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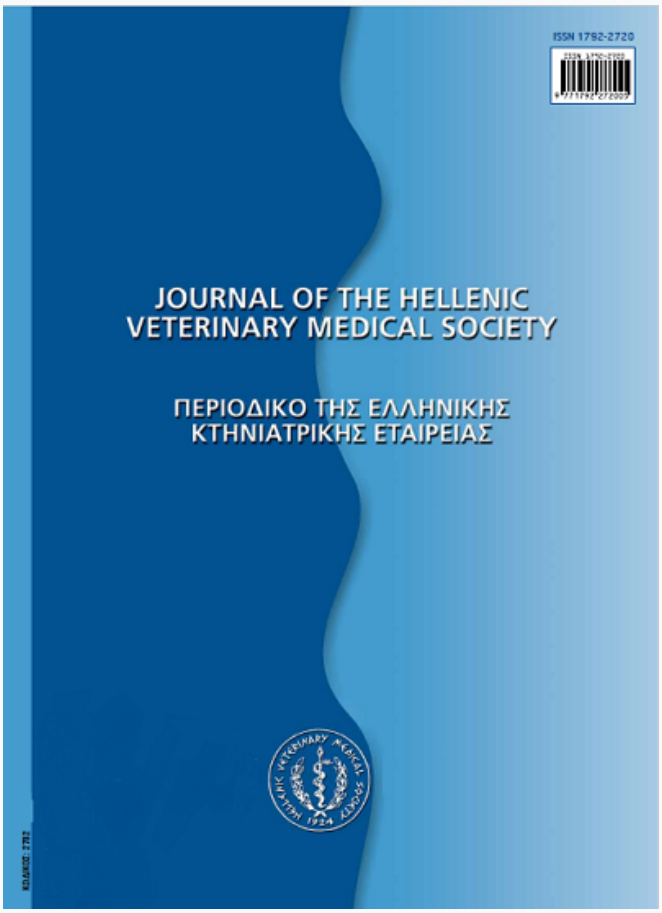

The Effect of Lyophilized Pomegranate Extract on Epididymal Sperm Quality, Oxidative Stress and Spermatogenic Cell Density in Rabbits

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The Effect of Lyophilized Pomegranate Extract on Epididymal Sperm Quality, Oxidative Stress and Spermatogenic Cell Density in Rabbits

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ABSTRACT. The aim of this study was to investigate the effect of lyophilized pomegranate extract on spermatological features, pathology of testes and total antioxidant/oxidant status in rabbits. Adult male rabbits were divided into four groups containing six rabbits each. For 8 weeks, Group I received standard diet and 1 ml % 0.5 carboxymethyl cellulose (CMC), Group II received 25 mg/kg/day pomegranate extract + 1 ml % 0.5 carboxymethyl cellulose (CMC), Group III received 50 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC and Group IV received 100 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC. Rabbits were sacrificed by using xylazine 5 mg/kg+ketamine 35 mg/kg anaesthesia and were euthanized by 150 mg / kg intraperitoneal thiopental sodium at the end of the eighth week. Spermatozoon motility, abnormal sperm rate, sperm membrane integrity, total antioxidant/oxidant level and spermatogenic cell density were investigated. All analyses were done only once at the end of study period. Data were compared by analysis of variance (ANOVA) and the degree of significance was set at ($p<0.05$). Sperm motility and membrane integrity increased significantly ($p<0.05$) in groups II, III and IV; abnormal sperm rate decreased significantly ($p<0.05$) in groups III, IV; total oxidant status decreased significantly ($p<0.05$) in group IV in comparison to the control group. Seminiferous tubule diameter increased significantly ($p<0.05$) in all groups compared to the control group. Germinal cell layer thickness significantly increased ($p<0.05$) in group IV compared to the control group. Results of this study suggest that 50 mg/kg/day + 1 ml % 0.5 CMC and 100 mg/kg/day + 1 ml % 0.5 CMC improve sperm parameters in rabbits.

Keywords: Oxidative stress, Pomegranate extract, Rabbit, Sperm characteristics, Spermatogenic cell density

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INTRODUCTION

As a member of the Punicaceae family, pomegranate has been known since the ancient era. It is emphasized in religious scripture as the symbol of abundance, productivity and chance, while its Latin name is Punica granatum which is the derived from Pomum (apple) and Granatus (seedy). Pomegranate (Punica granatum) is used for protection against diseases as folk medicine in the Middle East (Gurib-Fakim, 2006), and 52% of total mass of pomegranate is edible, of which 78% juice and 22% is seed (Türk et al., 2008a).

