



## Effects of pomegranate seed oil on oxidative stress parameters and lipid profiles in ovariectomized rats

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### Abstract

**Objective-** This study was designed to determine the effect of pomegranate seed oil (PSO) treatment on oxidative stress and lipid profiles in ovariectomized (OVX) rats.

**Design-** Experimental study

**Animals-** 24 female Wistar rats

**Procedures-** The rats were randomly divided into four groups: sham-operated (SHAM), SHAM with PSO treatment (SHAM + PSO), ovariectomized (OVX), and ovariectomized with PSO treatment (OVX + PSO). OVX and SHAM rats were treated with PSO (200 mg/kg/d) or normal saline solution (in a volume similar to PSO), orally using a stomach tube for 8 weeks.

**Results-** Although a significant decrease ( $p < 0.01$ ) in serum total antioxidant status (TAS) concentration in the OVX group was observed when compared to the SHAM group, no significant difference was observed for TAS between OVX + PSO and SHAM groups. Rats of the OVX group presented a significant increase in LDL cholesterol concentration ( $p < 0.05$ ), when compared to SHAM + PSO group. However, no significant difference was seen for LDL cholesterol between OVX + PSO and SHAM + PSO groups. No significant differences were seen for superoxide dismutase, glutathione peroxidase, malondialdehyde, triglyceride, total cholesterol and HDL cholesterol between groups.

**Conclusions and Clinical Relevance-** The results of the present study showed that PSO might have some beneficial effects on the antioxidant status and LDL concentration after ovariectomy in rats.

**Keywords-** pomegranate, oxidative stress, lipids, ovariectomy, rats.

### Introduction

Oxidative stress is a biochemical disequilibrium propitiated by excessive production of free radicals and reactive oxygen species (ROS)<sup>1</sup>. There is increasing evidence suggesting that oxidative stress is responsible for the pathophysiology of the aging process and may participate in the pathogenesis of atherosclerosis, neurodegenerative diseases, cancer, and diabetes<sup>2</sup>. Estrogens have antioxidant properties and can inhibit lipid peroxidation in vitro<sup>3</sup>. It is reported that oxidative stress is increased and some antioxidants are decreased in relation to menopause in which estrogen diminishes<sup>4,5</sup>. It has been found that oxidative stress may play a role in the pathology of postmenopausal

complications and that supplementation with antioxidants may be beneficial in the treatment of this condition<sup>6</sup>.

After menopause, the incidence of cardiovascular disease increases<sup>7</sup>. It is well known that lipid and lipoprotein metabolism is markedly altered in postmenopausal women where elevated total cholesterol, low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and elevated lipoprotein (a) values have been observed<sup>8</sup>. There is extensive evidence that links hypercholesterolemia with increased oxidative stress<sup>9</sup>.

Estrogen replacement therapy in ovariectomized women and female rats prevents the deleterious effect of ovariectomy. However, large controlled clinical trials have not shown benefits of estrogen replacement therapy, or have even shown deleterious effects, such as an increment of venous thromboembolic disease, stroke and breast cancer incidence<sup>10</sup>. Therefore, new therapeutic strategies to prevent oestrogen deprivation-induced deleterious effects on the cardiovascular system will be valuable<sup>11</sup>.

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*Punica granatum* (Punicaceae), commonly called pomegranate, is a rich source of bioactive compounds<sup>12,13</sup>. Some of the Flavonoids, due to their phenolic structure, are known to be involved in the healing process of free radical mediated diseases including diabetes<sup>14-15</sup>. Several alkaloids, flavonoids, polyphenolic compounds (such as delphinidin, cyanidin and pelargonidin) and hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin, gallic and ellagic acid esters of glucose) which possesses strong antioxidant properties have been reported from pomegranate<sup>16</sup>. An in vitro assay using four separate testing methods demonstrated pomegranate juice and seed extracts have 2-3 times the antioxidant capacity of either red wine or green tea<sup>17</sup>. In addition, a pilot study in type 2 diabetic patients with hyperlipidemia found pomegranate juice significantly reduced total and LDL cholesterol, and improved total/HDL and LDL/HDL cholesterol ratios<sup>18</sup>.

