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The protective role of grape seed extract against chronic toxicity of benzene in swiss albino mice

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Abstract. Benzene is an aromatic hydrocarbon often used for industrial purposes. The objective of the present study was to investigate the possible *in vivo* protective effects of grape seed extract (GSE) against benzene-induced toxicity in Swiss albino mice. 36 mice were divided into six groups of six animals of each, having free access to food and water *ad libitum*. The control group received distilled water alone, whereas mice in the treatment groups received GSE alone (50 or 150 mg/kg of body weight), benzene alone (250 mg/kg of body weight), or GSE + benzene for 50 weeks. All mice (100%) survived until the end of experiment and were sacrificed at the end of 24 hours. Bone marrow tissue was analyzed for micronucleus (MN) frequency, chromosomal aberrations (CAs), and pathological damages. Benzene alone–treated mice presented higher frequencies (P<.05) of MNs, CAs, and abnormal metaphases compared with the controls; moreover, the mitotic index was lower than in controls (P<.05). Oral treatment with GSE significantly ameliorated the indices of hepatotoxicity, nephrotoxicity, lipid peroxidation, and genotoxicity induced by benzene. Both doses of GSE tested provided significant protection against benzene induced toxicity, and its strongest effect was observed at the dose level of 150 mg/kg of body weight. Consequently, it was found that GSE has a significant protective effect against benzene-induced toxicity, and its protective effect is dose dependent.

Keywords: Benzene, chromosomal aberrations, grape seed, micronucleus, oxidative stres.

1. Introduction

Benzene, a volatile, colorless, highly flammable liquid, was first discovered in 1825 [1]. Benzene is an important pollutant compound, present in both occupational and general environment [2]. It is a clastogenic and carcinogenic agent [3]. It is classidied as a "Known" carcinogen "Category A" under the risk assessment Guidelines of 1986 [4]. Many industrial applications have been found for benzene, and since its discovery it has been widely used as an industrial solvent [1]. Potential exposure to benzene can be higher in certain industries, such as the plants for the production of organic chemicals, shoe factories and leather manufacturing, printing companies, elevator manufacturing, petrol stations and the petrochemical industry [2]. The main sources of environmental exposure to benzene are gasoline, automobile exhaust, and diesel fuel. Furthermore, lifestyle factors, such as smoking is the main source of benzene exposure for many people [1]. It can cause serious, negative health effects in humans depending upon both the amount and duration of the exposure [3]. Humans with acute inhalation exposure to benzene may experience drowsiness, dizziness, headache and a host of eye, skin, and respiratory tract irritations. At higher levels, people may even fall unconsciousness [5]. Chronic

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exposure at high concentrations might cause erythroid leukemia, non-Hodgkin's lymphomas, myelomas, and myelogenous leukemias in humans, and also it is known to induce structural and numerical changes in lymphocytes and bone marrow cells of man [6,7]. Benzene can cause an increase in the frequency of DNA double-strand breaks, which may remain unrepaired or result in the initiation of DNA recombinational repair mechanisms. It is also affects many enzyme activities in the liver and other tissues [3]. Benzene, being a lipid soluble, is transported through the blood and absorbed by red cell membranes. It tends to accumulate in tissues with high lipid content and about 50% of the absorbed dose is eliminated, unchanged, through exhalation [5]. In experimental animals, benzene is a potent bone marrow toxin and carcinogen. In animals, benzene induces genotoxic and cytotoxic damage in bone marrow presumably via toxic metabolites [8]. Many experimental animal studies, both inhalation and oral, also support the evidence that exposure to benzene increases the risk of cancer in multiple organ systems including the haemopoietic system, oral and nasal cavities, liver, fore stomach, preputial gland, lung, ovary and mammary gland [4]. Tumors reported in mice exposed to benzene include malignant lymphoma, pulmonary adenoma/carcinoma, Zymbal gland squamous cell carcinoma, Harderian gland adenoma, preputial gland squamous cell carcinoma, ovarian neoplasms, and mammary gland carcinoma or carcinosarcoma [6]. In animal tests, adverse effects on the developing fetus have also been observed [5].

