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Original Article

The effect of *Phoenix dactylifera* pollen on the expression of *NRF2*, *SOD2*, *CAT*, and *GPX4* genes, and sperm parameters of fertile and infertile men: A controlled clinical trial

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Editor-in-Chief:

Aflatoonian Abbas M.D.

Abstract

Background: Oxidative stress is caused by the imbalance occurring between the creation and clearance of the reactive oxygen species (ROS), which is responsible for 30–40% of male infertility. The positive impact of *phoenix dactylifera* pollen (Date palm pollen, DPP) on the improvement of sperm parameters has been well documented in animal models.

Objective: For evaluating the effect(s) of DPP on sperm parameters, ROS levels, expression of antioxidant genes, and activity of antioxidant enzymes of infertile men.

Materials and Methods: In this controlled clinical trial, a total of 60 male case with infertility and 20 normospermic fertile men were recruited. Before and after the treatment with DPP, the case were administered 400 mg/kg of gelatinous capsules daily for 30 consecutive days and semen samples were taken. Quantitative real-time polymerase chain reaction was applied for the evaluation of the mRNA expression levels of Nuclear factor erythroid 2-related factor 2(*NRF2*), superoxide dismutase (*SOD2*), glutathione peroxidase 4(*GPX4*), and catalase (*CAT*) genes.

Results: The *mRNA* expression levels of *NRF2, SOD2, GPX4*, and *CAT* ($p < 0.05$ for all) and significantly increased after treatment with DPP. The increased expressions of all antioxidant genes and enzymes significantly correlated with improvement in semen parameters including count ($p = 0.01$), motility ($p = 0.05$), and morphology ($p = 0.01$) of sperm. A significant correlation between the alteration of *SOD2* gene expression and SOD activity, *GPX4* and GPX, *and CAT* were also observed ($p = 0.05$).

Conclusion: DPP can increase the expressions of *NRF2, GPX4, SOD2*, and *CAT* genes and also improve the semen quality in infertile men.

Key words: DPP, Male infertility, SOD2, NRF2, GPX4, CAT, ROS, Spermatozoa.

This article has been extracted from Ph.D. Thesis. (Soghra Fallahi)

Registration ID in IRCT: IRCT2015020120895N2

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1. Introduction

As stated by the World Health Organization (WHO) guidelines, male infertility factors are responsible in roughly half of these couples (1). Moreover, male infertility has been proposed as one of the multifactorial disorders due to environmental, genetic, non-genetic factors, or a combination of these (2, 3). Several factors including structural reproductive tract abnormalities, mutations in mitochondrial DNA, chromosomal disorders, endocrine disturbances, hypogonadism, erectile dysfunction, chronic illnesses, several medications, exposure to radiation, reproductive tract infections, and cryptorchidism have previously been proposed as causes of male infertility (4, 5). However, the male infertility is still unknown in approximately 50% of the reported cases (4).

Accumulating evidence has shown oxidative stress (OS) as a major cause of the male infertility (6). OS has been reported to play an independent role as it has been considered as one of the conditions reflecting an imbalance between the production of oxygen-derived free radicals called ROS and the body's ability to the quenching of the ROS (7). In normal physiological conditions, ROS is generated at a low level in spermatozoa and plays a fundamental role in various biological mechanisms, namely capacitation, hyperactivation, and motility of sperms, acrosome reaction, and subsequent sperm–oocyte fusion and fertilization (8). Increased levels of ROS can pose a threat to sperms by causing peroxidation of sperm cellular membrane lipids, damage to sperm DNA integrity, decrease in sperm motility, oocyte–sperm fusion efficacy, and overall semen quality. Therefore, ROS plays a dual role as both deleterious and beneficial depending on their concentration in spermatozoa (9).

Moreover, enzymatic antioxidants like *SOD*, *CAT*, and *GPX*, which are abundant in the midpiece, make up a great proportion of human sperms (10). Nuclear factor erythroid 2-related factor 2 *(NRF2)*, as a key protein in antioxidant defense system, acts as nuclear transcriptional factor and regulates the expressions of *GPX, SOD*, and *CAT* genes (11). Altered expressions of *NRF2, GPX4*, *SOD2*, and *CAT* genes can be associated with increased OS, which could contribute to impaired fertilization capacity and male infertility (12).

