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Genotoxic and histopathological aspects of treatment with grape seed extract on cancer induced with cyclophosphamide in mice

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Abstract: Cyclophosphamide (CP) is an alkylating agent widely used in cancer chemotherapy**.** The present study was undertaken to evaluate the natural protective efficacy of methanolic grape seed extract (GSE) against CP-induced genotoxicity and histopathologically changes of liver and colon cancer cells in mice. After the mice were sacrificed on day 30 liver and colons were removed, the histopathological studies and expression of colon cyclooxygenase-2(COX-2), was immunohistochemically assessed. High dose of GSE had a significant reduction of micronuclei and chromosomal aberration frequency than low dose, Mice treated with grape seeds (low & high) dose displayed an improvement in colonic histopathology compared to cyclophosphamide-only mice. Histopathological results showed the presence bridging necrosis with early fibrosis between portal tract and central veins with dysplastic hepatocytes in the liver tissue, also cellular infiltration, epithelial disruption, aberrant changes in the form neoplasia, dysplasia of the colonic mucosa. However, grape seed extract treatment to cyclophosphamide treated rats greatly restored normalcy in the liver and colonic histoarchitecture, with no apparent signs of neoplasia or displisia**.** Immunohistochemistry of COX-2 showed an intense staining in the liver colon tissues; however treatment with grape seed reduced the expression of COX-2. Treatment with grape seed showed time -dependently accelerated liver and colonic healing. In conclusion, the results of our experiments strongly suggest the cancer chemoprotective effect of the methanolic Grape seeds extract and this may be due to the stimulation of the antioxidant effect.

Keywords: Cyclophosphamide, Chromosome, Histopathology, Grape Seed Extract, Mice

1. Introduction

Colorectal cancer is a malignant tumor recognized as the third most common cancer worldwide with high morbidity and mortality (Haggar and Boushey, 2009). Cyclophosphamide (CP) is an alkylating agent widely used in cancer chemotherapy (Baumann and Preiss, 2001, Fleming, 1997). The injury of normal tissues is the major limitation of using CP, which gives rise to numerous side effects (Bukowski 1999, Fraiser et al., 1991) CP administration has been shown to affect oxidative status in the circulation and different tissues of rats, such as liver as well as small and large bowel which enable this model to be used for an evaluation of antioxidant activities of natural or pharmacological compounds and their involvement in colon carcinogenesis. It has been reported that oxidative stress mediated disruption of redox balance after CP exposure generates biochemical and physiological disruptances (Das et al., 2002, Ghosh et al., 2002, Haque et al., 2001) Cancer chemotherapeutic prodrug – CYP is metabolized by liver cytochrome P450 enzymes namely, CYP3A4 and CYP2B6 to yield therapeutically active, cytotoxic metabolites (Roy and Waxman, 2006). The cytotoxic metabolites formed in the liver are distributed to different tissues by systemic circulation. Oxidative stress is reported to play a role in CYP induced tissue damage (Haque et al., 2003; Manda and Bhatia, 2003).

COX-2 is significantly overexpressed in variety of tumors, including colon cancer. Pharmacologic and genetic elimination of COX-2 activity reduces the progression of spontaneous and hereditary noninflammatory intestinal

cancer, both in animal models and in patients (Gupta and DuBois, 2001). Deletion of the Cox-2 gene in Apc (adenomatous polyposis coli) gene-mutant mice, a model for human familial adenomatous polyposis, results in significant reduction in both the number and the size of polyps (Oshima,. et al. 1996). Numerous studies have also demonstrated that COX-2-specific inhibitors suppress intestinal polyp formation in Apc-knockout mice, in a chemical carcinogen-induced tumor model and in xenograft colon cancer cell growth (Oshima, et al. 2002). The results of animal experiments demonstrating suppression of tumor formation in Apc mutant mice (Oshima,. et al. 1996) were confirmed in a clinical trial of familial adenomatous polyposis patients treated with COX-2 inhibitors (Steinbach, et al. 2000).

Grape seed extract is an industrial derivative from whole grape seeds that have a great concentration of vitamin E, flavonoids, linoleic acid, and Proanthocyanidin (OPC). Typically, the commercial opportunity of extracting grape seed constituents has been for chemicals known as polyphenols, including oligomeric proanthocyanidins recognized as antioxidants. The quantity, structure, and degree of polymerization of grape proanthocyanidins differ, depending on their localization in the grape tissues (Monagas et al., 2003).