An ovariectomized rat is the most frequently used animal model for postmenopausal complications in humans<sup>19-22</sup>. Interesting results have been reported in studies evaluating the role of antioxidants in ovarioectomy-induced oxidative stress<sup>6,23-25</sup>. However, there is no study available that reported the effect of pomegranate treatment on oxidative stress and lipid metabolism following ovarioectomy. Strong antioxidant principles and the presence of sterols in pomegranate seed oil<sup>12</sup> prompted us to design the present study to investigate whether management with pomegranate seed oil (PSO) has any protective effect on oxidative stress parameters [serum total antioxidant status (TAS), superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA)] and lipid profile (triglyceride, total cholesterol, LDL cholesterol and HDL cholesterol) in ovariectomized rats.

## Materials and methods

### *Animals and Administration Procedure*

24 female Wistar rats, aged 12 weeks, were obtained from colonies maintained at the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad. The animals were acclimated in an environmentally controlled animal laboratory and fed commercial food (Javaneh Khorasan Co, Mashhad, Iran) at a room temperature of 25 °C, with free access to distilled water for 1 week.

At 13 weeks of age, bilateral ovariectomy was performed via a two dorsolateral incision under general anesthesia with isoflurane and rats were ovariectomized (OVX) or sham-operated (SHAM).

The next day of the surgery, OVX and SHAM rats were treated with pomegranate seed oil (PSO, 200 mg/kg/d) or normal saline solution (in a volume similar to pomegranate seed oil), orally using a stomach tube for 8 weeks. Rats were randomly divided into four groups:

(1) SHAM, (2) SHAM + PSO, (3) OVX, and (4) OVX + PSO. Rats in the SHAM and OVX groups received only normal saline solution orally.

PSO was obtained from Orum Narin Ltd, Urmia, Iran. The estrogen content of PSO was determined using radioimmunoassay analysis and was 1300 pg/ml.

Rats were randomly divided into four groups: (1) SHAM, (2) SHAM + PSO, (3) OVX, and (4) OVX + PSO. Rats in the SHAM and OVX groups received only normal saline solution orally. The experiment was approved by the Animal Welfare Committee of the School of the Ferdowsi University of Mashhad.

### *Blood sampling*

At the end of experiment (eight weeks after PSO administration), anesthesia was performed with isoflurane and 5 ml blood sample was collected from heart prior to euthanasia. From each rat two blood samples were collected, one in a tube containing ethylenediamine-tetraacetic acid dipotassium salt (EDTA-K2) and the second in the tube without the anticoagulant for subsequent serum collection. The blood samples anticoagulated with EDTA were used for preparation of erythrocyte hemolysate. The serum was separated after centrifugation at 1,800 g for 10 min and stored at -20°C until analysis. For preparation of erythrocyte haemolysate, blood samples were centrifuged at 800×g for 15 min at 4 °C. The plasma and buffy coats were removed by aspiration. The sediment containing blood cells was washed three times by resuspending in isotonic phosphate-buffered saline, followed by recentrifugation and removal of the supernatant fluid and the buffy coats. One volume of the crude red cells was lysed in nine volumes of ice-cold distilled water to prepare a 10% erythrocyte hemolysate.

### *Biochemical analysis*

The amounts of triglyceride, total cholesterol, LDL cholesterol, HDL cholesterol and TAS in serum samples were measured by commercial kits [Pars Azmoon, Iran for triglyceride, total cholesterol, LDL cholesterol and HDL cholesterol; Randox, Antrim, UK for TAS] using an autoanalyser (Biotechnica, Targa 3000, Rome, Italy). Control serum (Randox control sera, Antrim, UK) was used for controlling measurement accuracy.

### *Antioxidant enzyme activities*

The activities of erythrocyte GPx and SOD were determined in erythrocyte haemolysate obtained immediately after sampling from anticoagulated blood. GPx activity was measured by commercially available kits (Ransel test kit, Randox Laboratories Ltd. G.B.). This method is based on that of Pagalia and Valentine<sup>26</sup>. GPx catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione

reductase and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance at 340 nm is measured.

SOD activity was measured by a modified method of iodophenyl nitrophenol phenyl tetrazolium chloride (INT) (Ransod test kit, Randox Laboratories Ltd. G.B.). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride (INT) to form a red formation dye. The SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the assay conditions.

