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Investigation on the Protective Effect of Grape Seed and Linseed Oils Against Cyclophosphamide Induced Genotoxicity in Mice

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Abstract: The protection conferred by grape seed oil and linseed oil against cyclophosphamide induced bone marrow chromosomal aberrations and sperm abnormalities as well as DNA fragmentation had been evaluated in adult Swiss albino mice. Cyclophosphamide induced genotoxicity indicated by increased number of aberrant cells and different types of structural chromosomal aberrations (gap, break, fragment and deletion) and numerical aberrations (hypoploidy and hyperploidy). In sperm morphology cyclophosphamide induced sperm abnormalities for both head and tail abnormalities. Pretreatment with grape seed oil (0.1ml/kg b.w. /day) or linseed oil (0.1ml/kg b.w. /day) for 14 days prior to an interperitoneal dose of cyclophosphamide (25mg/kg b.w) reduced ($P<0.05$) the number of chromosomal aberrations and sperm abnormalities and also reduced the percentage of DNA fragmentation caused by cyclophosphamide. It could be concluded that each of grape seed oil and linseed oil acts as a potent antioxidant that prevented genotoxicity of bone marrow cells and sperm abnormalities as well as DNA fragmentation.

Key words: Grape seed oil · Linseed oil · Chromosomal aberrations · DNA fragmentation · Sperm morphology

INTRODUCTION

Exposure to various environmental factors can lead to free radical formation. The most common form of free radicals is oxygen. When an oxygen molecule (O2) becomes electrically charged, it tries to steal electrons from other molecules, causing damage to the DNA and other molecules and therefore cause mutagenesis. A critical factor in mutagenesis is cell division [1]. When the cell divides, an unrepaired DNA lesion can give rise to a mutation. To protect against oxidative damage, animals have many different types of antioxidants defenses, these antioxidants decrease mutagenesis and thus carcinogenesis, in two ways: by decreasing oxidative DNA damage and by decreasing cell division [2].

Grape seed oil and linseed oil were reported to have antioxidant activities [3, 4]. Grape seed oil is a vegetable oil pressed from the seeds of various varieties of *vitis vinifera* grapes. Grape seeds contain antioxidants (polyphenols, including proanthocyanidins) [5], sufficiently high amounts of resveratrol which can be extracted commercially [6]. Resveratrol (3,5,4-trihydroxystilbene) a polyphenolic phytoalexin, primarily found in the skin in muscadine grapes as well as in the seeds [7]. Also, grape seed oil contains nutritionally useful essential fatty acids and tocopherols (vitamin E) [8]. Moreover, it is a highly concentrated source (76%) of linoleic acid [9]. Some studies suggested the use of grape seed oil as a chemopreventive and cytoprotective agent [10]. It was found that grape seed extract can protect bone marrow chromosomes against genotoxic substances by reducing the total number of aberrant cells and different types of structural chromosomal aberrations [3], other studies demonstrated no toxicity and mutagenecity for this substance [11]. Also, grape seed extracts preventing damage to human liver cells caused by chemotherapy medications [12].

Linseed oil is derived from the dried ripe seeds of the flax plant (*Linum usitatissimum*, Linaceae). Regular Linseed oil contains between 52 and 63 % alpha linolenic acid [13]. The linolenic acid has beneficial effect in reducing inflammation leading to atherosclerosis. Linseed oil may reduce cardiovascular risk through platelet function and inflammation [14], has positive effect on femur bone mineral content, bone mineral density and lumbar vertebrae [15]. Linseed oil supplementation was found to achieve a greater reduction in lung and total metastases [16]. High doses of linseed oil could also delay in the growth of mammary cancers [17].

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In this study, the modulatory effects of grape seed and linseed oils were examined through monitoring the incidence of cyclophosphamide (CP) induced genotoxicity in mice bone marrow, sperm abnormalities as well as DNA fragmentation.

