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Experimental Research

Pharmacology Section

Effects of Pomegranate Seed Oil on the Fertilization Potency of Rat's Sperm

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ABSTRACT

Background: Pomegranate has been taken great scientific attention in recent years due to its health benefits. Pomegranate seed oil is a rich source of 9-cis, and 11-trans conjugate linolenic acid. The aim of this study was to evaluate the effect of dietary pomegranate seed oil on the fertilization potency of rat's sperm.

Materials and Methods: Twenty-four male Wistar rats were divided into four groups. The first group, which served as the control group, received 1 mL of corn oil for seven weeks. Groups II, III, IV served as the experimental groups received 200, 500 and 1000 mg/kg of pomegranate seed oil, for the same period of time respectively. After seven weeks, all of the rats were sacrificed, and their epididymis sperm was collected and added to IVF medium (T6) containing metaphase II oocytes. Almost 21 oocytes had been removed from every female rat oviduct. In this medium, oocyte fertilization, cleavage rates, and embryo development into blastocysts, were evaluated by inverted microscopy.

Results: Levels of LD50 in the oral route in male rats were more than 5000 mg/kg body weight. Our data showed that the rates of fertilization, cleavage and embryo development into blastocysts were higher in the groups that had received 500 and 1000 mg/kg body weight of pomegranate seed oil.

Conclusion: This study demonstrated that pomegranate seed oil had a positive effect on the fertilization potency of male rats. These beneficial effects may be useful in assisted reproductive technology.

Keywords: Blastocyst, Embryo development, Fertilization potency, Oocyte

INTRODUCTION

The pomegranate fruit is native to Iran, and it has gradually spread to other parts of the world including Asia, the Middle East, and the Mediterranean countries [1]. The pomegranate was known in ancient times. The Greek philosopher and botanist Theophrastus (371-287 BC) wrote several botanical books which included a description of the pomegranate. In earlier times the pomegranate was considered to be a symbol of wealth and reproduction because it contains a substantial number of seeds, and it was also valued for its medicinal properties [2].

Because of antioxidant activities of pomegranate [3], the fruit has gained greater acceptance with the general population, therefore pomegranate production has increased significantly in the United States and it is now cultivated in the hot arid zones of California and Arizona. The pomegranate contains a high percentage of water, with the edible portion consisting of 78% juice and 22% seeds. The fruit contains a number of different chemical compounds that research has shown maybe beneficial for a variety of medical conditions [4]. Pomegranate juice is high in vitamin C, as well as polyphenols such as anthocyanins, punicalagin, ellagic and gallic acids. The fruit has increased in popularity for its ability to curb cell growth, apoptosis, inhibit HIV-I entry, microbicidal, cardioprotective and antihyperlipidemic properties. Moreover, a number of researchers have found that pomegranates have free radical scavenger and potent antioxidant properties [5]. Dried pomegranate seeds contain the estrogenic hormone estrone and the phytoestrogens; genistein, daidzein and coumestrol, in addition to amino acids such as glutamic and aspartic acids [6].

Research focused on pomegranate seeds indicates that they are rich in nutrients and antioxidants. Supplements made from pomegranate seeds may be useful in the prevention of DNA damage, lowering the risk of cancer and alleviating menopausal symptoms [7].

Fadavi and Azizi (2006) investigated the fatty acid composition of pomegranate seed oils from 25 different Iranian varieties. The oils contained 66.3-193 g/Kg dry matter, with a fatty acid consisting of linolenic acid (31.8–86.6%), linoleic acid (0.7-24.4%), oleic acid (0.4-17.4%), stearic acid (2.8-16.7%) and palmitic acid (0.3-9.9%). Small amounts of saturated fatty acids; myristic acid (0.1-4.7%) and behenic acid (0.0-3.9%), were also found in several of the cultivars [8].

Free radicals are produced as a normal part of sperm cell development, but problems may appear when the levels of free radicals are too high. Sperm production processes include capacitation, hyper activation, acrosome reactions, oocyte fusion and fertilization. Excess reactive oxygen species (ROS) production can result in cell damage and problems such as DNA fragmentation, lipid peroxidation, decreases in acrosome reactions and fusogenic ability, reduced spermatozoa mobility and lower IVF pregnancy rates. Furthermore, previous research has suggested that oral antioxidant treatment improves ICSI-IVF outcomes in couples with male infertility issues, by strengthening the structure of the sperm's DNA [9].