#### MDA concentration

The concentration of MDA was estimated in serum according to the method of Placer et al.<sup>27</sup>. The reaction mixture consisted of 0.2 ml of serum, 1.3 ml of 0.2 M Tris-0.16 M KCl buffer (pH 7.4) and 1.5 ml of thiobarbituric acid reagent. The mixture was heated in a boiling water bath for 10 min. After cooling, 3 ml of pyridine/nbutanol (3:1, v/v) and 1 ml of 1N sodium hydroxide were added and mixed by vigorous shaking. A blank was run simultaneously by incorporating 0.2 ml distilled water instead of the serum. The absorbance of the test sample was read at 548 nm. The nmol of MDA

per ml of serum was calculated using  $1.56 \times 10^5$  as extinction coefficient.

#### Statistical analysis

Statistical analysis was conducted using SPSS for windows (release 16, SPSS Inc, Chicago, Ill) with a P value of <0.05 as statistically significant. Data were expressed as mean  $\pm$  standard deviation (SD). One way ANOVA was used to compare means among the different groups. Following analysis of variance, significant between-group differences were detected by the Tukey Honestly Significant Difference (HSD) test.

#### Results

The mean and SD of the biochemical parameters in different groups are presented in Table 1. A significant decrease in serum TAS (p < 0.01) concentration in the OVX group was observed when compared to SHAM group (Table 1). Rats of the OVX group presented a significant increase in LDL cholesterol concentration (p < 0.05), when compared to SHAM + PSO group. LDL/HDL ratio tended to be higher in the OVX group compared to the SHAM + PSO group (p =0.053). No significant differences were seen for other parameters between groups.

**Table 1.** Effect of PSO treatment on the amounts of some blood oxidative stress markers and lipid profile in ovariectomized rats

|                                  | OVX+PSO                        | OVX                          | SHAM + PSO                    | SHAM                           |
|----------------------------------|--------------------------------|------------------------------|-------------------------------|--------------------------------|
| <b>Total Cholesterol (mg/dl)</b> | 66.5 $\pm$ 6.80                | 74.5 $\pm$ 16.33             | 64.33 $\pm$ 6.26              | 72.16 $\pm$ 14.95              |
| <b>Triglyceride (mg/dl)</b>      | 75.50 $\pm$ 32.38              | 70.16 $\pm$ 16.93            | 60.66 $\pm$ 16.65             | 62.66 $\pm$ 22.43              |
| <b>HDL cholesterol (mg/dl)</b>   | 33.58 $\pm$ 2.34               | 37.78 $\pm$ 5.525            | 36.78 $\pm$ 7.48              | 39.73 $\pm$ 6.10               |
| <b>LDL cholesterol (mg/dl)</b>   | 23.23 $\pm$ 5.44 <sup>ab</sup> | 28.8 $\pm$ 8.69 <sup>a</sup> | 15.96 $\pm$ 3.87 <sup>b</sup> | 24.95 $\pm$ 9.79 <sup>ab</sup> |
| <b>LDL/HDL ratio</b>             | 0.69 $\pm$ 0.19                | 0.75 $\pm$ 0.17              | 0.44 $\pm$ 0.13               | 0.63 $\pm$ 0.25                |
| <b>SOD (IU/ml)</b>               | 2700 $\pm$ 741                 | 3034 $\pm$ 745               | 2891 $\pm$ 593                | 3026 $\pm$ 496                 |
| <b>GPX (IU/ml)</b>               | 6.22 $\pm$ 1.61                | 5.01 $\pm$ 1.67              | 3.83 $\pm$ 1.04               | 4.07 $\pm$ 1.93                |
| <b>TAS (mmol/L)</b>              | 2.69 $\pm$ 0.54 <sup>ab</sup>  | 1.79 $\pm$ 0.51 <sup>a</sup> | 2.79 $\pm$ 0.69 <sup>ab</sup> | 3.72 $\pm$ 1.42 <sup>b</sup>   |
| <b>MDA (nmol/ml)</b>             | 2.75 $\pm$ 1.12                | 3.18 $\pm$ 1.17              | 2.96 $\pm$ 0.99               | 2.49 $\pm$ 0.78                |

In each row, means with different superscript show significant differences (P<0.05)

OVX: ovariectomized

SHAM: sham-operated

PSO: pomegranate seed oil

All parameters were measured in the blood serum samples, except for SOD and GPX that were measured in the erythrocyte hemolysate.