MATERIALS AND METHODS

Experimental Animals and Treatments: Ninety male adult Swiss albino mice weighing between 20-25 grams were used in this study. These animals were obtained from the Animal House of the National Research Center. All animals were fed a common diet and water *ad libitium*. Animals were divided into six separated groups. Each group contains 15 mice. Animals of each group were divided into two subgroups; 10 animals for the chromosomal aberrations analysis and DNA fragmentation and 5 animals to be sacrificed at the day 35 from the last treatment or injection and then examined for sperm shape and count.

Group 1 was the untreated control. Animals of group 2 were intraperitoneal injected with single dose of cyclophosphamide (25 mg/kg body weight) according to Shukla and Taneja [18] and Seehy [19] and considered as positive control.

Animals of group 3 orally administered grape seed oil (0.1 ml/animal/day) based on Al-Attar [20] and group 4 received linseed oil (0.1ml/animal/day) based on Bhatia [4] for 14 consecutive days.

Animals of group 5 and 6 orally administered grape seed oil and linseed oil for 14 consecutive days prior to interaperitoneal (i.p) injection with cyclophosphamide (25mg/kg body weight), respectively. Animals then were scarified after 48 hour.

Materials: Grape seed oil and linseed oil were obtained from the Unit of Squeeze and Extraction of Natural Oils in National Research Center, Dokki, Egypt.

Chromosomal Aberrations for Bone Marrow Cells: The method of Yosida and Amano [21] was used with some modifications. Mice were injected intraperitonealy with colchicine at a final concentration of 3 mg/kg b.wt. Mice were anesthetized with ether 2h. after colchicine treatment and the bone marrow from both femurs was collected in a centrifuge tube containing 0.075 M Kcl. cell suspension were incubated for about 30 min. at 37°C then centrifuged at 1500 rpm for 10 min. the cells were fixed in 3:1 methanol: glacial acetic acid which was freshly prepared. The fixative was added gently to the cells. Fixation was done twice, cell suspension were spread onto slides that had just been removed from the freezer. Five to seven slides were prepared for each mouse. Slides were stained with 10% Giemsa in phosphate buffer ph 6.8. Scanning slides for mitotic metaphase spreads was conveniently accomplished with a 25x magnification objective and analyzed with a 100 X oil objective. Ten mice were taken for each treatment and 100 well spread metaphases were analyzed per mouse.

Sperm Morphology and Count: The method of Wyrobek and Bruce [22] was used for investigating sperm morphology and count. Animals were killed 35 days after the last injection. Sperm were sampled from the caudae epididymis. Both epidiymes from each mouse were minced together with small scissors in physiological saline (0.9%NaCl), pipetted up and down and then filtered in a small test tube. The volume is made up of 2 ml. smears were prepared on clean dry slides. Five mice were taken for each treatment and at least five slides were prepared for each mouse to study sperm abnormalities. The slides were stained with eosin nefrosin stain. Sperm were examined under microscope for each group of animals more than 1000 sperm were examined for morphological abnormalities of the sperm shape. Some drops of the sperm suspension were put on haemocytometer to count the sperm.

DNA Fragmentation: The method of DNA fragmentation was carried out according to Perandones [23]. A bout 0.25g of the liver tissues were mechanically dissociated in 400 µl hypotonic lysis buffer (10mM tris, 1mM EDTA and 0.2% triton X-100, ph 8.0).

The cell lysate was centrifuged at 12.000 Xg for 15 min. the supernatant containing small DNA fragments was immediately separated as well as the pellet containing large pieces of DNA, were used for the diphenylamine (DPA) assay. The pellet was resuspended in 400 µl of hypotonic lysis buffer. 400µl 10% trichloroacetic acid (TCA) was added to both the supernatant and the resuspended pellet and incubated at room temperature for10 min. The tubes were centrifuged at 2000 rpm for 15 min. at 4°C. After discarding the supernatant, the precipitate was resuspended in 400 µl 5% TCA, incubated at 80°C for 30 min. and then allowed to cool at room temperature. After centrifugation, one volume of the extracted DNA was added to two volumes of colorimetric solution (0.088 M diphenylamine (DPA), 98% V/V glacial acetic acid, 1.5%V/V sulphoric acid and 0.5% V/V 1.6% Acetaldehyde solution). The samples were stored at

4°C for 48h. The colorimetric reaction was quantities spectrophotometrically at 578 nm. The percentage of DNA fragmentation was expressed by the formula:

$$\text{DNA fragmentation percentage} = \frac{\text{O.D supernatant}}{\text{O.D supernatant+O.D pellet}} \times 100$$

Statistical Analysis: The experiment followed complete randomized (C R D). The obtained data were subjected to analysis of variance (ANOVA) according to Snedecor and Cochran [24]. Least significant differences (L S D) were used to compare between means of treatments according to Walter and Duncan [25] at probability 5%.

