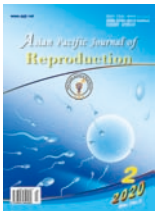

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Influence of *N*-acetylcysteine on pituitary-gonadal axis hormones and protamine expression level in streptozotocin-induced diabetic male rats

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ABSTRACT

Objective: To study the influence of *N*-acetylcysteine on the pituitary-gonadal axis hormones and protamine expression level in streptozotocin-induced diabetic male rats.

Methods: Forty-two adult male Wistar rats were divided into 6 groups, with 7 rats in each group. The control group left untreated; the streptozotocin group only received 50 mg/kg body weight streptozotocin intraperitoneally for 5 days to induce diabetes; the *N*-acetylcysteine group only received 200 mg/kg body weight *N*-acetylcysteine intraperitoneally, and the streptozotocin+*N*-acetylcysteine groups 1, 2 and 3 received 50 mg/kg streptozotocin intraperitoneally for 5 days to induce diabetes and then received 100, 200 and 400 mg/kg body weight doses of *N*-acetylcysteine intraperitoneally for 28 days, respectively. Enzyme-linked immunosorbent assay was used to measure the serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone, and real-time PCR was applied for measuring protamine expression level.

Results: Compared to the control and *N*-acetylcysteine groups, a significant decrease in the body weight, testicular weight and levels of testosterone and protamine expression was observed in the streptozotocin group and the streptozotocin+*N*-acetylcysteine groups 1 and 2. On the contrary, the levels of LH and FSH increased significantly. In the streptozotocin+*N*-acetylcysteine group 3, the body weight, testicular weight and expression level of protamine were significantly higher than those of the streptozotocin group. In the streptozotocin+*N*-acetylcysteine groups, testosterone and LH levels were significantly higher than and lower than the streptozotocin group, respectively. In the streptozotocin+*N*-acetylcysteine groups 2 and 3, the level of FSH was significantly lower than that of the streptozotocin group and streptozotocin+*N*-acetylcysteine group 1. Furthermore, a significant increase in the expression level of protamine was observed in the streptozotocin+*N*-acetylcysteine groups 2 and 3 when compared to the streptozotocin group and streptozotocin+*N*-acetylcysteine group 1.

Conclusions: *N*-acetylcysteine in an optimal dose of 400 mg/kg body weight has a protective influence on the pituitary-gonadal axis hormones and also on the expression level of protamine in diabetic male rats.

KEYWORDS: *N*-acetylcysteine; Diabetes; Testosterone; Protamine; Rat

1. Introduction

The pituitary-gonad system is one of the most important and active physiological axes in the body. It controls not only the reproductive functions but also regulates many physiological aspects of an individual, including sexual differentiation, development of secondary sexual characteristics and sexual behavior by synthesis and the secretion of androgens[1]. Successful spermatogenesis requires a series of precise epigenetic events that remove histones from the chromatin, and substitutes the transitions of proteins and protamine, and ultimately the chromatin density of the sperm[2]. Sufficient and correct expression and transcription of proteins and protamine are very important in the successful compression of the sperm nucleus and the fertility of the males. The studies conducted on protamine 1 and protamine 2 in mice indicate that mutation in

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one of these protamine alleles prevents the production of functional sperms[3]. Also, the incorrect ratio of protamine 1 and protamine 2 at the level of proteins has important implications on the fertilization rate and the fetus's growth and is associated with increased levels of DNA damage in the sperm[4].

Diabetes is a chronic disease and one of the most common endocrine disorders due to the impaired pancreas *i.e.* incorrect insulin secretion, insulin malfunction or both. In type Ⅰ diabetes or insulin-dependent diabetes, pancreatic beta cells responsible for insulin production in the body are eliminated or inactivated[5]. According to various studies, one of the systems most affected by diabetes is the reproductive system. It has been determined that in diabetic men, the gonadotropin-releasing hormone signaling is interrupted. Also, in men with type Ⅰ diabetes, leptin levels are reduced, which is associated with impaired hypothalamic-pituitary-gonadal axis activity[6]. It has also been shown that diabetes leads to reduced sperm motility and changes in sperm morphology. Moreover, diabetes weakens the Leydig cell functions and lowers testosterone secretion through excessive production of free radicals. Decreasing testosterone levels leads to reduced fertility in diabetic men[7]. Furthermore, diabetes induces testicular weight loss and reduces the mean number of spermatogonial cells, spermatocytes, spermatids and Sertoli cells[8].

