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Original Article

Investigating the effect of hydro-alcoholic extract of pomegranate seeds on sperm parameters and Testosterone level in male mice

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ABSTRACT

The most common cause of male infertility is men's inability to produce a sufficient number of normal sperms. The aim of this study was to investigate the effect of hydro-alcoholic extract of pomegranate seeds on testosterone level and some sperm parameters in male mice. Forty male mice were divided into 5 groups including control, control-sham and three group treated with doses of 125, 250 and 500 mg/kg/bw of hydro-alcoholic extract of pomegranate seeds. The extract was administered for 30 days by intra peritoneal injection. At the end of the 30th day of treatment period, serum testosterone levels were measured. The weight of the testes and parameters of the sperm count, the percentage of sperm viability, immature sperms and sperms with damaged DNA were measured. The increase in the weight of the testes, the number of sperms, the percentage of fertilization and the decrease in the percentage of sperms with DNA damage were significant only in the treatment group with a dose of 500 mg/kg /bw ($P<0.05$). In addition, the increase in the serum level of testosterone, the percentage of sperm viability and the decrease in the percentage of immature sperms in the treatment groups with the dose of 250 and 500 mg/kg/bw were significant ($P<0.05$). Antioxidant compounds and androgenic properties of hydro-alcoholic extract of pomegranate seeds improve the quality of sperm parameters in male mice.

اثر عصاره هیدروالکلی هسته انار بر شاخص های اسپرمی و تستوسترون موش سوری نر

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چکیده

شایع ترین علت ناباروری مردان ناتوانی در تولید تعداد کافی اسپرم طبیعی می باشد. هدف از این مطالعه بررسی اثر عصاره هیدروالکلی دانه انار بر سطح تستوسترون و برخی پارامترهای اسپرم در موش‌های سوری نر بود. عصاره هیدروالکلی دانه انار با دوزهای ۱۲۵، ۲۵۰ و ۵۰۰ میلیگرم در هر کیلوگرم وزن بدن به مدت ۳۰ روز با تزریق داخل صفاقی تجویز شد. در پایان دوره ۳۰ روزه درمان، سطح سرمی تستوسترون اندازه گیری شد. وزن بیضه ها و پارامترهای تعداد اسپرم، درصد زنده ماندن اسپرم ها، اسپرم های نابالغ و اسپرم های دارای DNA آسیب دیده اندازه گیری شد. افزایش وزن بیضه ها، تعداد اسپرم، درصد لقاح و کاهش درصد اسپرم های دارای آسیب DNA تنها در گروه تیمار با دوز ۵۰۰ میلی گرم بر کیلوگرم وزن بدن معنی دار بود ($P<۰/۰۵$). همچنین افزایش سطح سرمی تستوسترون، درصد زنده‌مانی اسپرم و کاهش درصد اسپرم‌های نابالغ در گروه‌های تیمار با دوز ۲۵۰ و ۵۰۰ میلی‌گرم بر کیلوگرم بر وزن بدن معنی‌دار بود ($P<۰/۰۵$). میتوان نتیجه گرفت که ترکیبات آنتی اکسیدانی و خواص آندروژنیک عصاره هیدروالکلی دانه انار میتواند باعث بهبود کیفیت پارامترهای اسپرم در موش های نر شود.

واژه های کلیدی: هسته انار، اسپرم، موش سوری، آنتی اکسیدان، تستوسترون

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INTRODUCTION

Infertility is one of the important issues in modern life. According to the available statistics, 50% of infertility cases are related to men. Various factors are effective in male infertility. The most important factor is the quality of the sperms. The factors that reduce the quality of sperms by increasing free radicals include the use of antibiotics, toxic substances, stress, insufficient intake of vitamins etc. [1, 2]. Today, natural products are being considered by researchers as an alternative source of pharmaceutical compounds. The use and investigation of medicinal fruits and plants as therapeutic and preventive agents against diseases is increasing due to their low adverse effects, cheapness and safety. That is why some drugs such as digoxin, quinine and vinca alkaloids have been formulated with the help of herbal medicine [3, 4, 5].