Brannan and Mah (2007) suggested that GSE at 0.1% is an effective antioxidant in both raw and cooked meat systems during refrigerated and frozen storage. They added that Grape seed extract inhibits lipid oxidation in muscle from different species during refrigerated and frozen storage and oxidation catalyzed by peroxynitrite and iron/ascorbate in a pyrogallol red model system Supplementation of aged rats with grape seed extract showed increased memory performance and declined reactive oxygen species production, decreased protein carbonyl levels and improved thiol levels. These findings demonstrated that grape seed extract enhanced the antioxidant status and decreased the incidence of free radical induced protein oxidation in aged rats thereby protecting the central nervous system from the reactive oxygen species (Balu et al., 2005). The present study was undertaken to evaluate the natural protective efficacy of methanolic grape seed extract against CP-induced genotoxicity and pathologically liver and colon cancer cells in swiss albino mice.

2. Materials and Methods

Animals. Adult (6-8 weeks) Swiss albino male mice (13 ± 2 g), bred in the animal colony of The National Research Centre, Cairo, were maintained at controlled temperature under an alternating light and dark condition. Standard food pellets, and drinking water was provided *ad libitum*. The experiments were carried out following strictly the Scientific's Guideline for the Care and Use of Laboratory Animals.

Animal Husbandry:

Chemicals. Cyclophosphamide was purchased from Pharmacological Co. The grape seed extract was prepared in kindly at the laboratory of Medicinal Chemistry, National Research Centre, Cairo, Egypt.

Preparation of methanolic grape seed extract. One hundred grams of squashed air-dried seed (collected from grape fruits), were incubated with 100 ml of 100% methanol. In order to prevent the solvent evaporation, the container is covered with Para film and foil. Next, it is kept in the fridge for 24 hours. After being sifted through the 0.22- micrometer Millipore filter, Incubation and filtration takes place four times in 4 days. The extracts were then concentrated to dryness under reduced pressure at 45 ^{o}C, using a rotary evaporator (EYELA, N-N Series, Rikakikai Co. Ltd. Tokyo, Japan). The crude extracts were weighed to calculate the yield and stored in a refrigerator (–4 ^{o}C), until used (Sultana *et al*., 2008).

Experimental design. Six mice were considered as negative control. Fifty Mice were injected with 40 mg kg^{-1} body weight of cyclophosphamide for consecutive 5 days, 30 days later insuring that mice were infected with gastric and colon cancer by histological examination, treated mice divided into 3 main groups.

- First group: mice considered as positive control
- Second group: Mice received 75 mg/kg/day of grape seed extract as drinking solution for 14 consecutive days; each mouse received 0.5 ml / day (Low dose)
- Third group: Mice received 150 mg/kg/day of grape seed extract as drinking solution for 14 consecutive days; each mouse received 0.5 ml / day (High dose)

Sample collection. From the first group, sample collected after 30 days of the last treatment of cyclophosphamide. From the second and Third groups, samples were collected during treatment after 1, 7 and 14 days, and after two weeks (16 days) as a recovery period after the extract treatment stopped.

2.1. Experimental Tests

2.1.1. Cytogenetic Tests

Micronucleus test. Bone-marrow cell suspensions were prepared as described by (Schmid, 1973), and then stained with May–Gruenwald and Giemsa at pH 6.8. The smears were dried, and the slides were screened under an oil immersion objective. Slides were screened for Polychromatic erythrocytes (PE's) cells (1000 PEs / mouse, 4 animals/ treatment). The significance of experimental from control data was calculated using the differences between two proportions test (Daniel, 1974) in the case of polychromatic erythrocyte percentage, and the tables of (Kastenbaum and Bowman, 1970) for micronucleated polychromatic erythrocytes test.

Somatic chromosomal aberrations. For somatic cells preparations, animals from the different groups were injected i.p. with colchicine, 2h before sacrifice. Chromosome preparations from bone marrow cells carried out according to the method of Yosida and Amano, (1965)

with some modifications. 100 well spread metaphases were analyzed per mouse in four mice per group. Metaphases with chromosome or chromatid abnormalities were recorded in bone marrow cells. All results are presented as mean ± S.E. The statistical significance of the difference for mitotic and meiotic results was analyzed through *student's t-test*. $P< 0.05$-0.01 was considered significant.

Histopathologic examination. Liver and colon from all experimental were fixed in 10% neutral buffered formalin. The fixed samples were dehydrated in ascending series of ethanol, cleared in zylene, and embedded in paraffin wax. Sections 5 µm thickness was prepared using a microtome, stained with hematoxylin and eosin (H & E), and examination under a light microscope (Bancroft. & Stevens. 1990).