Experts have employed herbal remedies for treating various diseases especially male infertility since ancient times (13). Because of various antioxidants constituents, *Phoenix dactylifera* [Date Palm Pollen (DPP)], a good source of hormones, minerals, enzymes, vitamins, proteins, and fatty acids, has been used as an herbal remedy in the treatment of male infertility but very few data are available about its molecular nature (14). Besides, its efficacy has been studied in animal models (15), but few studies have addressed the effectiveness of DPP on humans. In a very recent study, DPP administration was proven effective on testosterone and follicle-stimulating hormone (FSH) levels, as well as sperm motility; however, the subjects of this study were sub-fertile (16). In addition, although some studies have evaluated the effect of DPP on sperm parameters (17), no previous study has addressed the influence of DPP on male fertility at genomic level.

Therefore, this research aimed at the determination of the impact(s) of DPP on the levels of *mRNA* expression of antioxidant genes (*NRF2, GPX4, SOD2*, and *CAT*) in infertile male.

2. Materials and Methods

2.1. Study subjects

This research randomized, single-blind, and comparative clinical trial included 60 case

diagnosed with male infertility who were referred to the Om-e-Leila Fertility and Infertility Center, Om-e-Leila Hospital, Bandar Abbas, Iran, during 2016 (March)-17 (June) (Figure 1). We excluded the case with these criteria from the research: history of genetic and systemic disorders, reproductive tract abnormality, testicular trauma, alcohol and substance abuse, and fertility medications at least for six months before participation. DPP powder 400 mg/kg in gelatinous capsules was used to treat the eligible subjects daily for 30 consecutive days and all of the case finished the study course. DPP powder was purchased from palm farmers in autumn and formed into capsules in the laboratory. Additionally, 20 fertile males who had fathered a child within the last 2 yr and their semen samples were recruited as the controls. Initially, the controls comprised of the same number of case as the case group, however, due to the inconvenience of sample collection for semen analysis, some case failed to cooperate and were excluded from the study. At the time of enrollment, demographic data were obtained from all case using a structured interview form.

2.2. Sample collection and isolation of spermatozoa from seminal fluid

Semen samples were obtained twice from cases, before and after the treatment period 30 consecutive days, and once from controls. Then, we collected the fresh semen samples in the sterile plastic containers by masturbation after three to five days of sexual abstinence. Then, we liquefied the samples at 37°C for 30 min and semen analysis (semen volume, pH, viscosity, sperm morphology, concentration, and motility) was immediately performed with the use of the Sperm Quality Analyzer IIC (SQA IIC, United Medical Systems Inc., Santa Ana, CA, USA) based on the WHO guidelines (18). In addition,

in this randomized, single-blind, and comparative clinical trial, Semen analysis was performed by the blind technician. After purification of spermatozoa by Goodrich methods (19), BSA-free Ham's-F10 medium was used to wash the samples twice and then stored in RNALater solution (Qiagen: Germany) at a temperature of −80°C until RNA extraction. Sperms with smooth, rimmed, oval-shaped heads, 2.5–3.5 μm wide and 5–6 μm long, free of large vacuoles, an acrosome that covers 40–70% of the head, a midpiece of nearly the same length as the head but much slimmer, and un-coiled 45 μm-long tail thinner than the head and midpiece, and with no defects in the tail or head under light microscope are considered as having normal morphology.

2.3. Purification and measurement of free 8-Isoprostane

An affinity column (Cayman Chemical, Ann Arbor, MI, USA) available in the market was used to purify the free 8-Isoprostane in duplicate. For isolating of precipitates, all specimens were first centrifuged at 15000 g. Then, we used column buffer to dilute supernatant 1:5 and performed the next procedures based on the manufacturer's protocol. Concentration of 8-Isoprostane was assessed using 50 ml from specimens at a wave length of 405 nm by the enzyme-linked immuno-sorbent assay (ELISA) reader (STAT FAX 2100, USA).

2.4. RNA extraction and cDNA synthesis

NucleoSpin® RNA Midi kit (Macherey-Nagel, Düren, Germany) was used to extract the total RNA from the samples and then RNase-free DNase I (Thermo Scientific, USA), based on the manufacturer's manual, was used to treat the

RNA yield. After that, agarose gel electrophoresis was employed for confirming the quality of the extracted RNA. Its quantity was confirmed using Nano Drop® ND-1000 Spectrophotometer (Thermo Scientific, USA). In accordance with the company's guidelines, we applied the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) to reverse transcribe 20 ng of total RNA to cDNA.