A number of investigators have reported that pomegranates have free radical scavenger and potent antioxidant abilities. The sperms' plasma membranes contain high levels of unsaturated fatty acids, making it susceptible to peroxidative damage. Lipid peroxidation undermines the lipid matrix structure in spermatozoa membranes and these results in a loss of motility and compromises membrane integrity. Therefore, cell permeable ROS scavengers may improve sperm function [5]. In this study we examined the effect of pomegranate seed oil on improvements of oocyte fertilization potency in rat's sperm.

MATERIALS AND METHODS

This study was carried out in cellular and molecular research center at Yasuj University of Medical Sciences in Iran in 2010-2012. All animal experiments were performed according to the animal ethics committee of Yasuj University of Medical Sciences

Extraction of pomegranate seed oil

Ten grams samples of crushed dry pomegranate seeds were refluxed with 300mL of petroleum benzene in weighed flasks using a Soxhlet apparatus [10]. The oils were recovered by distilling the solvent in a rotary evaporator at 45°C, then dried to a constant weight in a vacuum oven at 90°C for 1 hour and then weighed [8].

Total phenol and antioxidant activity assay

The total phenol (TP) of the pomegranate seed oil (PSO) was measured according to the previously described procedure [11]. In summary, the reaction mixture contained 250 µL of fresh Folin–Ciocalteu (FC) reagent, 750 µL of 20% Na_2CO_3, 50 µL of PSO, and 3 mL of pure H_2O. The absorbance was determined at 765 nm after 2 hours of reaction at an ambient temperature and then the absorbance was used to calculate the phenolic contents in the seed oil according to the gallic acid standard curve. The reactions were conducted in triplicate and the results were reported as gallic acid equivalents (GAE) per gram of pomegranate seeds.

The antioxidant activity of PSO was measured by 2, 2-diphenyl-1-picrylhydrasyl (DPPH) and trolox equivalent antioxidant capacity (TEAC), assays. In brief, each reaction mixture contained 100 µL of PSO and 100µL of 0.2 mmol/L DPPH solution. Six concentrations of each extract were analyzed in order to estimate their EC50 value against DPPH. The EC50 value is the amount of antioxidant needed to neutralize 50% of the radicals in the solution being investigated. DPPH solution was added to each well to initiate the reactions, and the absorption rate at 515 nm was determined after 40 min of reaction. The blank contained only 200 µL of solvent, and the control consisted of 100 µL of solvent and 100 µL of 0.2 mmol/L DPPH. The following equation was used to calculate the percentage of radicals remaining after 40 min:

DPPH% remaining = {(Asample _ Ablank)/(Acontrol _ Ablank)} ×100%,

Asample, Ablank, and Acontrol stand for the absorbance of sample, blank, and control reactions at 40 min of antioxidant – radical reaction, respectively. The EC50 value for each sample was obtained by plotting the percentage of DPPH remaining at 40 min against the pomegranate extract concentrations [7]. The TEAC method was conducted according to Hua-Bin Li (2008) [12].

The LD50 of the PSO was determined by routine procedures according to Akbartabar Toori (2013) [13].

Aspartate aminotransferase (AST) and alanine amino transferase (ALT) activities were measured by AST activity assay kit (MAK055 SIGMA) and ALT activity assay kit (MAK052 SIGMA), respectively.

Animals, supplements and sample collection

Twenty-four healthy male rats (6 to 8 weeks old) were randomly divided into four groups. The first group, which served as the control group, received 1 mL of corn oil. Groups II, III, IV served as the experimental groups received 200, 500 and 1000 mg/kg of pomegranate seed oil respectively. Both the corn oil and the pomegranate seed oil were given by gavage daily for seven weeks. This administration period was necessary in order to determine the effect of the pomegranate seed oil on sperm production, because the rats need a period of 48-52 days for the entire spermatogenic cycle including spermatocytogenesis, meiosis and spermiogenesis.

The rats were sacrificed at the end of seven weeks. Blood samples were collected from their hearts via sterile injectors and the serum was separated for evaluating of liver enzymes. The epididymis were also removed and weighed.

The epididymal sperm count was determined with a haemocytometer. Caudate epididymis were excised and submerged in 200 µL T6 medium. The spermatozoa were allowed to disperse for 5-10 min.

Preparation of the eggs (MII oocytes): Six week old Wistar female rats were superovulated by an intraperitoneal (IP) injection of 10 IU pregnant mare's serum gonadotropin (PMSG), followed by an IP injection of 10 IU human chorionic gonadotropin (HCG) 48 hours later. At 13-15 hours after the HCG injection, the rats were sacrificed and the MII oocytes, almost 21 oocytes from every rat, were collected from their oviducts and transferred to 200 µL T6 medium.