Immunohistochemical assessment of COX-2. Immunohistochemical staining of COX-2 antibody was performed with sections of four µm thick were deparaffinized and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. COX-2 positive cells were determined with streptavidin biotin-peroxidase staining method. For immunohistochemistry examinations were used monoclonal COX-2 antibody as the primer antibody at a1:100 dilution, and biotinylated secondary antibody (DAKO-Universal). The binding sites of antibody were visualized with DAB (Sigma) The specimens were counterstained with Haematoxyline and evaluated by high-power light microscopic

3. Results

3.1. Micronucleous Test

3.1.1. Induction of Polychromatic Erythrocytes (Pes)

It can be shown as in fig.1 that the high dose of GSE was more effect than the low dose of GSE in reducing percentage of polychromatic erythrocytes (PEs) which indicated to the toxicity of cyclophosphamide through the experimental period, especially after one month which reduces the toxicity of CP to the control level.

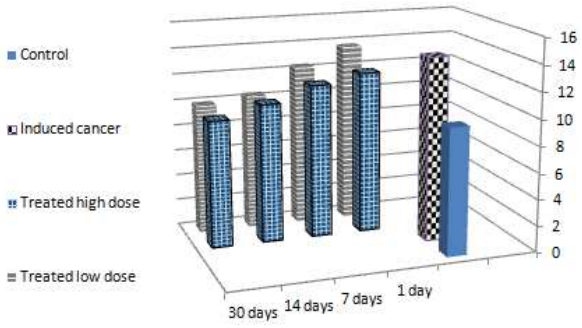


Figure 1. *Percentage of plychromatic erythrocytes (PE's) in mouse bone marrow cells, -ve control. Infected with cyclophosphamide, and treated with grape seed extract (low and high doses), 1, 7, 14 and 30 days.*

3.1.2. Induction of Micronuclei In Polychromatic Erythrocytes (Micronucleated Pes)

In order to investigate the effect of either low or high doses of GSE in comparison on mice induced with cancer cells as illustrated in fig.2, it can be indicated that is high dose of GSE was highly significant reducer than low dose of the micronucleated Polychromatic erythrocytes recorded 2.14% and 1.68% respectively after 30 days comparing to 9.62% as induced mice with cancer.

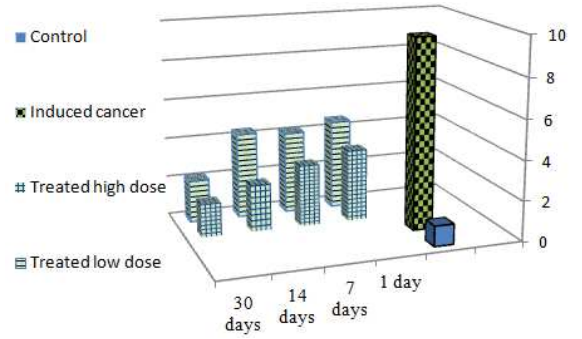


Figure 2. *Percentage of micronucleated (PE's) in mouse bone marrow cells, -ve control. Infected with cyclophosphamide, and treated with grape seed extract (low and high doses), 1, 7, 14 and 30 days.*

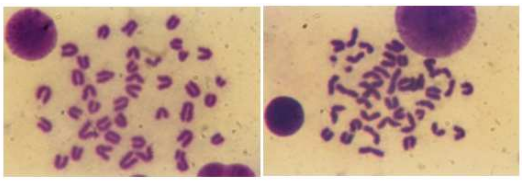
3.1.3. Chromosomal Aberrations

Table 1. *Percentage of chromosomal aberrations and types of aberrations induced with cyclophosphamide and treated with GSE on bone marrow cells of mice.*

Treatment	Sample time (days)	Scored metaphases	Meataphases with					Total	Mean %± S.E.
			Del	Centr. atten.	Chromatid gap	Chrom.break	Polyploid		
Control (non-treated)	0	300	3	2	4	1	0	10	3.33 ± 0.12
Infected mice with cancer	0	350	22	17	11	9	16	75	21.43 ± 1.2 **
Treated mice with GSE									
Low dose	1	350	20	16	9	5	13	63	18.1 ± 0.92 **
	7	350	18	11	7	4	11	51	14.5 ± 1.1 **
	14	350	12	7	3	3	7	32	9.14 ± 0.96 **
	30	350	4	4	3	2	1	14	4.00 ± 0.76
High dose	1	350	13	10	3	6	8	40	11.43 ± 1.08 **
	7	350	11	13	3	6	5	38	10.86 ± 0.41 **
	14	350	7	5	3	5	4	24	6.86 ± 1.02 *
	30	350	4	3	3	2	1	13	3.71 ± 0.98

Abbreviations: Del. : Deletion; Centr. Atten. : Centromeric attenuation; Number of mice = 5 per each treatment
* Significant at ($p < 0.05$) ** Significant at ($p < 0.001$)

Table 1 illustrated that high dose of GSE had a significant reduction of chromosomal aberration frequency than low dose, recorded 11.43% and 3.7% while 18.1% and 4% as low dose after one and 30 days, respectively, comparing to 3.33 and 21.43% as negative control and cancer induced mice. Fig. 3 (a, b) illustrated some of the chromosomal aberrations which recorded during the experiments (deletion and fragments).