Pomegranate has rich content comprising phenolic compounds and flavonoids (composition of anthocyanins, catechins and other flavonoids) and tannin (punicic acid, punicalagin, gallic acid, ellagic acid) (Afaq et al., 2005). Pomegranate seeds are rich in multiple unsaturated fatty acids, vitamins, minerals, polyphenols and polysaccharides. Eighty percent of pomegranate seed oil consists of punicic acid, phytoestrogens and estrone. Moreover, there are great amounts of polyphenols in pomegranate juice (Yılmaz, 2010, Teixeira et al., 2013).

In comparison to other parts of the fruit, pomegranate peel has higher antimicrobial properties (Tehranifar et al., 2011). Polyphenols, anthocyanins mines and ellagitan are the main antioxidants in its juice (Gil et al., 2000). Ninety two percent of antioxidants in pomegranate are ellagitannins, which are concentrated in the peel. Punicalagin is a basic ellagitannin which decomposes into ellagic acid and other small polyphenols in vivo (Seeram et al., 2004). Pomegranate's capacity of iron reduction and activity of free radical-scavenging is three times higher than those in usage of red wine and green tea for antioxidant purposes (Fuhrman et al., 2000). Furthermore, pomegranate content of antioxidant is higher than those of orange juice, blueberry, grape, and grapefruit (Seeram et al., 2006).

Pomegranate has recently become popular in usage for prevention of cancer (Afaq et al., 2005, Lansky et al., 2005)the search for novel agent(s, antiproliferative and apoptotic purposes (Seeram et al., 2005), and as an inhibitor of HIV-I and a microbicide (Neurath et al., 2005). Moreover, many studies have reported that pomegranate and its by-products have a strong activity of free radical scavenging, as well as effective antioxidant properties (De Nigris et al., 2005, Rosenblat et al., 2006). Reactive oxygen species (ROS) are highly oxidized reactive free radical compounds. ROS production in organs like the testes is a commonplace physiological event, whereas increased synthesis of ROS brings about DNA damage in cells and oxidation (Sikka, 1996). Antioxidants usually fight against lipid peroxidation and formation of ROS, minimize DNA damage and cellular lysis, as well as repairing sperm functions (Garcia-Perez et al., 2009).

Extract of pomegranate has been demonstrated to inhibit cancer cell growth in mice with prostate cancer, as well as inducing apoptosis in such cells (Malik and Mukhtar 2006). Singh et al. (2002) reported using model systems of DPPH and β-carotene-linoleate that methanol extract in pomegranate seeds had much lower antioxidant power than the peel. According to Tzulker et al. (2007) the homogenates taken from the entire fruit carried about 20 times higher antioxidant activity than shown in aril juices. Ahmed et al. (2014) reported that pomegranate extract showed natural protective instrument behavior in brain injury induced by ischemia/reperfusion in rats due to its effects as an anti-inflammatory, antioxidant, anti-apoptotic and ATP replenishing agent. In this study, pomegranate extract, an effective antioxidant, was orally administered to rabbits for 8 weeks, and the effects of spermatological parameters, oxidative stress parameters, and germ cell density were analyzed.

MATERIALS AND METHODS

Pomegranate Extract and Chemicals

The extract was prepared in Gazi University Faculty of Pharmacy, Department of Pharmacognosy. The fresh fruit was squeezed using a fruit processor. The resulting pomegranate juice was frozen at -80 oC and powdered by drying in a lyophilizer. The extract was given to experimental animals in three different doses. All chemicals supplied the Sigma-Aldrich Corporation (St. Louis, MO, USA).

Animals and the Design of the Experiment

This study used 2-4 kg on average, five-month-old twenty-four healthy adult male New Zealand White (NZW) rabbit bucks. Steps of the experiments were agreed by Afyon Kocatepe University's Committee of Animal Care and Use (2015-49533702/53) and they

complied with the codes of laboratory animal usage and care published by the National Institute of Health. The rabbits were held in galvanized wire cages separately with a light cycle of 12/12 day/night, temperature in the range of 18 to 25˚C and they were provided free access to ad-libitum feeding and fresh water. Four groups containing 6 rabbits each were randomly formed. Group I was given a standard diet and 1 ml % 0.5 carboxymethyl cellulose (CMC), Group II was fed with 25 mg/kg/day pomegranate extract + 1 ml % 0.5 carboxymethyl cellulose (CMC), Group III received 50 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC and Group IV was provided 100 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC. This period of administration (8 weeks) was required to reveal the effects of pomegranate extract on production of sperm as rabbits require a period of 40 to 50 days for the completion of spermatogenic cycle together with spermiogenesis, meiosis, as well as spermatocytogenesis.