RESULTS

Analysis of Bone Marrow Chromosomal Aberrations: The frequencies of different chromosomal aberrations observed in bone marrow cells of control and all treated groups are presented in Table 1. A significant increase (P<0.05) in the mean frequency of the total aberrations was noted in cyclophosphamide treated group (112.4±2.4) as compared with the control group (12.4±0.98). However, in grape seed oil and linseed oil groups the frequencies of total aberrations were slightly and insignificantly higher (16.8±1.35) and (14.8±1.35) than the control. In addition there was a significant (P<0.05) decrease in the mean percentage of total aberrations of the two combined groups (grape seed oil + cyclophosphamide) and (linseed oil+cyclophosphamide) (68.4±1.720) and (40.0±1.549), respectively as compared with cyclophosphamide group (112.4±2.4).

These results suggested that the two oils had protective effect against cyclophosphamide as monitored by decreasing the incidence of aberrations.

Sperm Morphology: Table 2 represents the abnormalities of sperm which are demonstrated in two types of abnormalities, head and tail abnormalities. The head abnormalities included amorphous, without hock, small, big and banana head. Tail abnormalities included the coiled tail. A significant increase (P<0.05) in the mean frequency of total abnormalities was noted in cyclophosphamide group (4.58±0.196) as compared with the normal control (1.50±0.130). However, in grape seed oil and linseed oil groups the frequencies of total abnormalities were significantly different (1.74±0.051) and (1.64±0.103), respectively than the control. In addition to,

Table 1: Chromosomal aberrations induced by the different types of treatments

	Structural aberrations						Numerical aberrations			
Treatments	Gap	Break	Deletion	Fragment	Centric fusion	Total	Hypoploidy	Hyperploidy	Total	Total aberrations
Control	2.8 c ± 0.490	2.8 d ± 0.490	2.4 b ± 0.748	1.2 d ± 0.490	0.4 ± 0.400	9.6 d ± 1.166	2.8 c ± 0.490	0.0 c ± 0.000	2.8 c ± 0.490	12.4 d ± 0.980
Grape	2.8 c ± 0.490	3.2 d ± 0.490	1.6 b ± 0.400	2.8 d ± 0.490	0.8 ± 0.490	11.2 d ± 0.800	5.2 b ± 0.490	0.4 c ± 0.400	5.6 b ± 0.748	16.8 d ± 1.356
Linseed	1.6 c ± 0.748	0.4 e ± 0.400	4.4 a ± 0.748	2.4 d ± 0.400	1.6 ± 0.400	10.4 d ± 0.980	3.6 bc ± 0.748	0.8 bc ± 0.490	4.4 bc ± 0.748	14.8 d ± 1.356
Cyclo	10.4 a ± 0.748	58.0 a ± 0.894	6.0 a ± 0.894	22.8 a ± 1.020	0.8 ± 0.490	98.0 a ± 2.098	12.4 a ± 0.980	2.0 a ± 0.000	14.4 a ± 0.980	112.4 a ± 2.400
Grape+ Cyclo	8.0 b ± 0.632	28.4 b ± 0.980	5.2 a ± 0.490	20.0 b ± 0.632	0.4 ± 0.400	62.0 b ± 1.414	5.6 b ± 1.166	0.8 bc ± 0.490	6.4 b ± 1.600	68.4 b ± 1.720
Linseed+ Cyclo	2.8 c ± 0.490	12.4 c ± 0.748	2.4 b ± 0.400	15.6 c ± 0.748	0.8 ± 0.490	34.0 c ± 1.549	4.4 bc ± 0.400	1.6 ab ± 0.400	6.0 b ± 0.000	40.0 c ± 1.549