2.5. Quantitative real-time polymerase chain reaction

Based on the company's instructions, we carried out duplicate real-time PCR with the gene-specific primers by means of SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara Bio Inc., Shiga, Japan) produced by the Rotor-Gene™ 6000 system (Corbett Research: Australia). Thermal cycling consisted of a round of denaturation for 30 sec at 95°C, which was followed by 40 more 5-sec cycles at 95°C. Each primer pair was heated at their specific temperatures (shown in Table I) for 15 sec and another 20-sec round at 72°C. Using serial dilution of cDNA, standard curves were drawn and thus level of the genes' expression level was normalized based on the mean expression of β-actin (the housekeeping gene). No template negative control was used.
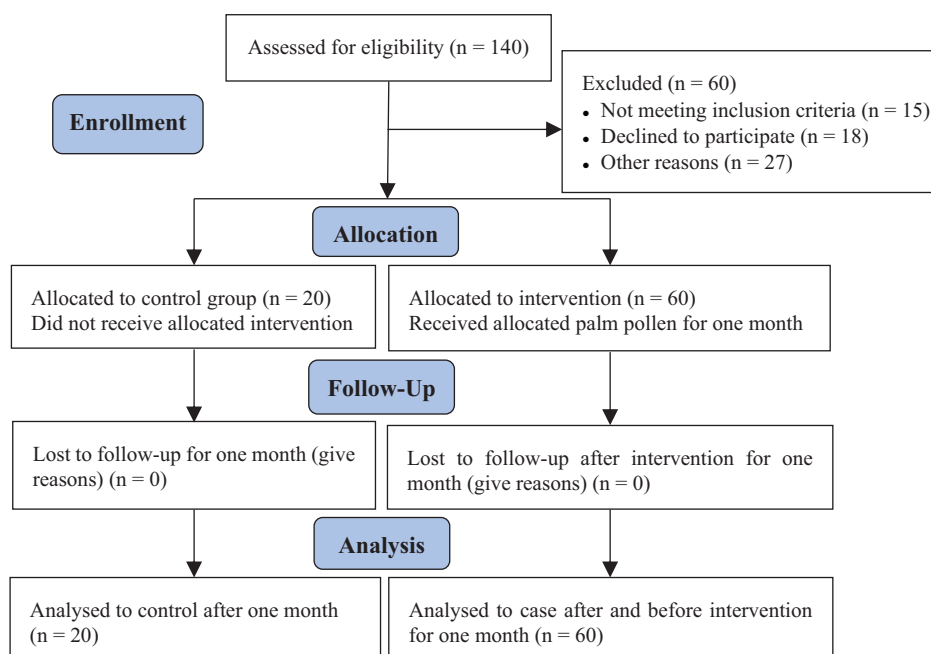

Figure 1. Consort flow chart.

Table I. Primer sequences and real-time PCR condition

Gene	The primers sequences (5'→3')	Amplicon (bp)	Annealing Temp. (°C)
SOD2	F-TTGACAAGTTTAAGGAGAAGC R-CTGAAGGTAAGCGTGC	197	59
GPX	F-CCGATACGCTGAGTGTGGTT R-CTCCCTGGCTCCTGCTTCC	72	61
CAT	F-CTGGGACTTCTGGAGCCTAC R-TCTCGCCGCATCTTCAACA	211	59
NRF2	F-CCAGCACATCCAGTCAGAA R-CGTAGCCGAAGAAACCTCA	151	58
β-actin	F-GCCTTTGCCGATCCGC R-GCCGTAGCCGTTGTCG	90	59

SOD2: Superoxide dismutase 2, GPX: Glutathione peroxidase, CAT: Chloramphenicol acetyltransferase, NRF2: Nuclear factor erythroid 2-related factor 2

2.6. Ethical considerations

The Ethics and Human Rights Committee of Hormozgan University of Medical Sciences (under the ethics code: HEC-93-12-4) verified this research, and then the written informed consents were received from all participants after the purpose of study was explained based on the declaration of Helsinki.