Collection of the Sample

The rabbits were put down with 35 mg ketamine and 5 mg xylazine per kg for anesthesia and 150 mg/kg for euthanasia at the end of the period of treatment. For histological analyses, the first testis tissue was fixed in Bouin's solution, followed by the other testis tissue stored at -20 ºC until biochemical evaluations for each rabbit.

Evaluation of Epididymal Sperm Parameters

The percentage of forward progressive sperm motility was evaluated using a phase contrast microscope with heated stage as described by Sönmez et al (2005). For this process, a slide was placed on a phase contrast microscope with a heated stage warmed up to 37 ºC and then several droplets of Tris buffer solution [0.3 m Tris (hydroxymethyl) aminomethane, 0.027 m glucose, 0.1 m citric acid] were dropped on the slide, a very small droplet of fluid obtained from left cauda epididymis with a pipette was added to the Tris buffer solution and mixed by a cover-slip. The percentage of forward progressive sperm motility was evaluated visually at 200 x and 400 x magnification. Motility estimations were performed by three different fields in each sample. The mean of three successive estimations was used as the final motility score.

The osmotic resistance was assessed by modified cumulative analysis of hypoosmotic eosin staining test (HE-test), using the eosin exclusion test and the Hypoosmotic Swelling Test (HOST) (Ducci et al., 2002, Fukui et al., 2004, Mansour, 2009). Semen samples were diluted 1:10 (v/v) in 100 mOsm fructose solution including 1% (w/v) eosin-Y, and were incubated in a water bath at 35ºC for 30 minutes. The sperm suspension smears were prepared by 10 µL of mixed samples, and 100 spermatozoa were observed in each slide under a phase contrast microscope at 400× magnification and they were classified into four types (Type I: tail swollen and head white, HOS+/E-; Type II: tail non-swollen and head white, HOS-/E-; Type III: tail swollen and head red, HOS+/E+; Type IV: tail non-swollen and head red, HOS-/E+) according to staining status of sperm head and curling of the sperm tail in smears.

Morphologically abnormal acrosomes were estimated on a wet mount slide using 2 - 3 semen drops thinned in Hancock's solution (Hancock, 1952) using a phase contrast microscope (Olympus CX 31, Olympus Optical Co., Ltd., Japan) and spermatozoa ratios were recorded.

Biochemical Studies

Measurement of Total Antioxidant Status in Epididymal Rabbit Sperm

Seminal plasma was separated from ejaculates by centrifugation at 5000 rpm for 10 min. The supernatants were transferred into Eppendorf tubes, recentrifuged 5000 rpm 10 min. to eliminate the remaining cells and the supernatant was stored at -20 ºC before being assayed. The status of the serum total antioxidant capacity (TAC) was determined using a new colorimetric measurement method which is automated (Erel, 2004). This technique was founded on the decolorizing of color characteristics of a ABTS (2,2'-azino-bis[3-ethylbenzothiazo-line-6-sulfonic acid]) radical cation with higher stability by antioxidants. The assay has exceptional values of accuracy by less than 3%. The method's results were stated in units of mmol Trolox equivalent/L.

Measurement of Total Oxidant Status in Epididymal Rabbit Sperm

Seminal plasma was obtained and treated as it is

above mentioned. The status of the serum total oxidant capacity (TOC) was determined by a colorimetric measurement method which is automated (Erel, 2005). In the usage of this technique, the ferrous ion-o-dianisidine complex was oxidized into ferric ion by oxidants in the sample. The reaction of oxidation was improved by glycerol molecules, which are richly existing in the medium of reaction. The ferric ion and xylenol orange gave rise to a colored complex in the acidic medium. The spectrophotometrically measurable color intensity was in relation with oxidant molecules' total amount in the sample. Hydrogen peroxide was used for the calibration of the assay and the results were stated in units of micromolar hydrogen peroxide equivalent per liter (mmol H2O2 Equivalent/L).

