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Evaluation of the Antimutagenic Effect of Pomegranate Seed Oil Against Genotoxicity Induced by Nitrobenzene in Mice

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Abstract: In this study the protective effect of pomegranate seed oil (PSO) against nitrobenzene (NB) induced genotoxicity in mice was investigated. Mice were treated with 100, 200 and 400 mg/kg b.wt. PSO for one and seven days. After consumption of PSO animals were orally treated with 300 mg NB/kg b.wt.. Samples were taken 24 h after the treatment. Our results showed that the percentage of chromosome aberrations in animals treated with the tested doses of PSO in both somatic and germ cells is not significantly different from these in control animals. Significant inhibition in the percentage of chromosomal aberrations in bone marrow cells and spermatocytes induced by NB were demonstrated. Also, PSO reduced the percentage of sperm abnormalities induced by NB in a significant and dose-dependent manner. Overall, the results highlight the potential of pomegranate seed oil as a safe and effective chemopreventive agent against environmental pollution.

Key words: Pomegranate Seed Oil · Nitrobenzene · Genotoxicity · Chromosome Aberrations · Sperm- Shape Abnormalities.

INTRODUCTION

The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Epidemiological and experimental studies reveal a negative correlation between the consumption of diets rich in fruit and vegetables and the risks for chronic diseases, such as cardiovascular diseases, arthritis, chronic inflammation and cancers [1-4]

Pomegranate fruit (Punica granatum) has been used worldwide as an item of diet and medicine for millennia and has also been regarded as an important symbol in world religions and mythologies and of medicine itself [5]. Potent antioxidant for pomegranate seed oil (PSO) [6-7] as well as a wide range of human breast cancer suppressive properties in vitro, including promotion of apoptosis and inhibition of proliferation and invasion were demonstrated [8,9]. PSO has been shown to suppress chemically induced carcinogenesis and exert antiangiogenic activity [10,11].

Many environmental pollutants are carcinogens and their amounts entering the environment are continually increasing. Nitrobenzene (NB), a synthetic hydrocarbon, is widely used in industry including in the production of aniline and polyurethanes and in petroleum refining [12,13]. Nitrobenzene is a highly toxic environmental pollutant characterized by its stability. Chemical and physical properties of nitrobenzene are similar to many industrial wastes. It shows a lack of auto oxidation or any oxidative degradation via hydroxylation [14]. Nitrobenzene is toxic to human by inhalation and dermal exposure, with toxic effects on liver, bone marrow and spleen hematopoiesis. It can also cause methemoglobinemia, hypoxia, hepatic toxicity, petechial hemorrhages and neurotoxic effects [15,16]. Toxicity of

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nitrobenzene contamination have also been revealed in aquicolous animals such as water fleas (Daphnia magna), snails (Lymnaea stagnalis), medaka (Oryzias latipes), guppies (Poecilia reticulata) and euglena gracilis by aquatic ecological toxicity assays [17]. Moreover, nitrobenzene is carcinogenic in mammals (rats and mice) which can induce spectrum of benign and malignant neoplasias in lung, thyroid, mammary gland, liver and kidney [13,18- 20].

The objective of these investigations is to study the genotoxic effect of PSO in mice at different doses as well as the protective role of PSO in a trial to minimize the genotoxicity of NB.

MATERIALS AND METHODS

Chemicals: NB (C5H5NO2) with technical grade 99.9 %, Sisco Research Laboratory PVT LTD. The seeds of pomegranate -identified by staff members of the Hebarium of the Department of Botany, Faculty of Science, Cairo University- were collected from Menofia region, Egypt in August, air-dried, pulverized and stored for further use. All other chemicals used were of analytical grade.

Extraction of Seed Oils: 250 gm of grinded pomegranate seeds were covered with n-hexane and left for 3 days at room temperature. The process of extraction repeated for 3 times. The solvent from combined n-hexane extract was evaporated under reduced pressure. The residue obtained (77 gm) was in the form of oily substance was designated as a crud extract of n-hexane.

Animals: Laboratory-bred strain Swiss albino male mice 8-10 weeks old with an average weight of 27.5 ± 2.5g were obtained from the Animal House, National Research Center, Cairo, Egypt. Animals were housed in groups (5 animals/ group) and maintained under standard food and water *ad libitum*

Doses and Treatment

Single Treatment: Mice were orally treated (using a stomach tube) with 100, 200 and 400mg/kg b.wt. PSO and 300mg/-kg NB. Mice received 300mg NB simultaneously with tested doses of PSO. The animals were killed 24h after treatment.