2.7. Statistical analysis

According to the research design, SPSS 16.0 (SPSS Inc., Chicago, IL: USA) was employed for statistical analyses and finally we set $p < 0.05$ to be statistically significant. Then, we employed a paired t test for comparing the sperms' parameters before and after the treatment period. In addition, we applied student's t test to compare the sperm parameters between controls and case (after treatment). Analysis of the distribution of *NRF2, GPX4, SOD2*, and *CAT* genes expression levels with the use of the Kolmogorov–Smirnov test indicated the lack of normal distribution of the data. For this reason, we employed non-parametric statistical tests for comparing the data. The Wilcoxon two-sample test was applied to detect the significant differences found in the *NRF2, GPX4, SOD2*, and *CAT* genes expression levels before and after the treatment. To compare the expression levels of the genes between the treated and healthy control individuals, two-tailed Mann–Whitney U-test was used. Spearman's Rho correlation test and was also utilized to analyze the correlations between the *NRF2, GPX4, SOD2,* and *CAT* genes expression levels and the various sperm parameters and free 8-Isoprostane levels.

3. Results

Demographic information of the participants are demonstrated in Table II and also change information the sperm count, semen volume and other parameters of case significantly enhanced after treatment with DPP. Comparison of the mean concentration of free 8-isoprostane and its significant reduction after capsule treatment was one of the most significant results of this study ($p < 0.05$) (Table III). Next, we assessed the possible correlation between the 8-Isoprostane concentration and seminal parameters. The common frequency of abnormal parameters of the participants in this study is also mentioned (Table IV).

Comparative evaluation of mRNA expression of NRF2, GPX4, SOD2 and CAT genes before and after DPP treatment and in healthy individuals using quantitative real-time PCR and evaluation of the ratio of these genes at the mRNA level using the 2 - ΔΔct method significantly increased showed the expression of these genes. The mRNA expression levels of NRF2, GPX4, and CAT genes in healthy control was significantly higher than the case after the treatment ($p = 0.04$, $p < 0.05$, and $p = 0.03$) (Figure 2) However, the differences between the mRNA expression levels of SOD2 gene in healthy controls and case after treatment with DPP were not statistically significant ($p = 0.16$).

Examination of seminal enzymatic activity results of GPX4, SOD2 and CAT genes in in case and healthy controls showed that the mean activity of seminal GPX4, SOD2 and CAT enzymes were higher in controls compared with case, in addition Our findings showed that the mean activity of GPX4, SOD2, and CAT enzymes were higher in case after the treatment compared with case before the treatment (Table V).

The associations between fold changes of NRF2, GPX4, SOD2, and CAT genes expressions with sperm parameters in this article indicated that increase in the NRF2, GPX4, SOD2, and CAT genes expressions after treatment with DPP

is significantly associated with increased the sperms' count, motility, and volume, and improved morphology of sperm (Table VI).

Spearman's correlation analysis showed SOD2 mRNA expression was significantly correlated with count, motility, and morphology of sperm, moreover, we found a relationship between the GPX4 expression at mRNA level and sperm count, sperm motility, morphology of sperm, pH of semen fluid, and seminal plasma free 8-Isoprostane level. The CAT expression at mRNA level was significantly related to the count of sperm, motility, and morphology of sperm and seminal plasma free 8-Isoprostane level (Table VII). We also detected a positive correlation between the NRF2 mRNA expression levels and sperm count, sperm motility, appearance and morphology of sperm, and pH of semen fluid.

Table II. Demographic properties of case and healthy controls

Variable	Case			Control		
	N (%)	Mean ± SD	Range	N (%)	Mean ± SD	Range
Age (yr)						
≤ 30	40 (66.67)	27.62 ± 2.23	22.00–30.00	12 (60.00)	27.75 ± 2.00	24.00–30.00
> 30	20 (33.33)	34.35 ± 3.32	31.00–40.00	8 (40.00)	33.62 ± 1.92	32.00–37.00
Weight (Kg)	60 (100.00)	88.36 ± 1.49	60.00–110.00	20 (100.00)	85.95 ± 9.68	60.00–104.00
Height (M)	60 (100.00)	1.66 ± 0.08	1.40–1.85	20 (100.00)	1.70 ± 0.07	1.57–1.81
BMI*						
Normal	15 (15.00)	22.11 ± 2.19	18.52–24.86	6 (30.00)	22.26 ± 1.90	18.63–24.11
Overweight	35 (58.33)	27.13 ± 1.17	25.00–29.71	8 (40.00)	25.54 ± 1.82	22.81–29.21
Obesity grade 1	10 (16.67)	31.83 ± 1.52	30.92–34.48	4 (20.00)	33.01 ± 0.56	30.42–34.78
Obesity grade 2	–	–	–	2 (10.00)	39.21 ± 0.06	39.06–39.36

*BMI: Body mass index, Normal (<18 – ≤ 24.99), Overweight (≤ 25 – ≤ 29.99), Obesity grade 1 (≤ 30 – ≤ 34.99), Obesity grade 2 (≤ 35 – ≤ 39.99)