Pomegranate (*Punica granatum L.*), from the *Punicaceae* family, has traditionally been used as a medicinal fruit for thousands of years [6]. In traditional Chinese medicine, pomegranate is a symbol of fertility, generation, abundance and numerous children. Reducing fever, treating diabetes, anti-diarrhea, blood tonic, stopping bleeding and wound healing are other traditional uses of pomegranate. Pomegranate seeds contain gamma-linoleic acid, oleic acid and linoleic acid, alpha-linoleic, ponicic, eicosadinoic, palmitoleic fatty acids [7, 8]. Pomegranate seeds also contain tocopherol (5.3-12.0 μmol/g). Since the plasma membrane of mammalian sperm contains a large amount of polyunsaturated fatty acids (PUFA), mammalian sperms are very sensitive to oxidative stress. Also, the lipid composition of the sperm membrane plays a major role in the physiological changes that lead to fertilization [9].

Numerous studies reveal that oxidative stress weakens sperm function such as sperm motility and plasma membrane integrity [10]. On the other hand, it has been found that reactive oxygen species have harmful effects on sperms [11]. The bioenergetics function of sperm mitochondria plays an important role in the formation of reactive oxygen species (ROS), which are particularly important in sperm for capacitation and acrosome reaction [12]. However, ROS fluctuation antioxidant defenses, cause damage in sperm membrane, acrosome and DNA [13, 14]. Pomegranate is effective in cancer treatment [15] and known as an anti-inflammatory fruit. Pomegranate seed oil improves glucose metabolism [16, 17, 18], and has cardio protective effects in rabbits [19]. Pomegranate seed oil contains approximately 80% conjugated alpha-linolenic acid (CLnA), abundant amounts of the cis-9, trans-11, cis 13 C18:3 isomer of ponicic acid, which is a specific fatty acid for this oil. Pomegranate seeds are also rich in polyphenol compounds that have strong antioxidant and antimicrobial characteristic [20]. Feeding with CLnA sources can affect sperm fatty acid composition and improve sperm quality in mammals [21]. Previous reports show that CLnA feeding can positively affect sperm characteristics that are directly related to male fertility. Also, pomegranate seeds are rich in sugar, vitamins, polysaccharides, polyphenols and minerals [22]. Pomegranate seed oil contains high levels of phenolic compounds, including punicic acid, punicalagin, as well as important fatty acids such as gallic acid, and ellagic acid [23]. Ellagic acid is a polyphenolic compound with antioxidant and ant proliferative properties that is also present in many other fruits and plants such as raspberries, walnuts and strawberries, by inhibiting the expression of inflammatory enzymes, it shows anti-inflammatory and

antioxidant effects [24]. Therefore, in the present study, the effect of three different doses of hydro-alcoholic extract of pomegranate seeds on changes in sperm parameters and testosterone level in male mice were investigated.

MATERIALS AND METHODS

Preparation of hydro-alcoholic extract of pomegranate seeds

First the fruit seeds were separated and washed thoroughly. Then the seeds were dried and powdered by a mill. 10 g of the powder were soaked in 100 mL of alcohol and water solution (with a ratio of 70:30) and then transferred to the percolator for 48 hr. The product was shaken and filtered. The obtained liquid was concentrated by vacuum to reach 1/12 volume. The extract was transferred to –20 °C freezer and it was diluted with distilled water and used during administration [25]. Forty adult male mice (8 weeks old) weighing 30 ± 3.5 g were purchased from the animal house of Urmia University. The mice were randomly divided into five groups (n=8): 1) Control group: no intervention was done in mice; 2) Sham control group: received 0.2 mL of saline normal (I.P) for 30 days; 3) The group treated with low-dose (125) hydro-alcoholic extract of pomegranate seeds: received hydro-alcoholic extract of pomegranate seeds at a dose of 125 mg /kg/bw (I.P) for 30 days; 4) The group treated with medium (250) hydro-alcoholic extract of pomegranate seeds: received hydro-alcoholic extract of pomegranate seeds at a dose of 250 mg /kg/bw (I.P) for 30 days; 5) The group treated with high dose (500) hydro-alcoholic extract of pomegranate seeds: received pomegranate seed hydro-alcoholic extract at a dose of 500 mg /kg/bw (I.P) for 30 days.