(a) (b)

Figure 3. *Chromosomal aberration induced with cyclophosphamide. a) Chromatid break (arrow). and b) fragments.*

3.2. Hisopathological Results

3.2.1. Liver

The control livers of mice showed a normal lobular architecture with central veins and radiating hepatic cords are separated by narrow blood sinusoids (Fig.4 A). After 5 days of cycophosphamide treatment showed disruption of normal architecture of hepatic lobules, dilatation and congestion of hepatic sinusoids, vacuolation in some hepatocytes and bridging necrosis with early fibrosis between portal tract and central veins (Fig.4B). Also, large areas of extensive, mainly pericentral hepatic necrosis replaced by mononuclear leucocytic cells, vacuolar fatty change and inflammatory cell infiltration. Dysplastic hepatocytes, basophilic cells with large polymorphic and hyperchromatic with deeply stained shrunken pyknosis and apoptosis nuclei (Fig.4 D).

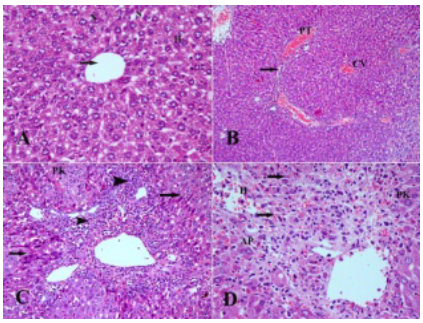


Figure 4. *A. A photomicrograph of the control liver of mice with central vein (CV) and surrounding hepatocytes (H), sinusoids (S) and nucleus (N). B. A photomicrograph of liver of mice treated with cycophosphamide showing congestion central vein (CV), bridging necrosis with early fibrosis between portal tract and central veins (PT & long arrow). C. A photomicrograph of liver of mice treated with cycophosphamide showing sever mononuclear inflammatory cell infiltration (arrow head) pyknotic (PK) and apoptotic nuclei (long arrow). D. A photomicrograph of liver of mice treated with cycophosphamide showing distortion of hepatic architecture (long arrow), pyknotic (PK) and apoptotic nuclei (long arrow) dilatation and congestion of hepatic sinusoids (H) (H & E X 400).*

The low and high dose of grape seeds extract with for 24 hrs did not prevent the toxic effect of cyclophosphamide, dilatation and congestion of hepatic sinusoids, large necrotic areas, dysplastic hepatocytes and basophilic cells were still present (Fig. 5A & B). The low and high dose of grape seeds extract with for 48 hrs also did not prevent the toxic effect of cyclophosphamide, dilatation and congestion of hepatic sinusoids, large necrotic areas, dysplastic hepatocytes and basophilic cells were observed (Fig. 5 C & D).

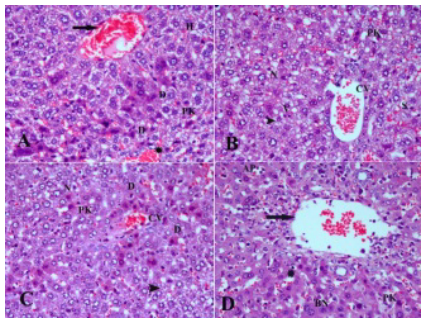


Figure 5. *A. A photomicrograph of liver of mice treated with grape seeds low dose cycophosphamide for 24 hrs showing congestion central vein (CV), pyknotic (PK), congestion of hepatic sinusoids (star) and mitotic division (D). B. A photomicrograph of liver of mice treated with grape seeds high dose cycophosphamide for 24 hrs showing pyknotic nuclei (PK), congestion of hepatic sinusoids (S) with vacuolation of hepatocytes (V). C. A photomicrograph of liver of mice treated with grape seeds low dose cycophosphamide for 48 hrs showing pyknotic (PK), and mitotic division (D). D. A photomicrograph of liver of mice treated with grape seeds high dose cycophosphamide for 48 hrs showing mild inflammatory cell infiltration around portal tract (long arrow), pyknotic (PK) and apoptotic nuclei (AP) (Hx & E x400).*