Table III. The comparison of sperm parameters and free 8-Isoprostane levels between the patients after treatment and healthy control

Variable	Cases after treatment	Controls	P-value*
Count	33.21 ± 25.94	77.15 ± 22.63	≤ 0.001
Volume	3.71 ± 1.32	2.65 ± 0.74	≤ 0.001
Appearance	–	–	0.99
Viscosity	–	–	0.56
Liquefaction	–	–	0.99
pH	7.58 ± 0.16	7.67 ± 0.27	0.26
Motility	34.06 ± 13.84	61.36 ± 11.89	≤ 0.001
Morphology	32.18 ± 9.98	61.90 ± 9.49	≤ 0.001
Free 8-Isoprostane	2.05 ± 2.01	1.90 ± 1.02	0.31

Data are expressed as the Mean ± standard deviation (SD), *Analyzed by Student's *t* test

Table IV. The comparison of sperm parameters and free 8-Isoprostane levels of case before and after the treatment

Variable	Before treatment		After treatment		P-value*
	N (%)	Mean ± SD	N (%)	Mean ± SD	
Count					
Normal (≥ 15 million/ml)	15 (25.00)	16.33 ± 1.30	49 (81.67)	39.18 ± 25.03	< 0.001
Abnormal (< 15 million/ml)	45 (75.00)	5.84 ± 4.10	11 (18.33)	6.62 ± 3.50	
Volume					
Normal (≥ 1.5 ml)	52 (86.67)	3.38 ± 1.33	60 (100.00)	3.71 ± 1.32	< 0.001
Abnormal (< 1.5 ml)	8 (13.33)	1.17 ± 0.17	–	–	
Appearance					
Normal	56 (93.30)	–	60 (100.00)	–	0.12
Abnormal	4 (6.70)	–	–	–	
Viscosity					
Normal	59 (98.30)	–	60 (100.00)	–	0.99
Abnormal	1 (1.70)	–	–	–	
Liquefaction					
Normal	57 (95.00)	–	59 (98.30)	–	0.5
Abnormal	3 (5.00)	–	1 (1.70)	–	
pH					
Normal	59 (98.30)	7.70 ± 0.22	60 (100.00)	7.58 ± 0.16	≤ 0.001
Abnormal	1 (1.70)	–	–	–	
Motility					
Normal (≥ 40%)	15 (25.00)	46.13 ± 11.23	20 (33.33)	49.05 ± 10.96	< 0.001
Abnormal (< 40%)	45 (75.00)	22.68 ± 8.15	40 (66.67)	26.57 ± 7.66	
Morphology					
Normal (≥ 4%)	6 (10.00)	42.00 ± 3.94	15 (25.00)	45.40 ± 5.08	< 0.001
Abnormal (< 4%)	54 (90.00)	24.35 ± 7.82	45 (75.00)	27.77 ± 6.78	
Free 8-Isoprostane (ng/ml)	60 (100.00)	4.37 ± 4.13	60 (100.00)	2.05 ± 2.01	< 0.001

*Wilcoxon test

Table V. The mean of SOD, GPX, CAT, and NRF2 genes expression and the activity of their enzymes in case (before and after the treatment) and healthy controls

Genes	Case		P-value*	Healthy controls	P-value**
	Before	After			
The mRNA expression levels					
SOD2	0.63 (0.01–1.29)	0.93 (0.11–2.22)	< 0.001	1.04 (0.54–1.69)	0.16
GPX	0.26 (0.01–1.06)	1.37 (0.48–3.70)	< 0.001	2.00 (1.09–4.00)	< 0.001
CAT	0.79 (0.03–1.55)	1.36 (0.24–2.96)	< 0.001	2.20 (0.98–4.53)	≤ 0.001
NRF2	0.89 (0.11–2.64)	1.60 (0.29–3.20)	< 0.001	2.16 (0.26–3.93)	0.04
The enzyme activity					
SOD2	75.96 (6.77–181.31)	82.78 (32.60–190.1)	0.030	112.29 (75.35–341.33)	≤ 0.001
GPX	53.22 (20.27–110.10)	73.70 (22.17–208.42)	< 0.001	107.39 (54.13–218.39)	< 0.001
CAT	1.69 (0.01–5.98)	2.53 (0.29–7.83)	< 0.001	4.08 (1.89–6.43)	< 0.001