The values represented Mean ± SE

Table 2: sperm abnormalities induced by different types of treatments

	Head abnormalities (%)						tail abnormalities (%)	total	Spermcount
Treatments	Amorphous	Head with hock	Small head	Big head	Banana shape	Total	coiled tail	abnormalities (%)	x10^6
Control	1.04 c ± 0.129	0.20 d ± 0.032	0.04 cd ± 0.025	0.02 b ± 0.020	0.06 b ± 0.025	1.36 c ± 0.154	0.14 b ± 0.025	1.50 c ± 0.130	24.2 a ± 0.41
Grape	1.16 c ± 0.051	0.26 d ± 0.040	0.04 cd ± 0.025	0.04 b ± 0.025	0.04 b ± 0.025	1.54 c ± 0.051	0.20 ab ± 0.032	1.74 c ± 0.051	19.8 b ± 0.19
Linseed	1.14 c ± 0.040	0.22 d ± 0.020	0.02 d ± 0.020	0.06 b ± 0.025	0.04 b ± 0.025	1.48 c ± 0.074	0.16 b ± 0.040	1.64 c ± 0.103	20.4 b ± 0.35
Cyclo	2.40 a ± 0.170	1.10 a ± 0.045	0.38 a ± 0.037	0.16 a ± 0.025	0.26 a ± 0.025	4.30 a ± 0.198	0.28 a ± 0.037	4.58 a ± 0.196	14.6 d ± 0.25
Grape+\ Cyclo	1.32 bc ± 0.097	0.84 b ± 0.025	0.14 b ± 0.025	0.06 b ± 0.025	0.10 b ± 0.032	2.46 b ± 0.093	0.14 b ± 0.025	2.60 b ± 0.078	17.7 c ± 0.49
Linseed+ Cyclo	1.56 b ± 0.068	0.58 c ± 0.037	0.12 bc ± 0.037	0.08 b ± 0.020	0.10 b ± 0.000	2.44 b ± 0.103	0.18 b ± 0.020	2.62 b ± 0.102	17.8 c ± 0.58

The values represented Mean ± SE

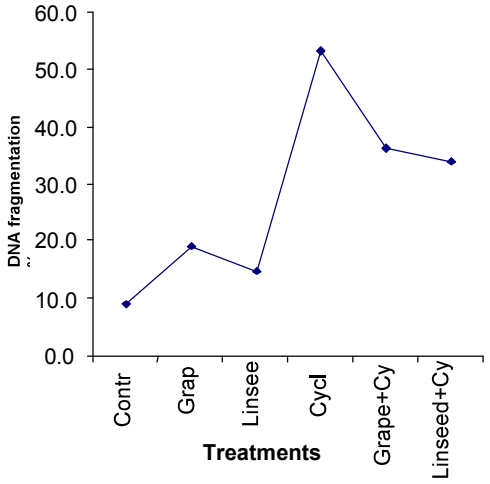

Chart 1: The percentages of DNA fragmentation for all treatments

there is a significant decrease ($P<0.05$) in the mean frequency of the total abnormalities of the two combined groups (grape seed oil+cyclophosphamide) and (linseed oil+cyclophosphamide) (2.60±0.078) and (2.62±0.102), respectively as compared with cyclophosphamide group.

Sperm Count: The frequencies of sperm count were demonstrated in Table 2. There was a significant decrease ($P<0.05$) in the mean frequency of the cyclophosphamide group (14.56±0.251), grape seed oil (19.77±0.187) and linseed oil (20.44±0.350) as compared with the control group (24.16±0.412). However, there were significance increase ($P<0.05$) in the mean frequency of combined group (grape seed oil+cyclophosphamide) (17.66±0.494) and (linseed oil+cyclophosphamide) (17.80±0.583) comparing with the cyclophosphamide group means that these two oils decrease the effect of cyclophosphamide on sperm count.