Sampling was done 30 days after treatment. On the 30th day, the mice were anesthetized with ketamine and xylazine [26], and blood was collected from the left ventricle of the heart and the samples were transferred to the test tubes. They were centrifuged for 5 minutes at a speed of 3500 revolutions per minute. Testosterone hormone level was measured by ELISA method.

Sperm collection method

The mice were euthanized by cervical dislocation. The abdominal cavity was opened and the tail of the epididymis was transferred to 1 mL human tubal fluid (HTF) medium in 5% Co2 incubator at 37 °C for 30 minutes. When the sperms of the tail of the epididymis entered into culture medium, the tail of the epididymis was removed from the culture medium and the sperms in the culture medium were evaluated. During this time, the testees were also removed from the abdominal cavity and (after separating the surrounding tissues) they were weighed using a scale with an accuracy of 0.001 g [27].

Evaluation of total sperm count

For this purpose, a 1:20 dilution of sperms (5 mL of sperm in the culture medium with 95 mL of diluent) was prepared. Then 10 mL of this dilution was completely mixed and transferred to the hemocytometer slide. Using the following formula, the number of sperm per milliliter of the culture medium was calculated. The number of sperm in 1 mL of the culture medium = 50000 × dilution factor× the number of sperm per 5 hemocytometer square [28].

Evaluation of the sperm viability

Sperm viability was examined using eosin-nigrosin staining. Sperms that were unstained were classified as live and those that showed red coloration were classified as dead [29].

Evaluation of the percentage of immature sperms

Aniline blue staining was used to determine mature and immature sperms. The smears were dried at room temperature and fixed by 3% glutaraldehyde solution (30 minutes). Then the smears were stained with 5% aniline blue (using 4% acetic acid for 5-8 minutes). Mature sperms with a colorless head and immature sperm with a blue head were visible under the light microscope [30].

Evaluation of DNA damage in sperms
Acridine orange staining was used to differentiate between sperms with healthy DNA and damaged DNA. Sperms with healthy DNA are visible under the fluorescent microscope in green color and sperms with damaged DNA are visible in orange to red color. For this purpose, smears were fixed with alcohol methanol, then the slides were stained with acridine orange [31].

Statistical analysis

The data were analyzed with SPSS software (version 21; IBM Corp., Armonk, USA) and one-way ANOVA test, by Tukey's post hoc test. The results of the study were presented as mean ± standard deviation and a $p < 0.05$ was considered significant.

RESULTS

In all treatment groups, an increase in blood testosterone levels was observed. Blood testosterone level in the medium and high dose treatment groups was significantly higher than the control and control-sham groups ($p<0.05$) (Table 1). In the medium and high dose treatment groups, an increase in the weight of the testes was observed. Weight of the testes in the high dose group was significantly higher than the control and control-sham groups ($p<0.05$) (Table 1). An increase in the sperm count in all three treatment groups was observed. Sperm count in the high dose treatment group was significantly higher than the other ($p<0.05$) (Table 1). The percentage of live sperms in the medium and high dose treatment group was increased. The rise in the medium and high dose treatment group is significantly higher than other groups ($p<0.05$) (Fig. 1 and Table 1). Evaluation of the percentage of immature sperms shows that the percentage of immature sperms has decreased in all treatment groups whereas in the high and medium dose treatment groups, this decrease was significant in contrast with the control and control-sham groups ($p<0.05$) (Table 1). In addition, the decrease in the percentage of immature sperm in the high dose treatment group was significant in contrast with medium dose group ($p<0.05$) (Fig. 2 and Table 1). A decrease in the percentage of sperms with damaged DNA was observed in the medium and high dose treatment groups. Only the high dose treatment group was significantly lower than the control and control-sham groups ($p<0.05$) (Table 1).