N-acetylcysteine is an amino acid species used as an antidote for acetaminophen poisoning. This amino acid has antioxidant properties[9]. It prevents acute renal failure in patients undergoing cardiac surgery[10]. Besides, the combination of *N*-acetylcysteine and *L*-carnitine improves the damages made in DNA from radiation by activating DNA repair genes[11]. Studies have also shown that *N*-acetylcysteine repairs the metabolic malfunction associated with lipid metabolism in adipocytes derived from bone marrow stroma[12]. Moreover, it has been found that this substance improves oxidative stress and inflammatory responses in patients with pneumonia[13], and its inhalation improves pulmonary function in patients with liver transplantation[14]. It also prevents toxic effects induced by human-made inorganic fibers in the epithelial cell lining of bronchi[15]. Treatment with *N*-acetylcysteine reduced memory damage and loss of hearing in the mouse senescence-accelerated prone 8 model[16]. Studies have shown that *N*-acetylcysteine reduces nephrotoxicity induced by colistimethate sodium[17]. It can also reduce the reactive oxygen species (ROS)-mediated oxidative damage of DNA in helicobacter pylori infection[18].

In a study by Shittu *et al*, it was reported that *N*-acetylcysteine has a protective effect on sperm quality in testicular toxicity induced by cyclophosphamide in Wistar rats[19]. Hu *et al* found that *N*-acetylcysteine prevents fluoride-induced testicular cell death through modulation of the nuclear factor erythroid-2-related factor 2 activity and inositol-requiring enzyme-1a/c-Jun *N*-terminal kinase signaling[20].

Considering the protective and antioxidant effects of *N*-acetylcysteine in the treatment of various diseases and the importance of spermatogenesis in male reproductive potential as well as the absence of a similar study in diabetic patients, we attempted to evaluate the possible effects of this amino acid on changes in the hormones of pituitary-gonadal axis and the expression level of protamine in streptozotocin-induced diabetic rats.

2. Materials and methods

2.1. Animals

Forty-two adult male Wistar rats weighing (270±10) g and aged 2.5 to 3.0 months old were provided and settled at Islamic Azad University of Kazerun, Iran. They were kept in standard cages at 22 ℃-20 ℃, 12-hour light and 12-hour darkness and 70% humidity. Also, the rats had free access to pellet diet and water *ad libitum*.

2.2. Study protocol

The rats were divided into 6 groups (n=7 in each group) as follows: the control group, the streptozotocin group (as positive control), the *N*-acetylcysteine group (as negative control) and three streptozotocin+*N*-acetylcysteine groups. This study was carried out in two periods *i.e.* 5 and 28 days. In the first period, the streptozotocin and all streptozotocin+*N*-acetylcysteine groups received 50 mg/kg streptozotocin (1 mg dissolved in distilled water) intraperitoneally for 5 days, to induce diabetes. At the end of the sixth day, their blood glucose levels reached 200 mg/dL. In the second period, the control and streptozotocin groups received no treatment (without administrating streptozotocin or *N*-acetylcysteine). The *N*-acetylcysteine group (without receiving streptozotocin in the first period) received only 200 mg/kg of *N*-acetylcysteine intraperitoneally for 28 days. The streptozotocin+*N*-acetylcysteine groups 1, 2 and 3 (all three groups received 50 mg/kg streptozotocin in the first period to induce diabetes) also received 100, 200 and 400 mg/kg of *N*-acetylcysteine intraperitoneally for 28 days, respectively.

2.3. Blood sampling and hormonal analysis

At the end of each test, the body weight of all animals was determined, and after 12 h of fasting, rats were anesthetized with diethyl ether (Merck, Germany). Then, their chests were cut open and a 2-mL syringe was used to take the blood sample from their left heart ventricle. Blood samples were poured into the test tubes without anticoagulant, and placed in an incubator (Memmert UNB 400, Germany) at 37 ℃ for 12 min. Following coagulation, the tubes were centrifuged (machine Hettich, Germany) at 2 800×*g* for 15 min. The supernatant (serum) was isolated by the sampler (Biopette, UK), and a new tube was used for storing it in the freezer at -20 ℃.

The serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone were measured by enzyme-linked immunosorbent assay (ELISA) using ELISA reader (Stat Fax 2100, USA). The Cusabio kit (Germany) was used to measure LH and FSH levels and testosterone level was determined using the DRG Instruments GmbH kit (Germany).

2.4. RNA extraction and quantitative determination of protamine expression

A surgical procedure to extract sperm from the testis was applied to the testes of all animals (testicular sperm extraction). With the help of Trizol (Invitrogen Inc., USA 0029), RNA was extracted from testicular cell suspensions and with appropriate kits (H Minus First-Strand cDNA Synthesis Kit, Thermo Scientific, USA), the complementary DNA (cDNA) was synthesized regarding the instructions of the manufacturer. Then, applying a specific primer for the *β-actin* gene as a house-keeping gene, the quality of cDNA, polymerase chain reaction (PCR) was conducted. When it was specified that the quality of cDNA was right and there was no genomic DNA, a template of cDNA was used for real-time PCR to assess the changes in the protamine expression level in tissues received from different groups.

With the help of a protamine gene-specific primer (5'-ATGGCCAGATACCGATGCTG-3' for forward and 5'-CACCTTCGCCGCCTCC-3' for reverse) and a Power SYBR green PCR master mix (Applied Biosystems, UK) and also an RT-PCR system plus step one (Applied Biosystems, UK), we conducted the real-time PCR reaction. The PCR reaction program had the following features: cycles of 95 ℃ for 10 min, 40 cycles of 95 ℃ for 15 min and 60 ℃ for 1 min. With the help of the $2^{-\Delta CT}$ method, the specific gene expression levels were quantified.

2.5. Statistical analysis

One-way analysis of variance and Tukey tests by least significant difference (SPSS for Windows, version 20, SPSS Inc., Chicago, IL, USA) were used for analysis. The results of the experiments were expressed as mean±standard deviation (mean±SD). $\alpha = 0.05$ was considered as statistically significant level. GraphPad Prism version 6 software was applied to present the corresponding statistical calculations in the diagrams (GraphPad Prism, Inc., San Diego, CA, USA).

2.6. Ethical approval

The Ethical Committee of the Islamic Azad University of Kazerun, Iran approved all the ethical considerations on animal care (Ethical Code No.: IR.Kiau.16330525961005, August 20, 2018). All experiments were undertaken based on Iran Veterinary Organization rules and regulations for working with laboratory animals.

3. Results

3.1. Body and testicular weight findings

No significant statistical differences in the body and testicular weight between the control and *N*-acetylcysteine groups were found ($P>0.05$). Compared to the control group, a significant decrease was observed in the body weight in the streptozotocin group and the streptozotocin+*N*-acetylcysteine groups ($P<0.05$), but compared to the streptozotocin group, a significant increase was seen in the streptozotocin+*N*-acetylcysteine group 3 ($P<0.05$) (Figure 1A). Compared to the control group, a significant decrease was found in the mean testicular weight in the streptozotocin group and the streptozotocin+*N*-acetylcysteine groups 1 and 2 ($P<0.05$), but compared to the streptozotocin group, a significant increase was seen in the streptozotocin+*N*-acetylcysteine group 3 ($P<0.05$) (Figure 1B).

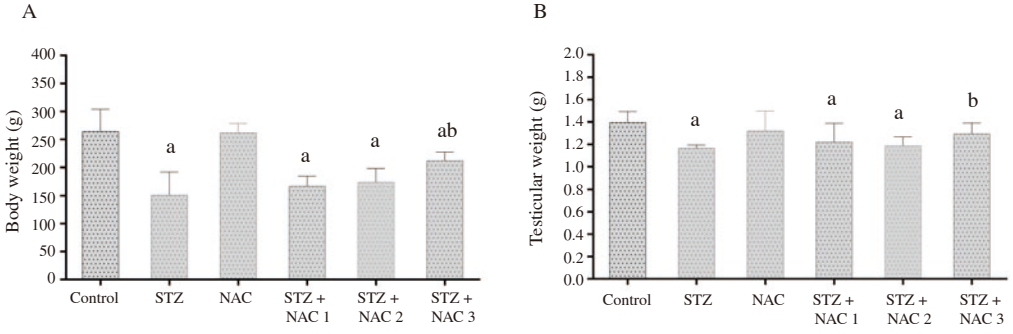

Figure 1. Comparing the mean body weight (A) and testicular weight (B) of rats in the groups receiving the indicated treatments. The letter a represents a significant difference compared to the control group at $P<0.05$; b represents a significant difference compared to the streptozotocin (STZ) group at $P<0.05$. NAC: *N*-acetylcysteine.