Data presented as mean (min–max), *Analyzed by Wilcoxon test, **Analyzed by Mann–Whitney test, SOD2: Superoxide dismutase 2, GPX: Glutathione peroxidase, CAT: Chloramphenicol acetyltransferase, NRF2: Nuclear factor erythroid 2-related factor 2

Table VI. The mean of *SOD*, *GPX*, *CAT*, and *NRF2* genes fold change in relation to abnormal seminal fluid parameters before and after the treatment

	SOD2			GPX4			CAT			NRF2		
	Before	After	P-value*	Before	After	P-value*	Before	After	P-value*	Before	After	P-value*
Count	0.6371	0.9500	< 0.001	0.2840	1.3989	< 0.001	0.8089	1.3980	< 0.001	0.8867	1.6698	< 0.001
Volume	0.4925	0.8750	0.012	0.2450	1.7300	0.012	0.8963	1.5425	0.012	0.7900	1.8750	0.01
Appearance	0.6450	0.8800	0.066	0.2425	1.4900	0.068	0.6775	1.7175	0.068	0.4400	1.2650	0.06
Liquefaction	0.2633	0.6337	0.109	0.2237	1.3567	0.109	0.5467	1.2900	0.109	0.3233	1.0967	0.10
Motility	0.6904	0.9740	0.012	0.8633	1.3460	0.024	1.1209	1.3662	0.041	1.2360	1.6782	0.03
Morphology	0.6309	0.9624	< 0.001	0.2706	1.3828	< 0.001	0.7872	1.3881	< 0.001	0.8717	1.6439	< 0.001

*Analyzed by paired *t* test, SOD2: Superoxide dismutase 2, GPX: Glutathione peroxidase, CAT: Chloramphenicol acetyltransferase, NRF2: Nuclear factor erythroid 2-related factor 2

Table VII. The correlation between *SOD2*, *GPX4*, *CAT*, and *NRF2* genes expression levels with sperm parameters

	SOD2		GPX4		CAT		NRF2	
	rs	P-value	rs	P-value*	rs	P-value	rs	P-value*
Count	0.339	0.0001*	0.610	0.0001*	0.410	0.0001*	0.477	≤ 0.001
Volume	0.016	0.853	0.036	0.675	0.029	0.733	0.149	0.07
Appearance	−0.076	0.373	−0.161	0.057	−0.148	0.081	−0.203	0.01*
Liquefaction	−0.153	0.073	−0.128	0.131	−0.162	0.071	−0.159	0.06
Viscosity	−0.142	0.094	−0.096	0.262	−0.093	0.272	−0.109	0.19
PH	−0.142	0.094	−0.194	0.022*	−0.088	0.300	−0.281	≤ 0.001
Motility	0.205	0.015*	0.422	0.001*	0.211	0.012*	0.282	≤ 0.001
Morphology	0.261	0.002*	0.485	0.0001*	0.273	0.001*	0.243	≤ 0.001
8-Isoprostane	−0.108	0.202	−0.259	0.002*	−0.194	0.021*	−0.119	0.16

*Analyzed by Spearman's Rho, SOD2: Superoxide dismutase 2, GPX: Glutathione peroxidase, CAT: Chloramphenicol acetyltransferase, NRF2: Nuclear factor erythroid 2-related factor 2, rs: Spearman's correlation coefficient
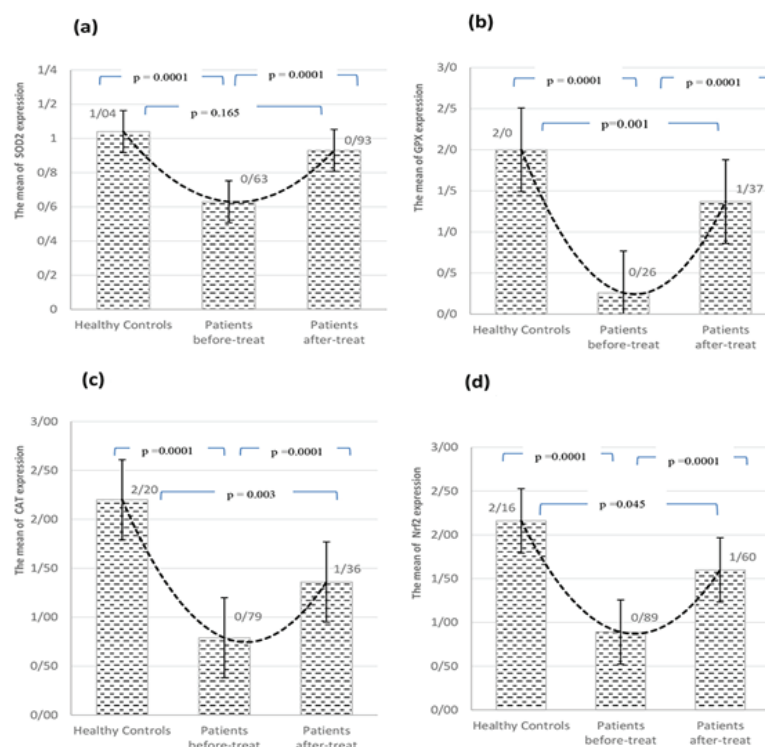