Table 1. Comparison of the average sperm count per ml of culture medium ($\times 10^6$), the percentage of sperm viability, immature sperm and sperm with damaged DNA in different groups (Mean ± SD).

	Group				
Parameter	Control	Sham-control	Low dose pomegranate seed treatment (125 mg/kg/day)	Medium dose pomegranate seed treatment (250 mg/kg/day)	High dose pomegranate seed treatment (500 mg/kg/day)
Testosterone level (nmol/l)	6.35±0.26^{a}	6.50±0.68^{a}	7.25±1.04^{a}	8.49±0.57^{b}	8.68±0.30^{b}
Weight of testes (mg)	115±4.09^{a}	113±4.48^{a}	114±3.88^{a}	119±4.07^{a}	129±2.95^{b}
Total number of sperms ($\times 10^6$)	52.35±4.11^{a}	51.05±2.56^{a}	55.31± 3.45^{a}	57.55±4.02^{a}	64.67±2.17^{b}
Percentage of live sperm	81.24± 2.31^{a}	82.30± 3.11^{a}	81.94±3.22^{a}	89.32±2.41^{b}	91.12±4.41^{b}
The percentage of immature sperm (%)	6.31± 0.36^{a}	6.12±0.17^{a}	5.57±0.61^{a}	4.32±0.23^{b}	3.87±0.18^{bc}
Percentage of sperms with damaged DNA (%)	11.04±0.97^{a}	10.81±0.53^{a}	9.94± 0.85^{a}	7.32±0.89^{b}	6.64±0.73^{bc}

Note: Different superscript letters ($^{a, b,\cdots}$) in each row indicates significant differences between the groups.

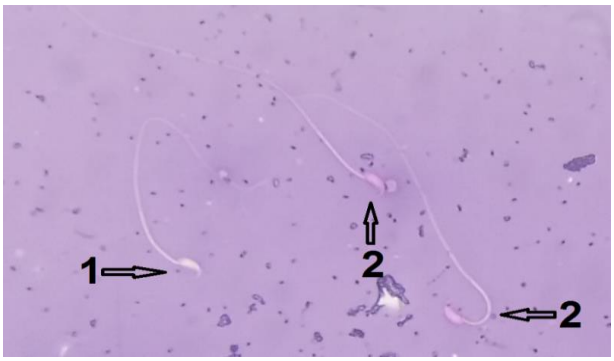

Figure 1: Live sperm with light head (1) and two dead sperm with red head (2) can be seen in the control group (Eosin-Nigrosine staining, 400x).

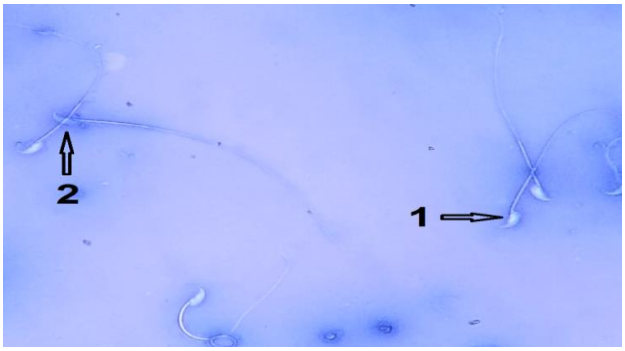

Figure 2: Mature sperms with light heads (1) and immature sperms with blue heads (2) can be seen in the pomegranate seed treatment group with a dose of 500 mg/kg/bw (aniline blue staining; 400x).

DISCUSSION

Our study showed that hydro-alcoholic extract of pomegranate seeds increased the serum concentration of testosterone. Testosterone is produced in the Leydig cells of the testes by the stimulation of LH hormone secreted from the pituitary gland. Probably the mechanism by which pomegranate seeds increase the testosterone hormone is through the effect on the pituitary gland and the increase of LH, or