Repeated Treatment: Mice received oral doses of 100, 200 and 400 mg PSO/kg b.wt. for 7 consecutive days. Samples were taken 24h after the last treatment. Other group of mice were treated orally with tested doses of PSO for 7 consecutive days, and on the last day with 300mg NB/kg b.wt. Mice killed 24h. after the last treatment.

For Sperm- Shape Abnormalities: Groups of five mice were orally treated with the tested doses of PSO simultaneously with 300 mg NB /kg b.wt. for 5 consecutive days. Animals were sacrificed 35 days after the first day of treatment by cervical dislocation.

One control group was treated with corn oil as a negative control.

Cytogenetic Parameters: For chromosome aberrations in somatic and germ cells, animals from the different groups were injected i.p. with colchicine, 2h before sacrifice. Chromosome preparations from bone marrow cells (somatic cells) carried out according to the method of Yosida and Amano [21]. Chromosome preparations from spermatocytes were made according to the technique developed by Evans *et al*. [22]. 100 well spread metaphases were analyzed per mouse in five mice per group. Metaphases with gaps, chromosome or chromatid breakage and fragments, were recorded in bone marrow cells and diakinesis metaphases-I with univalents were recorded in germ cells.

For sperm-shape abnormalities, the epididymides were excised and minced in 2ml physiological saline, dispersed and filtered to remove large tissue fragments. Smears were prepared and stained with 1% Eosin Y [23]. At least 1000 sperm / animal (5000 / group) were assessed for morphological abnormalities of the sperm abnormalities.

Statistical Analysis: The significance of the difference between experimental and control data was calculated using the t - test.

RESULTS

Chromosome Aberrations in Somatic and Germ Cells

Single Treatment: The percentage of chromosome aberrations in animals treated with the tested doses of PSO is not significantly different from these in control animals (Table 1, 2).

The protective effect of PSO, administered as a single dose, on the induction of chromosome aberrations in somatic and germ cells after oral treatment with different doses of PSO is represented in Tables (3, 4). The results showed that the three tested doses of PSO exerted a

Table 1: Number and mean percentage of the different types of chromosomal aberrations in mouse bone marrow cells after treatment with different doses of PSO.

Treatment and doses (mg/kg b.wt.)	Treatment day(s)	No. of metaphases with				Chromosomal aberrations Excluding gaps
		Gap	Frag. and/or break	Gap+(Frag. or break)	Rt.	Mean ± S.E.
Control (Corn oil)						
Single treatment	1	10	16	-	-	3.2 ± 0.42
PSO 100		7	15	5	1	4.2±0.37
PSO 200		11	16	3	-	4.6± 0.95
PSO 400		8	20	3	-	3.8 ±0.24
Repeated treatment						
PSO 100	7	12	14	7	-	4.2 ± 0.20
PSO 200		8	14	4	1	3.6 ± 0.23
PSO 400		9	13	3	-	3.2 ±0.30

The total number of scored metaphases is 500 (5 animals / group)

Frag.= fragment, Rt. = Robertsonian translocation.

Table 2: Number and mean percentage of metaphases with chromosomal aberrations in mouse spermatocytes after treatment with different doses of PSO.

Treatment and doses (mg/kg b.wt.)	Treatment day(s)	No. of different types of chromosomal aberrations			Total aberrations	
		XY univalent	Autosomal Univalent	XY+ autosomal univalent	No.	Mean % ± S.E.
Control (corn oil)						
Singl treatment	1	11	4	-	15	3.0 ± 0.23
PSO 100		12	4	-	16	3.2 ± 0.24
PSO 200		10	4	1	15	3.0 ± 0.2
PSO 400		12	3	1	16	3.2 ± 0.3
Repeated treatment						
PSO 100	7	9	5	1	15	3.0 ± 0.24
PSO 200		9	3	2	14	2.8 ± 0.2
PSO 400		8	4	1	13	2.6 ± 0.22

The total number of scored metaphases is 500 (5 animals /group)

Table 3. Number and mean percentage of the different types of chromosomal aberrations in mouse bone marrow cells after treatment with different doses of PSO plus NB.