Histologic Examination

For determination of changes in spermatogenic cell density, Bouin's solution was used to fix testis tissues for 48 h, sorted ethanol concentrations were used for dehydration, the tissues were fixed in paraffin wax, cut at 5 mm thicknesses and stained using Mayer's hematoxylin and eosin (H&E). For each section, ten seminiferous tubules (ST) were examined randomly and their thickness of germinal cell layer (starting from the lumen of the tubule to the basal membrane) and diameters were determined by an ocular micrometer under a light microscope, followed by the calculation of the mean ST size and germinal cell layer thickness.

Statistical Analysis

The data are demonstrated as mean and SEM (standard error of means). The significant level was chosen as $p < 0.05$. Tests of post hoc Tukey-HSD and one-way ANOVA (analysis of variance) were used to calculate the variances amongst the groups by all characteristics of sperm, biochemical parameters, as well as histological findings. The SPSS (Version 10.0; SPSS/PC, Chicago, IL) package software was used to carry out all the analyses.

RESULTS

Epididymal Sperm Characteristics

Table 1 presents the outcomes of different dosages of pomegranate extract on epididymal sperm motility, abnormal sperm rate, acrosome reaction rate and the hypoosmotic eosin staining test (HE-test). Group II, III and IV dosages of pomegranate extract increased sperm motility significantly ($p < 0.05$), while pome-

Table 1. Mean (± S.E.) spermatological parameters in epididymal rabbit sperm.

Groups	Motility (%)	Abnormal Sperm Rate (%)	Acrosome Rate (%)	H+/E- (%)	H-/E- (%)	H+/E+ (%)	H-/E+ (%)
Control	70±3.65^{b}	26.5±4.61^{a}	28.6±3.62^{ab}	64.2±2.12^{b}	30.3±2.56^{a}	2.3±0.49^{a}	3.2±0.79^{a}
SD+25 mg/kg/day	84±2.44^{a}	23.1±3.08^{ab}	33.1±4.29^{a}	72.6±1.24^{a}	25.2±1.62^{a}	0.6±0.40^{b}	1.6±0.24^{b}
SD+50 mg/kg/day	86±2.23^{a}	16.7±2.11^{b}	25.1±1.15^{ab}	72.8±2.94^{a}	25.3±2.77^{a}	1.0±0.25^{b}	0.8±0.47^{b}
SD+100 mg/kg/day	84±3.26^{a}	15.4±1.84^{b}	23.2±2.35^{b}	71.5±1.60^{a}	27.5±1.83^{a}	0.7±0.33^{b}	0.2±0.16^{b}

Different superscripts (a and b) within the same column showed significant differences among the groups. ($p < 0.05$)
HOS+/E-: tail swollen and head white; HOS-/E-: tail non-swollen and head white; HOS+/E+: tail swollen and head red; HOS-/E+: tail non-swollen and head red.
SD: Standard Diet

granate extract in both group III and IV decreased the ratio of abnormal sperms significantly in contrast to the control group. In contrast to the control group, all doses of pomegranate extract provided significant growth in the H+/E-($p < 0.05$) and significant decreases in H+/E+ and H-/E+ ($p < 0.05$).

Total Antioxidant and Oxidant Status Level

Table 2 shows the outcomes provided by pomegranate extract in different doses on epididymal sperm total antioxidant and oxidant status level. The total oxidant status level in the rabbits treated with group IV decreased in comparison to all other groups ($p < 0.05$). Degrees of total antioxidant status for the rabbits treated with all doses of pomegranate extract were like those of the control group.

Spermatogenic Cell Density

Table 3 demonstrates the influences of different dosages of pomegranate extract on epididymal sperm seminiferous tubule diameter and germinal cell layer thickness. In comparison to the control group, all doses provided significant growth in seminiferous tubule diameter and only the dosage of 100 mg/kg pomegranate extract per day increased the thickness of germinal cell layer significantly ($p < 0.05$) (Fig. 1).

DISCUSSION

In this study, daily pomegranate extract consumption for 8 weeks in different doses brought about increase in sperm motility, functional membrane integrity, seminiferous tubule diameter and germinal cell layer thickness. On the other hand, it decreased the abnormal sperm ratio and total oxidation level in NZW rabbits.