Figure 2. Changes in mRNA level of SOD2 (a), GPX (b), CAT (c), and NRF2 (d) genes before and after the treatment of infertile men with DPP in comparison with healthy controls and cases.

4. Discussion

As mentioned earlier, excessive levels of ROS can negatively affect the process of spermatogenesis and impair the ability of spermatozoa to fertilize ova (9). Antioxidants and antioxidant enzymes in the male reproductive tracts are the main antioxidant defense systems that protect spermatozoa from oxidative damage caused by ROS (6, 9). Therefore, antioxidant therapy could be beneficial in increasing the scavenging capacity of seminal plasma and improving the semen quality in infertile males. In traditional medicine, DPP has an extensive utilization as one of the folk remedies to treat the male infertility (14). This study was designed for investigating the *mRNA* expression levels of the antioxidant genes *NRF2, GPX4, SOD2*, and *CAT* in infertile males before and after treatment with DPP and healthy controls. We also investigated the impact of DPP on the sperm parameters of infertile man. We found that taking DPP in infertile males significantly improves the semen quality including sperm count, semen volume, morphology, and motility of sperm (p < 0.05) (Table IV). In accordance with our data, Al-Snafi reported that the sperms' motility and count enhanced in the infertile males who were given treatment with DPP (20). Similarly, evaluated the effects of DPP on the sperm parameters of infertile man; sperm motility, count, morphology, as well as forward-progressive motility significantly increased with DDP (21). Al-Dujaily also reported a significant improvement in the sperms' motility and count after the addition of DPP extract to sperm activation medium (22). In a study on adult male Sprague–Dawley rats, it was found that DPP can promote the count, motility, morphology, and DNA quality of sperms. Moreover, it increased the weight of testis and epididymis (23). Adaay and Mattar showed that after treatment with the extracts, a mixture of 3 plants (*P. dactylifera*, *Nasturtium officinale* and *Tribulus terrestris*), the sperm concentration and motility increased remarkably in Swiss albino male (24). These findings suggest that consumption of DPP in infertile males of human and animal models improves a number of semen parameters through various possible molecular mechanisms. Analysis of seminal plasma has revealed that human semen contains high concentrations of mineral ions such as calcium (Ca) and zinc (Zn) (24), which fundamentally contribute to spermatogenesis. In sperm cells, a major component of the signaling cascade leading to capacitation, hyperactivation, chemotaxis, and acrosome reaction is Ca. There exists convincing data supporting the critical role of the Zn in mediating of nuclear chromatin decondensation, sperm oxygen consumption, and acrosin activity (25). Additionally, it has been documented that the zinc finger proteins are involved in the expression of steroid hormone receptors (26). Selenium (Se), as essential micronutrients, acts as free radical scavenger and improves semen quality to the presence in antioxidant component of selenoprotein (27). DPP contains many minerals including Ca, Zn, and Se; therefore, it can positively influence the quality of semen. However, other possible mechanisms of sperm quality improvement by DPP need to be further looked into. Moreover, this might be the

reason for similar findings in previous studies of animal models which were in line with our results.