Treatment and doses (mg/kg b.wt.)		No. of metaphases with				Chromosomal aberrations	
Mean ± S.E.	Treatment day(s)	Gap	Frag. and/or break	Gap+(Frag. or break)	Rt.	Excluding gaps	Inhibition %
Control (corn oil)							
Single treatment	1	10	16	-	-	3.2 ±0.37	
NB 300		15	43	7	5	11.0 ± 0.26 **	
NB + PSO 100		13	24	3	-	5.4 ±0.71 ♦♦	50.9
NB + PSO 200		12	21	2	1	4.8 ±0.45 ♦♦	56.36
NB + PSO 400		9	15	4	2	4.2 ±0.58 ♦♦	61.81
Repeated treatment							
NB + PSO 100	7	9	17	3	1	4.2 ±0.31 ♦♦	61.81
NB + PSO 200		13	17	1	-	3.6 ±0.70 ♦♦	67.27
NB + PSO 400		16	15	1	1	3.4 ±0.50 ♦♦	69.09

The total number of scored metaphases is 500 (5 animals / group)

Frag.= fragment, Rt. = Robertsonian translocation.

** Significant at 0.01 level (t-test) comparing to control (non-treated).

♦♦ Significant at 0.01 level (t-test) comparing to treatment.

Table 4: Number and mean percentage of metaphases with chromosomal aberrations in mouse spermatocytes after treatment with different doses of PSO plus NB.

Treatment and doses (mg/kg b.wt.)	Treatment day(s)	No. of different types of chromosomal aberrations			Total aberrations		
		XY univalent	Autosomal Univalent	XY+ autosomal univalent	No.	Mean % ± S.E.	Inhibition %
Control (corn oil).							
Single treatment	1	12	4	1	17	3.4 ± 0.24	
NB 300		36	16	10	62	12.4 ± 0.44**	
NB + PSO 100		21	15	4	40	8.0 ± 0.7	35.48
NB + PSO 200		18	11	5	34	6.8 ± 0.54♦*	45.16
NB + PSO 400		16	11	4	31	6.2 ± 0.31♦♦	50.00
Repeated treatment							
NB + PSO 100	7	15	15	2	32	6.4 ± 0.5♦♦	48.38
NB + PSO 200		12	10	5	27	5.4 ± 0.58♦♦	56.45
NB + PSO 400		16	6	3	25	5.0± 0.37♦♦	59.67

The total number of scored metaphases is 500 (5 animals / group)

** Significant at 0.01 level (t-test) comparing to control (non-treated)

♦ Significant at 0.05 level (t-test) comparing to treatment

♦♦ Significant at 0.01 level (t-test) comparing to treatment

Table 5: Number and percentage of different types of sperm shape abnormalities in male mice after treatment with different doses of PSO plus NB.

Treatment and doses (mg/kg b.wt.)	Examined sperm No.	No. of sperms with Head abnormalities					Tail abnormalities	Abnormal sperm No	Abnormal sperms Mean % ± S.E..	Inhibition %
		Amorphous	Triangle	Without hook	Small	Big	Coiled			
Control (corn oil)	5000	58	23	27	17	5	18	148	2.96+0.25	
PSO100	5000	75	42	44	23	13	13	210	4.20+0.25	
PSO 200	5000	70	33	31	19	17	21	191	3.82+0.37	
PSO 400	5100	55	25	33	13	9	15	150	2.94+0.22	
NB 300	5006	124	96	65	40	26	60	411	8.2 1± 0.21 **	
NB + PSO 100	5000	95	51	80	13	11	20	270	5.4+00.43 ♦	34.23
NB + PSO 200	5050	84	61	33	24	19	30	251	4.97 +0.72 ♦	39.46
NB + PSO 400	5004	75	45	26	19	17	30	212	4.23 ± 0.5.5♦♦	48.47

** Significant at 0.01 level (t-test) comparing to control (non-treated)

♦ Significant at 0.05 level (t-test) comparing to treatment

♦♦ Significant at 0.01 level (t-test) comparing to treatment

significant reduction ($p < 0.01$) in the percentage of chromosome aberrations in somatic and germ cells induced by NP.