#### Discussion

Oxidative status depends on the balance between oxidants and antioxidants. The TAS concentration is an overall indicator of oxidative status<sup>28</sup>. This study revealed that TAS was significantly reduced in OVX group compared to the SHAM group. Similarly, Muthusami et al.<sup>4</sup>, Ha<sup>29</sup> and Prabhu et al.<sup>30</sup> showed that levels of antioxidants decreased in ovariectomized rats

compared with the control group. Muthusami et al.<sup>4</sup> suggested that antioxidant status is seriously compromised by ovariectomy in experimental animals. The absence of hormones caused by ovariectomy or menopause may induce or accelerate oxidative stress. Estrogens may exert potent antioxidant actions, which may contribute to the protective effect in females<sup>25</sup>. The antioxidant metabolism of estrogen protection has been suggested to be independent of receptor binding<sup>31</sup>. Estrogen declines precipitously after ovariectomy or

menopause. This ovarian hormone deficiency increases the generation of ROS and utilization of antioxidants<sup>24</sup>. Antioxidant enzymes are involved in the defense system against free radical mediated tissue or cellular damage. They metabolize either free radicals or reactive oxygen intermediates to nonradical products<sup>4</sup>. Both decreased<sup>4,5</sup> and increased<sup>32,33</sup> antioxidant enzymes are reported after ovariectomy in animals or menopause in females. However, in the present study the activity of antioxidant enzymes in OVX rats were almost same compared with the control group. The ovariectomy-induced ROS accelerate the activity of lipid peroxidation in the polyunsaturated fatty acids of the cell membrane, and the increased level of MDA, one of the end-products in the lipid peroxidative process, inversely influenced the activity of the antioxidant enzymes<sup>29,30</sup>. In the present study, the increased MDA concentration was not found in the ovariectomized rats and no significant differences were observed between different groups. Therefore, the lack of significant differences for enzyme activity between groups could be related to unchanged lipid peroxidation in the studied rats.

Pomegranate is a rich source of bioactive compounds and is used in folk medicine for the treatment of various diseases. Pomegranate extracts have been shown to scavenge free radicals and decrease macrophage oxidative stress and lipid peroxidation<sup>34</sup>. Studies in rats and mice confirm the antioxidant properties of a pomegranate, showing a 53-percent increase in reduced glutathione levels<sup>34</sup>. Guo et al.<sup>35</sup> found 250 mL of pomegranate pulp juice daily for four weeks given to healthy elderly subjects increased plasma antioxidant capacity from 1.33 mmol to 1.46 mmol. Current research seems to indicate the most therapeutically beneficial pomegranate constituents are ellagic acid, ellagitannins (including punicalagins), punicic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones<sup>12</sup>. In the present study, PSO treatment restored TAS to control levels and no significant differences were observed between OVX+PSO group and SHAM group. PSO is a rich source of punicic acid, ellagic acid and sterols<sup>12</sup>. The presence of both potent antioxidants and sterols in the PSO makes it a good option for treating the oxidative stress and other ovarian hormone deficiency complications after ovariectomy or menopause. *In vitro* assay of a cold pressed seed oil extract found the antioxidant capacity of it is superior to red wine and similar to green tea extract<sup>36</sup>.

Rats in the OVX group showed a significant increase in serum LDL cholesterol concentration compared to SHAM + PSO rats. In addition, LDL/HDL ratio tended to be higher in the OVX group compared to the SHAM + PSO group. It is well known that lipid and lipoprotein metabolism is markedly altered in postmenopausal women where elevated total cholesterol, LDL, VLDL,