The use of certain materials may help to decrease the toxicity created by benzene. Recently, biopolymer materials such as zinc, selenium and leaf extract of *Ocimum basilicum* L. have been used for specially decreasing the toxic effects of benzene [9,10]. Grape seed extract (GSE) is a natural extract obtained from the seed of grape [11]. Grapes and grape products are good sources of dietary flavonoids, which are powerful antioxidant compounds [12]. Grape seed's antioxidative activities are much stronger than those of vitamin C and vitamin E and may involve radical scavenging, quenching, and enzyme-inhibiting actions. GSE is a complex mixture of polyphenols containing dimers, trimers, and other oligomers (procyanidins) of catechin and epicatechin and their gallate derivatives together known as the proanthocyanidins [13]. Beyond their antioxidant powers, proanthocyanidins may protect against atherosclerosis, gastric ulcer, large bowel cancer, cataracts, and diabetes. Thus, grape seed and GSE may benefit people with heart disease and cancer [14]. GSE also is marketed as a dietary supplement in countries such as the United States and Turkey, owing to several health benefits mainly attributed to its antioxidant property. Several experimental studies have demonstrated that GSE is highly bioavailable and provides significantly greater protection against free radicals and free radicalinduced lipid peroxidation and DNA damage than vitamins C and E and b-carotene. GSE was also shown to demonstrate cytotoxicity towards human breast, lung, and gastric

adenocarcinoma cells, while enhancing the growth and viability of normal human gastric mucosal cells [15].

The aim of the present study was to investigate the protective role of GSE against chronic toxicity induced by benzene in mouse model.

2. MATERIALS AND METHODS

2.1 Animals

A total number of 36 adult male *Mus musculus* var. *albino* mice weighing 25-30 g were used in the current study. Healthy mice were obtained from the Animal Research Center of the Refik Saydam Hifzissiha Institute (Ankara, Turkey). All animals were housed in 26 x 15 x 50 cm stainless steel cages and kept under controlled laboratory conditions of 22±3 ^{o}C and 55±5% relative humidity with a 12-hour light–dark cycle throughout the experiment. The animals were allowed to acclimatize for 1 week before the planned experimental test and fed a standard pellet diet (Samsun Food Industry, Samsun, Turkey) and water *ad libitum*. In this study, the methods and techniques applied to mice were carried out according to the guidelines set by the World Health Organization (Geneva, Switzerland) and the ethical standards of the local ethical committee for animal experiments at Giresun University (Giresun, Turkey).

2.2 Products and Chemicals

GSE (formerly Grape Seed PCO Phytosome 50–120 tabs) was obtained from Health Genesis Corp. (Bay Harbor Island, FL, USA). Benzene [Sigma, St. Louis, MO, USA (CS reagent, ≥99.0%)] was purchased from Biostar A.S.(Ankara). Colcemid solution (10 lg/mL, catalog number 12-004-1) was purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). Fast Green FCF (Sigma, St. Louis, MO, USA) and Grunwald Giemsa (Sigma) stains were purchased from Biostar A.S.(Ankara).

2.3 Experimental Protocol

The animals were divided into six groups each containing six mice. Group I, controls orally treated with distilled water for 50 consecutive weeks; Group II, orally treated with 50

mg/kg of body weight GSE for 50 consecutive weeks; Group III, orally treated with 150 mg/kg of body weight GSE for 50 consecutive weeks; Group IV, orally treated in drinking water with 250 mg/kg of body weight benzene for 50 consecutive weeks; Group V, orally treated with 50 mg/kg of body weight GSE +250 mg/kg of body weight benzene for 50 consecutive weeks; and Group VI, orally treated with 150 mg/kg of body weight GSE + 250 mg/kg of body weight benzene for 50 consecutive weeks. For Groups V and VI, GSE application was started 7 days before exposure to benzene and continued together with benzene for 50 consecutive days. The dose of benzene used in this study was determined to be 250 mg/kg. This dose was chosen because it induced an increase in the frequency of toxicity that was essential to determine the protective role of GSE [16]. Also, GSE doses were chosen that were comparable to those daily consumption amounts recommended by practitioners of nutritional medicine to support optimal health, and 50 and 150 mg/kg of body weight were effective doses for protection by GSE [17].