The sperm suspension (1×10^6 motile spermatozoa/ml) was capacitated for 2 hour in T6 media and this capacitated sperm was added to T6 medium containing MII oocytes. They were assessed with an inverted microscope (Bar= 50 mm) for fertilization and development into the 2-cell stage after 24 hour, and finally into the blastocyst stage after 120 hour.

RESULTS

The amount of the extracted oil was 105.64-112.36 g/kg weight of dry seeds. The EC50 of the antioxidant activity for PSO in TEAC and DPPH assays were 0.30 and 0.48 mg/mL, respectively. The LD50 of PSO in the oral route in male rats was more than 5000 mg/kg body weight. The serum AST and ALT activity in all groups are shown in [Table/Fig-1]. There were no significant differences in ALT and AST activities between experimental and control groups.

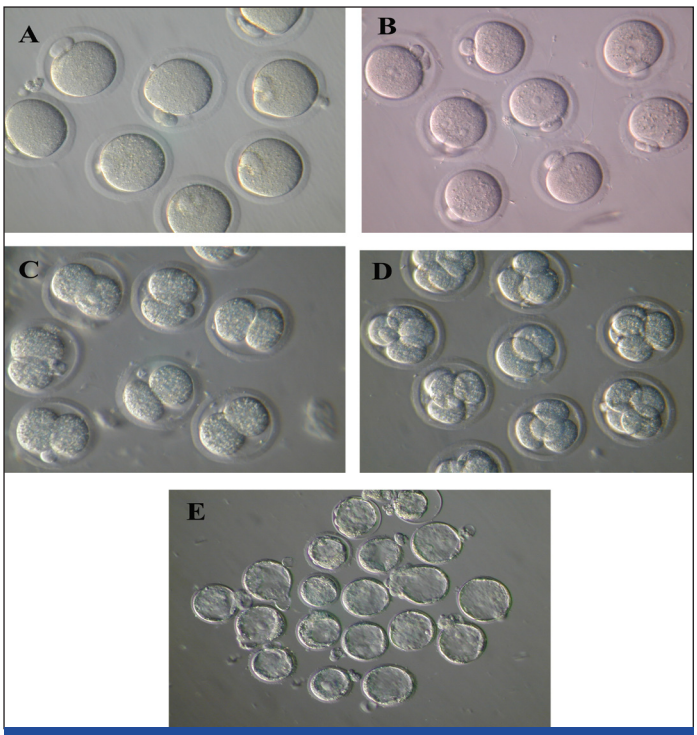
The total phenol of the extracted oil was equivalent to 1.05-1.49 mg of gallic acid in one gram of dry seeds. The antioxidant capacity of PSO in 1 mg/mL concentration, using DPPH and TEAC assays were 84% and 98%, respectively. The effect of pomegranate seed oil on invitro fertilization oocytes, and embryo development to blastocyst by the different treatment sperm groups are illustrated in [Table/Fig-2,3]. The results show that there were no significant differences between control (corn oil) and group II (200 mg/kg PSO) in terms of the rates of fertilization and development to 2-cells and blastocyst

Groups	ALT IU/ml	AST IU/ml
Control Group I (corn oil)	107±24.2	187±30.9
Experimental Group II (200 mg/kg PSO)	89.2±9.5	176±18.1
Experimental Group III (500 mg/kg PSO)	104.8±27.7	179±28.1
Experimental Group IV (1000mg.kg PSO)	83.2±11.5	178.8±37.8

[Table/Fig-1]: Liver enzymes activity
Groups II, III and IV were received 200, 500 and 1000 mg/kg body weight pomegranate seed oil

Groups	No. of MII	Pronucleus (fertilization)	2-Cell	4-Cell	8-Cell	Morula	Blastocyst
Control Group I (corn oil)	120	72.3(1.5)	67.9(2.1)	61.7(1.5)	58(2.0)	50.7(1.5)	45.2(4.1)
Experimental Group II (200 mg/kg PSO)	115	76.7(2.4)	72.3(2.8)	67.5(3.9)	63.7(3.8)	56.2(3.4)	49.6(4.0)
Experimental Group III (500 mg/kg PSO)	138	88.7(2.3)*+	87.6(3.6)*+	77.7(2.6)*	73.2(1.2)*	68.1(0.5)*+	66.1(1.3)*+
Experimental Group IV (1000 mg.kg PSO)	122	90.1(3.3)*+	88.8(2.8)*+	79.5(3.4)*+	74.4(1.2)*+	69.3(1.1)*+	66.3(2.6)*+