According to the findings, *mRNA* expression levels of antioxidant genes *SOD2, GPX4, CAT* and *NRF2* are significantly lower in infertile case in comparison with the healthy control. Moreover, *mRNA* expression levels of *NRF2, GPX4, SOD2,* and *CAT* genes increased in case after treatment with DPP (p < 0.05) (Figure 2; Table III). Similarly, reported a link between functional polymorphisms in *NRF2* promoters and dysfunctional spermatogenesis in humans. Due to the role of *NRF2* in the transcription of some antioxidant genes such as *GSTM1* and *SOD2 mRNA*, functional polymorphisms in *NRF2* promoters can decrease their levels (13). Previous findings have shown, that decreased fertility in male *NRF2* (–/–) compared to the wild-type is a result of knockout of *NRF2* transcription factor and the subsequent disruption in spermatogenesis (28). In light of these studies and our findings, sperm fertilization capacity is determined by the level of *NRF2* expression. The expression of *GPX4* appears to increase in testis during spermatogenesis (28). *GPX4* is predominantly localized in the mitochondria of spermatozoa and through the antioxidant activity against mitochondrial ROS and by maintaining the integrity of the structure of mitochondrial capsule, it plays an important role in sperm motility (29). Diaconu and coauthors reported a substantial decrease in expressing *GPX4* in spermatozoa of the human infertile males who have been diagnosed with oligoasthenozoospermia (30). Imai et al. reported an association between severe

abnormalities in spermatozoa and depletion of *GPX4* in spermatocytes (29). Furthermore, it has been shown that *GPX4* is involved in condensation of chromatin and protection of sperm DNA against oxidation, a necessary process in the maintenance of sperm quality (30). It has been reported that mitochondrial *GPX4* disruption causes abnormalities in spermatozoa (31). SOD2, mitochondrial SOD or Mn SOD, is one of the highly expressed genes that are regulated by the *NRF2-ARE* signaling pathway in human semen that are responsible for protecting the sperms from oxidative damages (32). Some have also shown that SOD contribute physiologically to the maintenance of a balance between H_2O_2 and O^{2-} and disruption in the expression of the enzyme relate to the impaired functions of the sperms (33). Another study reported the involvement of the decreased seminal plasma scavenger antioxidant capacity, especially as the lower SOD activity, in the male infertility (34). In fact, the existence of nearly 1 Ala-MnSOD allele (rs4880) enhanced the risks of infertility in male subjects (35). Other authors demonstrated the positive association between the seminal SOD activity and the sperm fertilization potential and male infertility (36). It has been showed that the presence of variant Val allele (Ala16Val polymorphism in the SOD2 gene) can alter the conformation of the secondary structure of the protein leading to decreased efficiency of transport into mitochondria which is believed to be associated with infertility (37). Moreover, a positive correlation between the protein expression of *CAT* and sperm motility and morphology was observed in a study performed

by Macanovic and colleagues (38). It has been reported that functional polymorphism in *CAT* gene (–262 C/T) alters the level of *CAT* in blood and influences the promoter activity (39). Sabouhi and colleagues demonstrated that this mutation is correlated to the males' infertility (40). Taken together, results reveal the fundamental contribution of expression and activity of antioxidants enzymes to the pathogenesis of male infertility. Results of a systematic review revealed that DPP can be an appropriate supplement for the treatment of infertility, in that it can decreases free radicals, increases sperm motility, and enriches sperms with important minerals (17). All in all, consistent with the previous studies, our results demonstrate a significant improvement of sperm parameters and antioxidant gene expression by DPP in case who suffer from male infertility.

It has been demonstrated that DPP contains notable amounts of polyphenols including phenolic acids, flavonoid glycosides, and anthocyanidins (41). Yeh and colleague reported that gallic acid (a phenolic acid) induces an accumulation of *NRF2* (42). Vari and colleague revealed that protocatechuic (another phenolic acid) can improve the antioxidant potential of the macrophage through a pathway in which *JNK-mediated NRF2* activation plays a critical role (43). Because of the regulation of expression of several antioxidant enzymes such as *GPX4*, *SOD2*, and *CAT by NRF2*, it is not surprising that the elevated expression of *NRF2* lead to increase in the expressions of antioxidant genes *GPX4, SOD2,* and *CAT*. One limitation of the current study was that the treatment was given to the infertile subjects for a duration of one month due to their lack of cooperation and limited resources for the study; however, the minimum time required for a complete cycle of spermatogenesis is 86 days. Therefore, future studies should consider a three-month treatment period for better results.

5. Conclusion

We could present further documents for supporting this hypothesis: altered expression of antioxidant genes *NRF2, GPX4, SOD2,* and *CAT* at *mRNA* levels are correlated to the greater risks of males' infertility. We also conclude that administering DPP in infertile males enhances the levels of expression of *NRF2, GPX4, SOD2,* and *CAT* genes and improves the semen quality including sperm count, semen volume, and morphology and motility of sperm.

Acknowledgements

Conflict of Interest

Hereby, it is declared that there is no conflict of interest regarding the research.

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