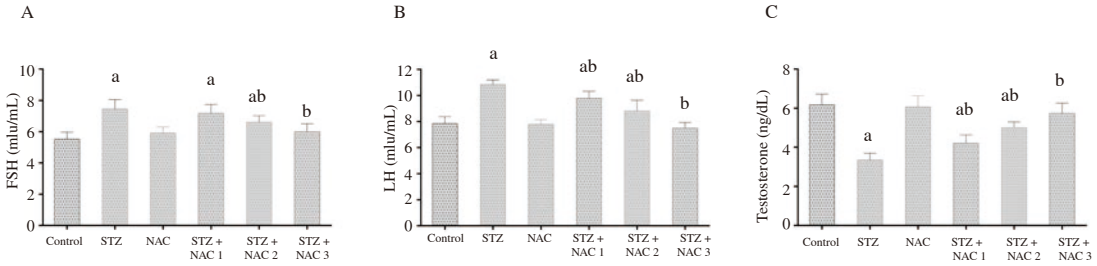

Figure 2. Comparing the mean serum levels of follicle-stimulating hormone (FSH) (A), luteinizing hormone (LH) (B) and testosterone (C) among the groups receiving the indicated treatments. The letter a represents a significant difference compared to the control group at $P<0.05$; b represents a significant difference compared to the streptozotocin group at $P<0.05$. STZ: streptozotocin; NAC: *N*-acetylcysteine.

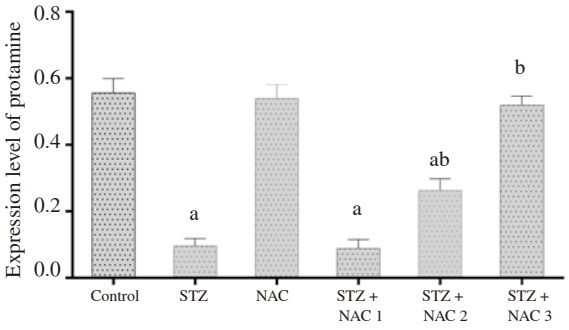

Figure 3. Comparing the mean protamine expression level among the groups receiving the indicated treatments. The letter a represents a significant difference compared to the control group at $P<0.05$; b represents a significant difference compared to the streptozotocin group at $P<0.05$. STZ: streptozotocin; NAC: *N*-acetylcysteine.

3.2. Hormonal findings

No significant statistical difference in the serum levels of FSH, LH and testosterone between the control and *N*-acetylcysteine groups was found ($P>0.05$). Compared to the control group, a significant increase was observed in the mean serum level of FSH in the streptozotocin group and the streptozotocin+*N*-acetylcysteine groups 1 and 2 ($P<0.05$), but compared to the streptozotocin group, a significant decrease was seen in the streptozotocin+*N*-acetylcysteine groups 2 and 3 ($P<0.05$) (Figure 2A). Compared to the control group, a significant increase was observed in the mean serum level of LH in the streptozotocin group and the streptozotocin+*N*-acetylcysteine groups 1 and 2 ($P<0.05$), but compared to the streptozotocin group, a significant decrease was seen in the streptozotocin+*N*-acetylcysteine groups 1, 2 and 3 ($P<0.05$) (Figure 2B). Compared to the control group, a significant decrease was observed in the mean serum level of testosterone in the streptozotocin group and the streptozotocin+*N*-acetylcysteine groups 1 and 2 ($P<0.05$), but compared to the streptozotocin group, a significant increase was seen in the streptozotocin+*N*-acetylcysteine groups 1, 2 and 3 ($P<0.05$) (Figure 2C).

3.3. Gene expression findings

Among the control and *N*-acetylcysteine groups as well as streptozotocin+*N*-acetylcysteine group 3, no significant statistical difference in the expression level of protamine was found ($P>0.05$). Compared to the control group, a significant decrease was observed in the mean expression level of protamine in the streptozotocin group and the streptozotocin+*N*-acetylcysteine groups 1 and 2 ($P<0.05$), but compared to the streptozotocin group, a significant increase was seen in the streptozotocin+*N*-acetylcysteine groups 2 and 3 ($P<0.05$) (Figure 3).