2.4 Erythrocyte Micronucleus Assay

The mouse erythrocyte MN assay, a modified mouse MN test that conventionally scores the MN frequencies in bone marrow polychromatic erythrocytes, was used throughout this study. In this assay, the MN frequency is scored in mature normachromatic erythrocytes in the circulating blood obtained from the tail of the mouse. The mouse erythrocyte MN assay was performed according to the protocol of Te-Hsiu et al [18]. In brief, each mouse was anesthetized with ether, and blood samples were collected from a small puncture on the tail vein of the mouse. Approximately 5 μL of peripheral blood collected from each mouse was immediately mixed with 3% EDTA solution in a cell well and smeared onto a clean glass slide. The erythrocytes were fixed in 70% cold methanol for 2 minutes, and the slides were allowed to air-dry overnight at room temperature. Then, smears of blood were stained with 5% May–Grunwald Giemsa for 15 minutes. Usually three slides were made and repeatedly double scored by two different observers. From the prepared slides, a total of 1,000 normachromatic erythrocytes were scored for the presence of MNs under oil immersion at x100 magnification using a binocular light microscope (model BX51, Olympus, Tokyo, Japan), and MNcells were photographed at a magnification of x500 [19].

2.5 MN Assay For Exfoliated Cell

The mice were fainted under ether anesthesia. Before sample collection, the mouth of each mouse was washed with tap water. After rinsing the mouth with tap water, the exfoliated cells were obtained by scraping the right/left buccal mucosa with a moist wooden spatula. The scraped cells were placed onto pre–cleaned slides and air–dried during 15–30 min. Then cells were fixed using 3:1 methanol: acetic acid (v/v) for 10 min. The slide smears were stained with Feulgen and counter–stained with Fast Green as described in literature [20]. From each slide, 1000 exfoliated cells were scored for the presence of MN as described above for erythrocytes. Mono-nucleated cells for each mouse were analyzed for the presence of MN using the scoring criteria described by Fenech et al. [21] : (i) the diameter of MN should be less than one–third of the main nucleus, (ii) MN should be separated from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary, (iii) MN should have similar staining as the main nucleus.

2.6 Preparation of Bone Marrow Cells For Chromosome Analysis

Mice were injected intraperitoneally with 0.025% colcemid before killing and sacrificed 2 hours later under ether anesthesia. The bone marrow from the femur was aspirated, washed in physiological saline, treated hypotonically (0.075M KCl), fixed in Carnoy's fixative, and stained with 5% Grunwald Giemsa stain [22]. CAs were scored using x100 oil immersion lens in randomly selected areas under the light microscope (model BX51, Olympus). CAs were classified according to the classification developed by Savage [23].

2.7 Mitotic Index, Abnormal Metaphase Number and CAs Analysis

The mitotic index (MI) was determined as the percentage of dividing cells among 1,000 nucleated cells in slides prepared for each group. The number of aberrant metaphases (AMNs) was counted as the number of damaged metaphases among 100 metaphases in slides prepared for each group. For all treatment groups 100 metaphases were counted for CAs such as chromatid breaks, fragments, and gaps, and they were considered to be equal.

2.8 Histopathological Examinations

For light microscopic examination, fresh tissue samples including the liver and kidneys were fixed in 10% neutral buffered formalin solution for routine processing, embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin (H-E). Histopathological changes were semiquantitatively assessed under the light microscope with an ocular with grids and 4x, 10x, and 40x objectives.

2.9 Statistical Analysis

The statistical analysis software SPSS for Windows version 10.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical data analysis. Statistically significant differences between the groups were compared using one-way analysis of variance (one-way ANOVA) and Duncan's test. The data were given as mean ±SD values, and values of $P < .05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

The frequency of MNs are presented in Table 3.1. and 3.2. There were no statistically significant differences in the MN numbers of erythrocytes and exfoliated cells of the control and GSE-treated groups ($P>.05$). The mean number of MNs was, for the control group, 0.33±0.52 in erythrocytes and 0.00±0.00 in exfoliated cells, for the 50 mg/kg of body weight GSE group, 0.00±0.00 in erythrocytes and 0.00±0.00 in exfoliated cells, and for the 150 mg/kg of body weight GSE group, 0.17±0.41 in erythrocytes and 0.00±0.00 in exfoliated cells. The frequency of MNs clearly increased in the benzene-treated mice, and the mean number of micronucleated cells was significantly higher in the benzene only-treated group than in the controls and GSE-treated groups ($P<.05$). In addition, the number of MNs was greater in the erythrocytes than in the exfoliated cells of buccal mucosa. The mean number of MNs, for the 250 mg/kg dose of benzene, was 59.33±10.03 in erythrocytes and 41.67±4.93 in exfoliated cells. The treatment of animals with GSE doses (50 and 150 mg/kg of body weight) showed a significant reduction ($P<.05$) in the frequency of MNs when compared with the group treated with benzene alone. The frequency of MN decreased with the rise in GSE dose. The MN frequency in the mice treated with the 150 mg/kg of body weight dose of GSE was lower than those in mice treated

with the 50 mg/kg of body weight dose of GSE. In brief, there was a highly significant dose–effect relationship between the MN frequency and GSE dose.