Various inquiries have revealed that oxidative stress occurring in the seminal fluid, that is, reactive oxygen species (ROS) in excess over antioxidant levels, reduces quality of sperm in two ways: 1) Some ROS acting as free radicals harm the cell membrane of sperms by lowering motility of sperm, 2) the sperm DNA may be damaged by the free radicals (Tremellen, 2008). The balance between the male reproductive tract's antioxidant defense system and ROS production is disturbed by oxidative stress, which causes defective sperm function to rise (Aitken and Sawyer, 2003). As there is an abundant amount of polyphenols in the fruit in P.

Table 2. Mean (± S.E.) total antioxidant/oxidant status in epididymal rabbit sperm

Groups	TAS (mmolTrolox Equiv./L)	TOS (μmol H2O2 Equiv./L)
Control	0.8±0.09[a]	56.4±2.97[a]
SD+25 mg/kg/day	1.02±0.06[a]	48.5±3.32[a]
SD+50 mg/kg/day	1.06±0.07[a]	48.7±2.72[a]
SD+100 mg/kg/day	0.86±0.11[a]	34.4±2.12[b]

Different superscripts (a and b) within the same column showed significant differences among the groups. ($p< 0.05$)
TAS: Total antioxidant status TOS: Total oxidant status SD: Standard Diet

Table 3. Mean (± S.E.) spermatological cell density in rabbit testis tissue

Groups	Diameter of ST (μm)	GCLT(μm)
Control	152.70±4.43[b]	33,20±1,03[b]
SD+25 mg/kg/day	182.80±6.80[a]	34,26±0,91[b]
SD+50 mg/kg/day	184.50±5.65[a]	35,34±0,53[b]
SD+100 mg/kg/day	191.90±7.31[a]	41,04±1,50[a]

Different superscripts (a and b) within the same column showed significant differences among the groups. ($p< 0.05$)
STs: seminiferous tubules GCLT: Germinal cell layer thickness SD: Standard Diet

Granatum which can act directly or indirectly as antioxidants based on their chemical structure, it expresses antioxidant activity. As the concentrations in the system are not high enough to have any direct and significant antioxidant outcome in vivo, the main concern is on the availability of polyphenols in consideration of their direct antioxidant effects. Hence, the inhibitive influence of polyphenols against, for instance, oxidative stress possibly comes from their ability to trigger the system of endogenous antioxidant defense; so, they work indirectly as antioxidants. By phase-2 enzymes, ortho-phenol groups containing polyphenols present in the fruit of pomegranate may be metabolized into electrophilic orthoquinones, which are crucial for inducing the system of endogenous antioxidant defense (Dinkova-Kostova and Talalay, 2008). Antioxidant

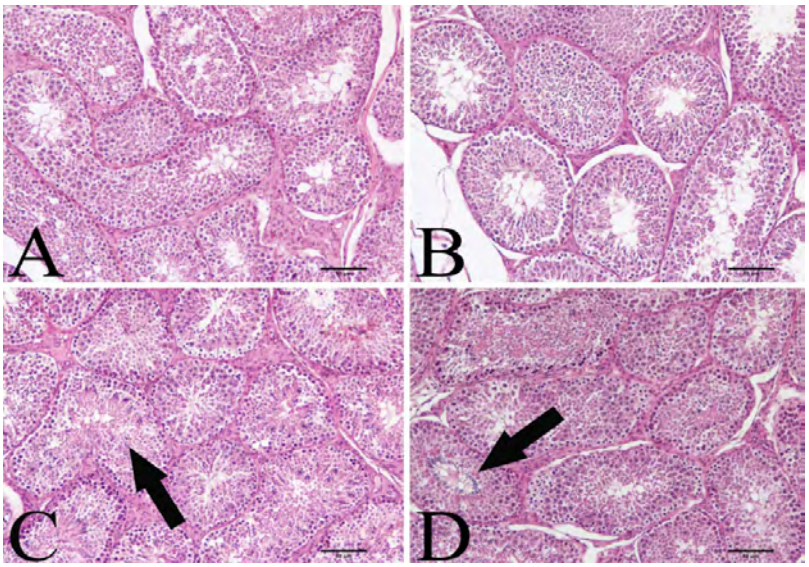

Fig 1. The effect of pomegranate extract in testis of rabbits. Representative figures were stained with Mayer's hematoxylin and eosin (H&E). A: Control group and 1 ml % 0.5 carboxymethyl cellulose (CMC) B: animals treated with 25 mg/kg/day pomegranate extract + 1 ml % 0.5 carboxymethyl cellulose (CMC) C: animals treated with 50 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC D: animals treated with 100 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC.