Repeated Treatment: Repeated treatment with the tested doses of PSO caused no significant alternations in the percentage of chromosome aberrations when compared with the control (Table 1, 2). The mean percentage of aberrant cells in animals treated with the different doses of PSO decreased significantly ($p < 0.01$). A 69.09% and 59.67% reduction in the percentage of chromosome aberrations in somatic and germ cells respectively was observed in animals treated for 7 consecutive days with 400 mg PSO /kg b.wt. (Tables 3, 4).

Sperm Shape Abnormalities: The percentage of sperm abnormalities reached 4.20 %, 3.82 %, 2.94% after treatment with the 3 tested doses of PSO respectively compared with 2.96 % for the control group (corn oil).

The results in table 5 also, demonstrate the percentage of sperm abnormalities was significantly reduced in all group of mice treated simultaneously with PSO and NP at the tested dose level.

DISCUSSION

Pomegranate seed oil consists of more than 80% conjugated fatty acids, the most important of which is octadecatrienoic acid and punicic acid. Punicic acid is

cytotoxic to mouse leukemia cells [24,25]. Also, PSO contains large amounts of 9cis, 11trans, 13transconjugated linolenic acid. Various conjugated linolenic acids have been shown to inhibit the growth of transplanted cancer cells or to exert cancer cell killing activity; in vitro. It is able to reduce tumor occurrence in mice and rats up to 87 % [10,11,26].

The current paper is focusing on safety assessment of PSO as well as its protective role against the genotoxicity of NB using different mutagenic end points (chromosome aberrations, in somatic and germ cells and sperm abnormalities).

The percentage of chromosome aberrations in animals treated with the tested doses of PSO in both somatic and germ cells are not significantly different from these in control animals. This results reveal that PSO had no genotoxic effect on somatic and germ cells. Meerts *et al.* [27] observed that PSO did not induce an increase in the number of reverting colonies in all strains tested in the absence and presence of metabolic activation. Therefore, it is not mutagenic in the Ames test.

The results indicated that the mean percentage of chromosome aberrations induced with 300 mg NP /kg b.wt. (after single treatment) reached 11.0 % and 12.4 % ($p > 0.01$) compared with 3.2% and 3.4% for the control in somatic and germ cells respectively. According to Bonacker *et al.* [28] NB induced genotoxicity in mammalian cells, with predominantly aneugenic activity. Chromosome aberrations are indirect effects of NB which give rise to DNA breakage involving either single or double strands such as promotion of free radical reactions in cells and/ or interference with DNA and protein synthesis [29]. Also, Guo *et al.* [30] found that chromosome aberrations frequencies of soybeans roots tip cells increased with NB concentration increasing from 5 to 50 mg/L, they suggested that NB had genotoxicity on soybean tip cells.

Vitamins play a beneficial role against the mutagenicity of some chemicals [31, 32]. PSO, as a natural product, used to minimize the genotoxicity of NB in somatic cells, germ cells and sperm abnormalities of mice. The obtained results revealed that the percentage of chromosome aberrations induced by NB decreased to a significant extent when mice were treated with NB plus PSO in somatic and germ cells. Also, the results indicated that repeated treatment with the highest dose of PSO (400 mg/ kg b. wt.) has the maximum protective effect against the chromosome aberrations induced by NB. The inhibition percentage reached 69.09 % and 59.67 % in mouse bone marrow cells and spermatocytes respectively.

Numerous studies have confirmed that NB is a testicular toxicant, with the most sensitive spermatic end-points being sperm count and motility, followed by progressive motility, viability and presence of abnormal sperm with, finally, the fertility index and tailless sperm meiosis of secondary spermatocytes being suppressed [33, 34]. The results showed that the different doses of PSO induced a significant reduction ($p < 0.01$) in the percentage of sperm abnormalities. It reached 34.23%, 39.46 %,and 48.47 % after treatment with the different doses of PSO respectively. PSO possess antioxidant and radical-scavenging properties which is related to the effect of flavonoids and phenolic compounds beside other components having major effects, such as triterpenoids, gamma-tocopherol, 17-aestradiol, estrogens ‘‘estrone and estriol’’, testosterone, b-sitosterol, coumesterol, campesterol, stigmasterol, punicic acid and flavonoids ‘‘genistein and diadzein’’ [7,35].

Overall, pomegranate seed oil appears to be a benign natural product with potential chemopreventive effect against different mutagens. More in-depth investigations, including clinical studies, are warranted to evaluate this hypothesis further.

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