because of its direct effect on the testicular tissue and the stimulation of testosterone secretion. [32] Considering that testosterone inhibits the release of LH hormone from the pituitary gland thus it is possible that pomegranate seeds increase testosterone directly and by affecting the testicular tissue [33]. Previous studies have shown that flavonoid compounds and fatty acids are effective on testosterone secretion by inhibiting aromatase enzyme activity. Inhibiting the activity of this enzyme causes an increase in androgens (testosterone and di hydro-testosterone) in the blood [34]. Pomegranate seeds, having fatty acid compounds, will probably be able to stimulate testosterone secretion in this way. Also, pomegranate seeds caused a significant increase in the weight of the testes of the high-dose treatment group. Studies show that plants containing flavonoid compounds increase the weight of reproductive organs and sperm quality [35, 36]. The weight of the testes is also influenced by testosterone [37, 38]. Therefore, due to the fact that pomegranate seeds have been able to increase the level of testosterone, it is possible that the weight of the testes increased with the same mechanism. Pomegranate seeds have a high content of conjugated alpha-linolenic acid (CLn), punicic acid (PA) and conjugated isomer of alpha-linoleic acid and have antioxidant, anti-inflammatory, kidney protection, liver protection, nerve protection, anticancer properties. They improve the body's immune system, increase the metabolism of carbohydrates and reduce insulin resistance [39]. Considering that oxidants and antioxidants have attracted attention in nutrition, biology and medicine research, production of pro-oxidants, including oxygen free radicals, has been found to be an essential feature of aerobic metabolism [40]. Disturbance in the pro-oxidant/antioxidant system is defined as oxidative stress. ROS are highly reactive molecules classified as free radicals due to the presence of an unpaired electron, such as superoxide ion (O─2), nitric oxide (NO), and hydroxyl radical (HO─). They are mainly confined in cellular compartments and are balanced by natural antioxidant molecules such as glutathione, glutathione peroxidase, superoxide dismutase, vitamin E, and vitamin C, which act as free radical scavengers [41, 42].

Mukherjee et al showed that Panic acid has the highest antioxidant activity at a concentration of 0.6% and reduces the peroxidation of unsaturated fatty acids in lipids and the formation of free radicals [43]. On the other hand, double bonds of panic acid and conjugated alpha-linoleic acid in pomegranate seeds can inhibit free radicals and increase the level of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). The antioxidant activity of pomegranate seeds is also related to tocopherols and the content of its polyphenolic compounds, and these factors that reduce oxidative stress in pomegranate seeds have the ability to inhibit singlet oxygen and release hydrogen [44, 45]. Cellular damage in semen is the result of an improper balance between the production and scavenging of ROS. When ROS production exceeds critical level, it can affect all antioxidant defense strategies of sperm and seminal plasma and cause oxidative stress [46, 47]. Therefore, ROS production and total antioxidant capacity (TAC) can be used as indicators of oxidative stress in semen which are related to male infertility. Infertile men show significantly lower ROS and TAC scores than the fertile men [48]. In the present study, the number of sperms in the high dose group of pomegranate seed and the percentage of live sperms, the percentage of mature sperms in the high and medium dose treatment

groups were increased in comparison with the control and sham control groups. These results were in line with previous findings, which reported that the maturation characteristics and chromatin health of sperms are affected by the oxidative status of seminal fluid [49]. Given the fact that pomegranate seeds contain high levels of fatty acids and phenolic compounds [22, 23], Pomegranate seeds can have anti-inflammatory effects by inhibiting the expression of inflammatory enzymes and oxidant elements [24]. Furthermore, Bouroshak's studies reveal anti-cancer and antioxidant effects for pomegranate seeds [50]. Also, flavonoid compounds and fatty acids increase testosterone secretion by inhibiting aromatase enzyme activity [7]. Probably these compounds of Pomegranate seeds are able to stimulate the secretion of testosterone. In agreement with these reports, the present study also showed an increase in serum testosterone level, testes weight, sperm count and health.

CONCLUSION

The present study has shown that the hydro-alcoholic extract of pomegranate seeds has positive effects on spermatogenesis and sperm parameters in male mice probably due to its antioxidant and androgenic activity in doses of 250 and 500 mg/kg/bw.

ETHICS

All ethical standards have been respected in this study.

CONFLICT OF INTEREST

None declared.

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