Table 3.1. Effects of grape seed exctract on the frequency of micronuclei induced by benzene in mouse erythrocytes

Groups	Number of cells scored	Minimum	Maximum	Average±SD
Group I (Control)	1000	0	1	0.33±0.52[d]
Group II (GSE 50)	1000	0	0	0.00±0.00[d]
Group III (GSE 150)	1000	0	1	0.17±0.41[d]
Group IV (Benzene)	1000	45	70	59.33±10.03[a]
Group V (GSE 50+Benzene)	1000	35	54	44.00±7.18[b]
Group VI (GSE 150+Benzene)	1000	25	40	31.00±5.44[c]

*Data are mean ± SD values (n = 6). GSE was used at 50 or 150 mg/kg of body weight, and benzene was used at 250 mg/kg of body weight. Statistical significance between means was assessed using one-way analysis of variance followed by Duncan's test as a post-analysis of variance test.
[abcd]Within a column, means not sharing the same letter are significantly different (P < .05).

Table 3.2. Effects of grape seed exctract on the frequency of micronuclei induced by benzene in exfoliated cells of buccal mucosa in mice

Groups	Number of cells scored	Minimum	Maximum	Average±SD
Group I (Control)	1000	0	0	0.00±0.00[d]
Group II (GSE 50)	1000	0	0	0.00±0.00[d]
Group III (GSE 150)	1000	0	0	0.00±0.00[d]
Group IV (Benzene)	1000	37	50	41.67±4.93[a]
Group V (GSE 50+Benzene)	1000	27	37	31.67±3.98[b]
Group VI (GSE 150+Benzene)	1000	15	25	19.50±3.27[c]

*Data are mean ± SD values (n = 6). GSE was used at 50 or 150 mg/kg of body weight, and benzene was used at 250 mg/kg of body weight. Statistical significance between means was assessed using one-way analysis of variance followed by Duncan's test as a post-analysis of variance test. [abcd]Within a column, means not sharing the same letter are significantly different (P < .05).

Table 3.1. presents the results of CAs analysis in mitotic chromosomes of bone marrow cells of Swiss albino mice treated with benzene alone, GSE alone, and GSE+benzene. The mice treated with 50 and 150 mg/kg of body weight doses of GSE did not show any significant difference in the total number of CAs, AMNs, and MI compared with the control group (P> .05). According to the classification criteria proposed by Savage [23], six structural CAs were detected in the treatment groups. We observed a high frequency of CAs such as chromatid break, chromosome break, acentric fragments, dicentric, gaps, and rings in mouse bone marrow cells. The mean number of chromatid breaks was greater than those of the other CAs. Treatment of these animals with benzene resulted in the induction of CAs and AMNs. As expected, mice

treated with benzene presented high frequencies of the total number of both CAs and AMNs compared with the controls (P< .05). A considerable decrease in MI after benzene exposure also was observed, and Duncan's test showed that this decrease was statistically significant (P< .05). However, treatment with GSE of mice exposed to benzene resulted in a significant reduction in the frequency of CAs and AMNs. The number of CAs and AMNs showed a tendency to decrease in mice treated with 50 and 150 mg/kg of body weight doses of GSE compared with the benzene only–treated group. With the 150 mg/kg of body weight dose of GSE, this reduction for CAs, such as chromatid and chromosome breaks was by at least twofold compared with the benzene group. In addition, the MI again presented a rising trend.

Table 3.3. Mitotic index and distribution of different types of chromosomal aberrations and abnormal metaphases observed in mouse bone marrow cells pretreated with grape seed exctract alone or in combination with benzene