characteristics of the most widespread among P. granatum's polyphenols are accordingly considered to be led by their indirect antioxidant functions. Ellagic acid, which is a potent scavenger of free radicals (O2 − and OH), inhibits oxidative damage on DNA (Türk et al., 2008b). It is thought that pomegranate has higher antioxidant activity than most other plant species (Thring et al., 2009). Pomegranate's antioxidant activity and ascorbic acid's ROS-quenching activity are close to each other. Although it is widely accepted that antioxidant mechanisms in biological matrices are highly complicated and several different factors may take a role, the functioning mechanism put in motion by antioxidant activity of these compounds has not yet been understood completely (Çam et al., 2009). According to Madrigal-Carballo et al. (2009), as phenolic hydroxyl groups rapidly provide hydrogen to reduction agents, polyphenolic molecules in pomegranate sustain redox reactions. Hussen and Arrack (2014) reported pomegranate peel extract to be effective in protection of rabbits against carbon tetrachloride (CCl4) toxicity by their antioxidant activity. In our study, improvements observed in total oxidant status may be explained by deterrence of excessive creation of free radicals formed by spermatozoa, with the help of the pomegranate extract's antioxidant properties.

Pomegranate juice consumption caused rise in sperm motility, functional membrane integrity, width of seminiferous tubule and thickness of germinal cell layer. On the other hand, it decreased the abnormal sperm ratio and total oxidation level in comparison to the control group. This finding agrees with those of many scholars. According to the study by Türk et al. (2008b), consumption of pomegranate juice in rats caused an increase in epididymal motility of sperm, seminiferous tubule diameter and thickness of germinal cell layer, while it reduced the rate of abnormal sperms in when compared with the control group. Similarly, Türk et al. (2010) suggested that ellagic acid has a defensive influence against testicular and spermatozoal toxicity brought about by cyclosporine A. This protecting effect of ellagic acid appears to be somehow related to curtailment of oxidative stress. Hence, after tissue transplantation and in autoimmune diseases, ellagic acid may be used in association with cyclosporine A to enhance cyclosporine A-induced injuries in terms of quality of sperms and oxidative stress parameters. Mansour et al. (2013) reported that administering P. Granatum extract to rats for 6 weeks lowered abnormal sperm rate and raised epididymal sperm concentration and sperm motility led to reduction in the amount of malondialdehyde, a by-product of lipid peroxidation, which means that antioxidant effect prevented lipid peroxidation in the spermatozoa membrane. In a rabbit study of Fayed et al. (2012), it was reported that a supplementary diet with pomegranate peel in different concentrations (0.5%, 1.0% and 1.5%) for eight weeks raised semen volume, motility and total number of spermatozoa, while the morphological abnormal and dead/live sperm ratio were decreased. On the other hand, in human the daily consumption of tablets of P. granatum extract for 3 months resulted in a significant increase of the sperm motility and volume (Fedder et al., 2014). According to Leiva et al. (2011), daily administration of 500 mg/kg pomegranate extract to rats exposed to lead poisoning for

35 days reversed the effects poisoning and increased sperm count and motility. Zeweil et al. (2013) showed in male rats exposed to temperature stress that, different concentrations of pomegranate peel extract added to diets of rats for 8 weeks increased sperm count, sperm motility and the amount of fructose in seminal plasma, while the rate of dead spermatozoon was decreased. Guo et al. (2008) stated that as pomegranate juice is consumed, plasma antioxidant capacity rises. Lowering malondialdehyde levels means decreasing lipid peroxidation in seminal plasma, evidenced by the rise in seminal plasma total lipids with pomegranate treatments. In this study, enhancements found in density of spermatogenic cells and quality of sperm can be referred to deterrence of too much formation of free radicals generated by spermatozoa, with the help of the antioxidant properties of pomegranate extract.

CONCLUSION

The results of study support that the daily consumption of pomegranate extract for eight weeks caused increased spermatogenic cell density, epididymal sperm motility, membrane integrity and decreased abnormal sperm rate in male rabbits. The total oxidant status level in the rabbits treated with 100 mg/kg/day of Pomegranate extract was decreased when compared to the control group. Thus, it can be say that Pomegranate extract at concentrations of 50 up to 100 mg/kg/day of body weight, is beneficial improving the epididymal sperm parameters in rabbits.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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