DNA Fragmentation: The frequency of the mean percentage of DNA fragmentation of the control and all treated groups are presented in chart 1. A significant increase ($P<0.05$) in the mean percentage of cyclophosphamide group (53.22±1.002) as compared with the control (8.99±1.51) was observed. However, in the two combined groups (grape seed oil+cyclophosphamide) and (linseed oil+cyclophosphamide) the frequencies of the mean percentage of the DNA fragmentation were significantly ($P<0.05$) decreased (36.38±4.44) and (33.82±2.19), respectively than the cyclophosphamide group (53.22±1.002).

DISCUSSION

There are several ways to reduce or prevent the action of mutagens. Chemicals which interfere with DNA repair or with mutagen metabolism can be used as effective antimutagens [26]. The present investigation was directed to study the possible protective activity of orally administered grape seed oil and linseed oil against cyclophosphamide induced cytotoxicity and genotoxicity towards mouse somatic cells and sperm morphology *In vivo*.

Pretreatment of mice with grape seed and linseed oils for 14 days and simultaneously with a single dose of cyclophosphamide significantly reduced the frequency of chromosomal aberrations of bone marrow cells and sperm abnormalities as well as DNA fragmentations. These results agree with Asita *et al.* [27] who reported that grape seed inhibit the mutagenic effects of cyclophosphamide and/or other mutagens in mice and rats, both in *vitro* and/or *In vivo*. Grape seed extract acts as a potent antioxidant prevented genotoxicity of bone marrow cells by reducing total number of aberrant cells and different types of structural chromosomal aberrations caused by mutagn [28].

Linseed oil also protect against cyclophosphamide-induced oxidative stress. Oxidative stress refers to the cytotoxic consequence of reactive oxygen byproducts: superoxide anions and hydroxyl radicals which are generated as metabolites of normal and aberrant metabolic processes that utilize molecular oxygen [4].

The protection afforded by linseed oil might be due to the antioxidative action of its important constituents, the lignans, glycosides of secoisolariciresinol as the major lignan, together with small amounts of matairesinol, isolariciresinol and pinoresinol. The lignan like secoisolariciresinol and pinoresinol were reported to have strong antioxidant nature [29].

Grape seed extracts prevented DNA oxidative damage in various tissues and DNA fragmentation induced by many agents [28, 30, 31].

Ray *et al*. [32] suggested that *In vivo* protection of DNA by Grape seed extracts might be due to detoxification of cytotoxic radicals and presumed contribution to DNA repair. The cause of the antigenotoxic effect of the grape seed extracts is the presence of a lot of biological active compounds in it, mainly antioxidants. From a cellular perspective, one of the most advantageous features of proanthocyanidins oligomers free radical scavenging activity is chemical structure, it is incorporated within cell membranes. This

physical characteristic along with its ability to protect against both water-and fat-soluble free radicals provides incredible protection to the cells against free radical changes on chromosomes [33].

Many authors found that genotoxicity and chromosomal instability induced by many agents are straightly correlated with the parameters of oxidative stress [34, 35].

Antigenotoxic activity of grape seed extracts is not sudden; it is well known that consumption of fresh fruits and vegetables is associated with decline in incidence of oncology [36]. The condition was attributed to many biologically active compounds which can trap the aggressive metabolites of carcinogens. It is well known that many carcinogens/mutagens act via radical mechanisms and hence damaging biologically important molecules, DNA in the first turn [37].

These experiments demonstrated that these two oils reduced the abnormalities of sperm. Linseed oil contains omega-3 fatty acids, especially alpha-linolenic acid. The deficient intake of this fatty acid is correlated with impaired sperm motility among subfertile men and cause sperm abnormalities. Lignans are precursors of enterolacton, which inhibits aromatase and reduces the ratio of 16-OH over 2-OH oestrogen metabolites. The resulting reduction in oestrogen load may favourably influence Sertoli cell function [38].

It was concluded that grape seed oil and linseed oil provided a protection against cyclophosphamide induced genotoxicity, however these oils may scavenge free radicals produced by mutagens and thus inhibit damage to cellular DNA. So it is recommended to eat balanced diet rich in natural antioxidants which must be taken in a moderate amounts to avoid its opposite effect.

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