and elevated lipoprotein a values have been observed<sup>8</sup>. Estrogen has been shown to decrease LDL and increase high density lipoprotein HDL levels<sup>37</sup>. These quantitative changes in lipoproteins are considered to account for one third of the cardioprotective effects of oestrogen<sup>37</sup>. Furthermore, during the menopause there is not only a reduction in the number of LDL liver receptors but also a decrease in the activity of the remaining receptors, and this may explain the increased lipoprotein and lipid concentrations in postmenopausal women<sup>38,39</sup>. Opposite to the OVX group, the increased LDL cholesterol was not observed in ovariectomized rats treated with PSO (OVX + PSO). These findings suggest that PSO treatment effectively prevented the OVX induced rise in serum LDL cholesterol. A pilot study involving type 2 diabetic patients, investigated the cholesterol-lowering effects of concentrated pomegranate juice for eight weeks. Statistically significant decreases were observed in total cholesterol, LDL cholesterol, total/HDL cholesterol ratio and LDL/HDL ratio. The authors attributed these effects to decreased absorption and increased fecal excretion of cholesterol, as well as possible effects on HMG-CoA reductase and sterol O-acyltransferase, two enzymes key to cholesterol metabolism<sup>18</sup>. There is extensive evidence that links hypercholesterolemia with increased oxidative stress. The oxidative modification of lipoproteins, particularly LDL, has emerged as a fundamental process in the development of atherosclerosis<sup>9</sup>. Improved antioxidant capacity, evidenced via increased TAS, and decreased LDL concentration following PSO treatment suggest that PSO could have protective effects against cardiovascular disease after menopause. Further research is required to evaluate atherosclerosis risk factors especially oxidized LDL, following PSO treatment in ovariectomized rats.

The results of the present study showed that PSO might have some beneficial effects on the antioxidant status and LDL concentration after ovariectomy in rats. This is the first report to show that the PSO could suppress the ovarian hormone deficiency-induced rise in serum lipids and oxidative stress, to our knowledge.

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## چکیده

### اثرات روغن دانه انار بر روی پارامترهای استرس اکسیداتیو و پروفایل چربی در موش صحرایی اواریکتومی شده

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**هدف-** در مطالعه حاضر اثرات روغن دانه انار بر روی استرس اکسیداتیو و پروفایل چربی در موش های اواریکتومی شده مورد ارزیابی قرار گرفت.

**طرح مطالعه-** مطالعه تجربی

**حیوانات-** ۲۴ موش صحرایی ویستار

**روش کار-** ۲۴ موش صحرایی ماده ویستار به صورت تصادفی به چهار گروه کنترل، کنترل روغن دانه انار، اواریکتومی و اواریکتومی همراه با تجویز روغن دانه انار تقسیم شدند. رت های اواریکتومی شده و کنترل به ترتیب روغن دانه انار (روزانه ۲۰۰ میلی گرم به ازای هر کیلوگرم وزن بدن) و نرمال سالین (در یک حجم برابر با روغن دانه انار) بصورت خوراکی و با استفاده از لوله معدی به مدت ۸ هفته دریافت نمودند.

**نتایج-** اگر چه کاهش معنی دار ( $p < 0.01$ ) ظرفیت تام آنتی اکسیدان سرم در گروه اواریکتومی در مقایسه با گروه کنترل مشاهده گردید، اما هیچ گونه تفاوتی بین گروه های اواریکتومی دریافت کننده روغن دانه انار و کنترل از لحاظ ظرفیت تام آنتی اکسیدانی مشاهده نگردید. موش های صحرایی گروه اواریکتومی افزایش معنی دار کلسترول لیپوپروتئین با چگالی پایین (LDL) را در مقایسه با موش های صحرایی گروه کنترل روغن دانه انار نشان دادند ( $p < 0.05$ ). اما هیچ تفاوت معنی داری بین گروه های اواریکتومی دریافت کننده روغن دانه انار و کنترل روغن دانه انار از لحاظ کلسترول لیپوپروتئین با چگالی پایین مشاهده نگردید. هیچ گونه تفاوت آماری معنی داری از لحاظ آنزیم های سوپراکسید دیسموتاز و گلوتاتیون پراکسیداز، مالون دی آلدهید، تری گلیسرید، کلسترول تام و کلسترول لیپوپروتئین با چگالی بالا (HDL) بین گروه های مختلف وجود نداشت.

**نتیجه گیری و کاربرد بالینی-** نتایج بدست آمده در مطالعه حاضر نشان می دهد که تجویز روغن دانه انار ممکن است در بهبود وضعیت آنتی اکسیدان و کاهش کلسترول لیپوپروتئین با دانسیته پایین بدنبال اواریکتومی در موش صحرایی مفید باشد.

**کلید واژگان-** انار، استرس اکسیداتیو، چربی ها، اواریکتومی، موش صحرایی.

