zarghami (31) who reported a high concentration of 8-isoprostane in males with asthenozoospermia, oligoasthenoteratozoospermia and asthenoteratozoospermia, by comparing with normospermic healthy controls.

In the current attempt, DPP-treated infertile males showed a significant reduction of 8-isoprostane concentration and this was correlated with a significant increase in sperm count. In line with our observation, previous investigations demonstrated a protective effect of DPP against cadmium and electromagnetic fields-induced OS in male rats (26, 32). Selenium is required for normal spermatogenesis and it serves as an antioxidant via the selenoprotein glutathione peroxidase which catalyzes reduction of lipid hydroperoxides and protects sperm cell membrane against oxidative damage (33). It has been reported that selenium supplementation led to improvement of sperm motility and successful induction of conception (33, 34). Antioxidant efficacy of zinc micronutrient in the improvement of sperm parameters of infertile men has also been previously reported (35). Therefore, containing selenium, zinc and vitamins, DPP has potential antioxidant properties to counteract excessive ROS. On the other hand, the aforementioned phenolic components and flavonoids can induce expression of nuclear factor-erythroid factor 2 (Nrf2) (19, 20, 36). Nrf2 encodes a transcription factor regulating the antioxidant gene expression via binding to the response elements in their promoters (37). In accordance with these data, it can be suggested that DPP induces high expression of antioxidant genes through Nrf2 transcription pathway and protects sperms from OS.

We found that expression level of PRDX1 and PRDX6 genes were significantly lower in the DPP group, by comparing with the healthy control group. In line with our finding, a decreased amount of PRDX1 and especially PRDX6 was detected in the men with idiopathic infertility in comparison with normospermic controls (13). Bumanlag et al. (38) demonstrated that absence of PRDX6 gene induced oxidization of lipid, proteins and DNA of mice spermatozoa which in turn led to an impairment of sperm motility and function. In the present study, DPP consumption resulted in significantly increased expression of the *PRDX1* and *PRDX6* genes in the infertile men. Increased expression of PRDXs was positively correlated with improved parameters of semen including count, motility, morphology and liquefaction in our study. Similarly, a significant association of *PRDX1* and *PRDX6* modifications with motility of sperm, DNA damage and lipid peroxidization was reported (13). There was no direct evidence to unveil the correlation between DPP consumption and increased expression of the PRDXs observed in our study, suggesting that phenolic and flavonoids components presented in DPP were responsible for the altered expression pattern of PRDXs. For example, Miyamoto et al. showed that quercetin could induce expression of Nrf2 and subsequently upregulate antioxidant *PRDX3* and *PRDX5* genes expression (37). However, further research are needed to disclose other potential pathways.

The current study limitation was its small sample size. Due to the limited resources for evaluation of gene expressions and the relatively unpleasant semen sampling for the participants, we were not able to include more individuals in the study. This limits the generalizability of our findings.

Conclusion

We found that DPP consumption has a positive effect on semen parameters in men with infertility. Moreover, our study was the first to demonstrate that DPP consumption could result in an increased expression of antioxidant *PRDX1* and *PRDX6* genes. However, the reason for this remains inexplicable and it should be further investigated.

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Authors' Contributions

K.M.; Supervisor, participated in study design, data collection, and drafting and statistical analysis. A.M.F.; Conducted data collection and contributed to all experimental work and manuscript draft preparation. S.F.; Contributed extensively in interpretation of the data and conclusion. Z.A., M.I.R.; Contributed to data collection and cooperate in molecular experiments. All authors are responsible for the final approval of the version to be published.

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