[Table/Fig-2]: Effect of pomegranate seed oil on invitro fertilization rat oocyte, and embryo development to blastocyst
Data expressed as mean percent (SD)
*Compared with control group $p<0.05$, + Compared with 200mg/kg experimental group $p<0.05$. Statistical analyses of meiotic maturation, IVF and blastocyst rates were made by one-way ANOVA followed by Tukey post hoc test. A p-value <0.05 was considered significant



[Table/Fig-3a-e]: (a) MII oocytes, (b) Fertilized oocyte, (c) 2-cell stage, (d) 4-cell stage and (e) blastocysts stage

stage. The rates of fertilization and development from the 2-cells to the blastocyst stage in the oocytes treated with 500 (group III) and 1000 (Group IV) mg/kg PSO, were significantly higher than those of the control and the Group II which was treated with 200 mg/kg ($p<0.05$). Although the oocytes treated with 1000mg/kg PSO showed higher rates of fertilization and development to blastocysts compared with those of the 500 mg/kg group, the differences were not statistically significant.

DISCUSSION

Pomegranate seed is a byproduct of pomegranate processing and consists of about 20% (w/w) of the whole fruit [14]. Pomegranate seeds have a range of health giving properties that may be the result of a range of biologically active compounds, particularly polyphenol antioxidants [15]. Moreover, significant levels of phenolic content were detected in pomegranate seeds in earlier studies [16].

Polyunsaturated fatty acids (PUFA) and other bioactive compounds are present at high levels in pomegranate seeds and this has garnered a great deal of interest from the scientific community [17]. Previous studies have also found that PSO may also contain significant levels of tocopherol antioxidants [18]. Evaluations of the bioactive components and antioxidant activities of local pomegranate seeds have been performed in Iran [14], Tunisia [16], the USA [17], and many other countries.

According to a study by Armand Zini using dietary antioxidants may reduce damage to sperm DNA, especially in cases where high levels of DNA fragmentation have occurred. The actual mechanism of how dietary antioxidants work has not been fully established, although invitro antioxidant supplements have been shown to have a positive effect on sperm DNA, by protecting them from exogenous oxidants. There are still unanswered questions regarding the ability of antioxidants to protect sperm from other stressors such as; endogenous ROS, gentle centrifugation, and cryopreservation [19]. A study on rat model of testicular torsion-detorsion has been shown that pomegranate juice can increase sperm concentration and decrease oxidative stress indexes like malondialdehyde [20].

A study by A Yüce et al., has suggested that the long-term use of cinnamon bark oil (CBO) produces improved sperm quality, along with decreases in apoptotic germ cells, which in turn decreases levels of testicular lipid peroxidation (LP); the outcome was increased antioxidant enzyme activities in rats. It was concluded that the daily ingestion of CBO for at least 10 weeks may be useful for azoospermic men. Cinnamon has a number of useful characteristics, including; antioxidant, anti-inflammatory and anti-diabetic properties [21].

Reactive oxygen species (ROS) can damage the delicate sperm plasma membrane, as it consists of polyunsaturated fatty acids. Antioxidant vitamins C (20 mm) and E (2 mm) may protect against dimethoate-induced ROS by decreasing LP and hyperactivity of superoxide dismutase (SOD) and catalase (CAT) in human erythrocytes. Using the same concentration of vitamins demonstrates that pretreating sperm cells with vitamin C and E improves sperm quality, and this shows that dimethoate-induced sperm damage can be reduced by vitamin supplementation [22].

In summary, free radical generation is associated with the impairment of semen quality. This is reflected in terms of alterations in the antioxidant enzyme system. Thus, it can be inferred that eliminating oxidative stress, through antioxidant treatment, and supplementation, could be useful in the diagnosis and prognosis of subjects showing sperm abnormalities, and improve their functional ability. Future research may include studies using markers of oxidative stress and the antioxidant system on a larger scale and exploring the subjects' genetic susceptibility to semen quality impairment [23].

In our study, we investigated the effects of pomegranate seed oil (PSO) on the sperm potency of oocyte fertilization, and because of the powerful antioxidant activity of PSO, it may affect sperm quality and its fertilization potency. Our data showed that feeding male rats with PSO for duration of approximately 48 days, may improve the fertilization potency of sperm. There were no abnormal results in liver test parameters (ALT and AST) after the administration of pomegranate seed oil, showing that this oil does not have any toxicity in animals.

CONCLUSION

The results of our study showed that pomegranate seed oil supplementation improved the rate of rat embryo development. This study also demonstrated that PSO supplementation at a dose of 1000 mg/kg had the most effect on the embryo development in rats. Although the exact mechanism of this effect is not clear, PSO supplementation may decrease oxidative stress in sperm and increase the rate of pregnancy in females. However, detecting the exact mechanism needs more research. For clinical application of these findings further human study need to be designed.

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