CAs type	Groups					
	I	II	III	IV	V	VI
Chromatid break	00.33±0.52^{d}	00.17±0.41^{d}	00.33±0.52^{d}	51.67±5.32^{a}	39.33±6.80^{b}	25.17±3.97^{c}
Break	00.00±0.00^{d}	00.00±0.00^{d}	00.00±0.00^{d}	37.33±4.37^{a}	28.50±4.51^{b}	17.00±4.15^{c}
Acentric fragment	00.33±0.52^{d}	00.50±0.55^{d}	00.33±0.52^{d}	21.67±4.55^{a}	14.50±3.94^{b}	07.33±2.34^{c}
Dicentric	00.00±0.00^{d}	00.00±0.00^{d}	00.00±0.00^{d}	18.33±4.13^{a}	11.67±3.67^{b}	05.50±2.81^{c}
Gaps	00.83±0.75^{d}	00.50±0.55^{d}	00.67±0.52^{d}	11.00±2.53^{a}	07.33±1.97^{b}	02.67±1.03^{c}
Rings	00.00±0.00^{d}	00.00±0.00^{d}	00.00±0.00^{d}	06.50±2.43^{a}	3.83±1.47^{b}	1.50±0.55^{c}
AMNs (%)	2.83±0.75^{d}	2.33±0.52^{d}	1.67±0.52^{d}	51.83±3.12^{a}	40.50±3.94^{b}	29.50±3.39^{c}
MI (%)	825.83±34.49^{a}	817.00±38.51^{a}	830.17±47.05^{a}	487.83±13.32^{d}	553.67±33.57^{c}	634.17±25.66^{b}

*Data are mean ± SD values (n = 6). GSE was used at 50 or 150 mg/kg of body weight, and benzene was used at 250 mg/kg of body weight. One hundred cells were analyzed per animal (six animals per group, for a total of 600 cells per treatment) for chromosomal aberrations and abnormal metaphases (AMNs). The mitotic index (MI) was calculated by analyzing 1,000 cells per animal (for a total of 6,000 cells per treatment), and percentage of the MI was calculated for each treatment group. Statistical significance between means was assessed using one-way analysis of variance followed by Duncan's test as a post-analysis of variance test.
[abcd]Within a column, means not sharing the same letter are significantly different (P < .05).

There was a dose–effect relationship between the MN frequency and dose of GSE. In previous studies, a significant increase in MN frequency after exposure to chronic doses of benzene was determined [6,8]. Also, benzene significantly induced mitotic abnormalities such as acentric fragments, dicentric, gaps, and rings. The most common abnormality observed in the present study was chromatid breaks. Pretreatment with oral doses of GSE caused a significant reduction in the frequency of MNs and CAs compared with the group treated with benzene alone. Morgan and El-Tawil [24] investigated the possible *in vivo* protective effects of GSE

against acrylonitrile-induced MN and CAs in male rats. They indicated that acrylonitrile significantly induced MN and CAs. Pretreatment with GSE significantly improved these mutagenic effects in a dose related manner. In addition, Yalcin et al.[17] showed that GSE significantly protected bone marrow chromosomes from doxorubicin-induced genotoxicity by reducing the total number of abnormal metaphases and the frequency of structural CAs. Besides, GSE treatment decreased the frequency of MNs and increased the mitotic index values.

The protective effect of GSE on chronic toxicity induced by benzene may be explainable with the antioxidant capacity of GSE, which is a vegetable product. A number of previous studies have shown that proanthocyanidin-rich extracts from grape seeds contain different types of polyphenolic components such as dimers, trimers, tetramers, and oligomers and monomeric flavanols like catechins and epicatechin derivatives that have antioxidant, anticancerogenic, and anti-inflammatory properties [14]. Also, many investigators have demonstrated the efficacy of GSE as an inhibitor of lipid peroxidation and as a powerful free radical scavenger in vitro as well as in vivo. Similarly, GSE was found to reduce the oxidation of polyunsaturated fatty acids in mouse liver microsomes and provide protection against lipid peroxidation and DNA fragmentation in mice [25]. Zhao et al. [26] suggested that dimerization and trimerization of monomers (catechins and epicatechins) could lead to anticarcinogenic effects in skin tumorigenesis possibly because of increase in their antioxidant activity. In a similar study, Arii et al. [27] have shown that oral administration of GSE in diet to mice inhibits APC mutationassociated intestinal adenoma formation. Raina et al. [28] showed that GSE inhibits in vivo growth of DU145 xenograft in nude mice via an inhibition of cell proliferation and an induction of apoptosis. In another study, researchers showed that oral administration of GSE exhibited marked amelioration of lipid peroxidation, antioxidative enzymes, and rate of hemolysis which returned to normal values during the course of GSE treatment [29].

CONCLUSION

In this study the cytotoxicity and genotoxicity of benzene were showed. Also the protective effect of GSE was determined. Antioxidant activity of GSE might be responsible for the protection against chronic toxicity induced by benzene. Therefore, the antioxidant role of GSE might be used as a ‘‘toxicity-limiting agent’’ to reduce effects on human health of chemical agents in the near future.

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