4. Discussion

Diabetes has unfavorable consequences on the reproductive function in the male gametes. Testicular function and spermatogenesis are influenced in both type 1 and type 2 diabetic men. A dramatically higher percentage of sperm with nuclear and mitochondrial DNA fragmentation in observed in diabetic men and the nature of this damage is oxidative[21]. Previous studies have shown that streptozotocin can severely reduce insulin secretion and induce diabetes through damaging beta cells of the Langerhans[22]. In streptozotocin-induced diabetic models, the damage in the male reproductive system is associated with increased oxidative stress in testicular tissues[23]. Similar to our results, the induction of diabetes causes weight loss, sex organ weight loss, and a reduction in the number and motility of the sperm[24]. Also, reduced levels of serum testosterone in diabetic rats have been reported[25]. Moreover, inducing streptozotocin-diabetes decreased serum testosterone levels, the weight of testes, the diameter of testicular tubes and the total sperm count[24]. These findings are similar to the results of the present study.

Considering the conducted studies, glucose metabolism is highly important for sperm cells and either type 1 diabetes or type 2 diabetes could have detrimental effects on male fertility especially on sperm quality, such as sperm motility, sperm DNA integrity, and the

ingredients of seminal plasma[26]. During spermatogenesis, diabetes may influence the epigenetic modification and these epigenetic dysregulation may be inherited through the male germ line and it is passed into more than one generation, which in turn the risk of diabetes in the offspring may be increased[27].

It was found that changes in the expression of the genes involved in DNA repair and replication were highly associated with the increase in sperm DNA fragmentation in diabetic men. Oxidative stress, ROS, and sperm DNA fragmentation have been shown to increase by numerous factors. In diabetic men, mRNA profiles indicated expression perturbations in the genes involved in stress response, DNA metabolism, and replication/repair, particularly due to their association with oxidative stress that plays an important role in many diabetic complications. Diabetes is all accompanied by multiple changes in the sperm proteome[28].

It was indicated that diabetic men have decreased fertility, but the main mechanism is still unclear. Subfertility could be either due to local influences of the hyperglycemia on the testes resulting in disruptions in spermatogenesis, or due to hormonal alterations influencing the hypothalamic–pituitary–gonadal (HPG) axis and subsequent spermatogenesis. In a human study, it was attempted to examine the function of HPG axis in diabetic men by measuring LH and FSH secretion in response to gonadotropin-releasing hormone (GnRH)[29]. Some studies indicate that LH secretion in response to GnRH pulses was lower in diabetics than in healthy controls. LH secretion in response to GnRH administration was also represented to be impaired in diabetic men with reduced testosterone levels compared to the controls, while LH and FSH levels still remained similar. Disruptions in the HPG axis have severe reproductive consequences. However, there is the chance that not only all the diabetic outcomes on fertility are mediated through HPG axis, but also by the detrimental effects of hyperglycemia and oxidative DNA damage to the testes and sperm cells[29,30].

In fact, different hypotheses have suggested that condensation of the sperm nucleus into a compact hydrodynamic shape, protection of the genetic message delivered by the spermatozoa, involvement in the processes maintaining the integrity and the repair of DNA during or after the nucleohistone–nucleoprotamine transition and involvement in the epigenetic imprinting of the spermatozoa were associated with the infertility in men[31]. Considering altered mRNA levels as a potential origin of altered protein levels could be generally well justified. This issue could be even more important in this model because the protamine genes must be transcribed and stored in spermatocytes and round spermatids for later translation in elongating spermatids when transcription is no longer active. One of the hypotheses for the function of protamines is that they could be involved in the protection of the genetic message delivered by the spermatozoa. Incomplete protamination could render the spermatozoa which are more vulnerable to attack by endogenous or exogenous agents such as nucleases, free radicals or mutagens[31,32].

Sperm dysfunction due to lipid peroxidation, decreased semen quality and sperm DNA damage are the result of increased pathological ROS generation. So, scavenging excess ROS is essential for normal spermatogenesis and fertilization. Spermatozoa are susceptible to oxidative damage because their plasma membranes are rich in polyunsaturated fatty acids and they have low concentrations of scavenging enzymes that exhibit significantly greater production of seminal ROS[33].

N-acetylcysteine, a derivative of amino acid *L*-cysteine, is currently used mainly as an antioxidant. *N*-acetylcysteine has free radical scavenging activity both *in vivo* and *in vitro*. Furthermore, daily treatment with *N*-acetylcysteine results in a significant improvement in sperm motility as compared to the control. It was also found that *N*-acetylcysteine has improved sperm concentration and acrosome reaction and has reduced ROS and oxidation of sperm DNA. On the other hand, *N*-acetylcysteine, as a thiol-based antioxidant, has an important role in protecting cellular constituents against oxidative damage[10,34].

The hypothetical action of *N*-acetylcysteine results from its ability to stimulate and maintain intracellular levels of reduced glutathione and also to detoxify ROS. The influences of *N*-acetylcysteine on ROS and sperm motility could be mediated by a modulation of mitochondrial activity[19,35]. There are a few numbers of reports on the effects of dietary antioxidant supplementation on sperm DNA integrity. Most of these clinical studies have evaluated men with high levels of sperm DNA damage. Treating with antioxidant supplements is generally associated with reduced levels of sperm DNA damage and/or improved fertility potential[9].

Spermatogenesis is highly controlled by the hormonal environment of the testis and any alteration in the hormonal profile, and also by influencing the rate and quality of spermatogenesis that may highly influence chromosomal ploidy and integrity of sperm chromatin[1]. The increased levels of pre-treatment gonadotropin found in patients are likely related to a state of hypogonadism, causing aberration in the process of spermatogenesis, which improves with *N*-acetylcysteine supplementation and has been confirmed by the increase in serum testosterone levels and improved serum testosterone after *N*-acetylcysteine supplementation. Increased testosterone provides a negative feedback to the hypothalamus and pituitary, leading to reductions in both GnRH pulse frequency and pituitary responsiveness to GnRH, and ultimately resulting in reduced release of gonadotropin[36].

A repairing effect on varicocele-induced oxidative stress and damage to DNA in men testicles can be caused by *N*-acetylcysteine, Barekat *et al* reported they found that *N*-acetylcysteine reduced the level of protamine deficiency, DNA fragmentation and oxidative stress, which is consistent with the results of this study[37]. In another study by de Silva *et al*, it was found that *N*-acetylcysteine can modify

the adverse effects of arsenic trioxide on the germ plasm of male rats. They showed that arsenic trioxide has reduced testosterone levels as well as seminal secretion. It was suggested that ROS can damage Leydig cells, and ultimately reduce testosterone levels. Treatment with arsenic trioxide resulted in damages to spermatogenesis, sperm motility, semen-sac weight, and serum testosterone level, and when *N*-acetylcysteine was administered, the animals showed improvement in sperm parameters and semen-sac weight[38]. Rao *et al* found that *N*-acetylcysteine prevents the programmed cell death in germ cells induced by methoxy acetic acid in male rats[39]. Also, Jallouli *et al* showed that *N*-acetylcysteine prevents steroidogenesis, the intoxication of the reproductive system and oxidative stress induced by dimethoate[40].

All of these studies have shown the protective effects of *N*-acetylcysteine on the intoxication of the reproductive system, and are consistent with the results of the present study. Increasing testosterone and protamine expression levels after treatment with *N*-acetylcysteine indicates the ability of this drug to increase the levels of these parameters, and elevating testosterone and protamine expression levels has a direct impact on fertility in men.

In conclusion, the present study showed that *N*-acetylcysteine has some protective effects on pituitary-testis-axis hormones and changes in the protamine expression level in diabetic rats in an optimal dose of 400 mg/kg body weight, and exerts these effects by lowering oxidative stress, reducing ROS and moderating DNA damage. However, more research is needed to achieve a firm deduction.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

Maryam Hassanzadeh Moghadam, Mehrdad Shariati and Mohammad Amin Edalatmanesh cooperated in concept, design and data collection of the study. Sirous Naeimi and Mohammad Amin Edalatmanesh performed data analysis. Maryam Hassanzadeh Moghadam and Mehrdad Shariati wrote the manuscript. Mehrdad Shariati and Sirous Naeimi conducted the critical review. Mehrdad Shariati approved the final manuscript.

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