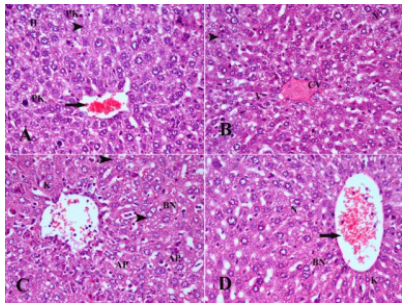


Figure 6. *A. A photomicrograph of liver of mice treated with grape seeds low dose cycophosphamide for one week showing pyknotic nuclei (PK) and degeneration some hepatocytes (H). B. A photomicrograph of liver of mice treated with grape seeds high dose cycophosphamide for one week showing congestion central vein (CV), necrotic area (arrow head) and vacuolation some hepatocytes (V). C. A photomicrograph of liver of mice treated with grape seeds low dose cycophosphamide for tow weeks showing necrotic cells around central vein (arrow head), apoptotic nuclei (AP), binucleated cells (BN) and activated kuppfer cells (K). D. A photomicrograph of liver of mice treated with grape seeds high dose cycophosphamide for two weeks showing dilated central vein, binucleatide cells (BN) activated kuppfer cells (K). (H & E x400).*

In the group of grape seeds at low and high dose and cyclophosphamide for one week showed mild hepatic damage, little liver necrosis with minimized the dysplastic changes. Also, congestion of central vein and deeply stained shrunken pyknotic nuclei also observed (Fig. 6 A & B).

Histopathological examination of liver treated with grape seeds at low dose for two weeks and cyclophosphamide showed preserved the normal histological picture of the liver tissue with pericentral hepatic necrosis replaced by mononuclear leucocytic cells. Also, number of binucleated hepatocytes cells and apoptosis nuclei was noticed (Fig. 6 C).

However, the high dose of grape seeds extract (150 mg/kg) for two weeks and cyclophosphamide reveled markedly ameliorated the cyclophosphamide induced hepatic injury and preserved the normal histological picture of the liver tissue, except few necrotic cells, dilatation and congestion of central vein with number of binucleated hepatocytes cells was observed (Fig. 6 D)

3.3. Colon

The control animals of colon mice showed the mucosa, clear submucosa muscularis lamina propria and intact crypt architecture, large number of goblet cells and show basal nuclei. The crypts are interspersed in the connective tissue layer (Fig. 7 A). Histopathological examination of colon mice received cyclophosphamide for five days was characterized by severe disruption of tissue architecture, massive mononuclear cellular infiltration (Fig. 7 B).

In the cyclophosphamide treated groups, well differentiated signs of dysplasia were observed. Nuclei were enlarged, thickening of epithelium cells with hyper-chromatic and showed increased mitotic activity was seen (Fig. 7C) Multiple foci of inflammation were present throughout the lamina propria and the submucosa, which consisted of mononuclear infiltration (Fig. 7D). Most of the goblet was absent.

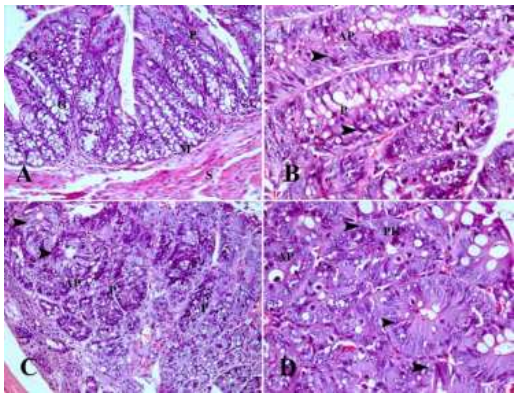


Figure 7. *A. Photomicrograph of section of colon of control mice showing its characteristic layers; mucosa, submucosa (SM), muscolosa (M), colonic crypts extending down to muscularis mucosa and straight, parallel. The lamina propria appears filling the space between the colonic crypts. Goblet cells (G) predominate in the glands and show basal nucleiB. Photomicrograph of colon section of mice received cycophosphamide showing crypt with dysplasia features and hyperchromatic nucleus (arrow head), pyknotic (P) and apoptotic nuclei (AP). C. Photomicrograph of colon section of mice received cycophosphamide showing crypt with dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P) and apoptotic nuclei (AP). D. Photomicrograph of colon section of mice received cycophosphamide showing crypt with dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P) and apoptotic nuclei (AP). (H & E x400).*

Mice treated with low and high dose of grape seeds daily for 24 hrs along with cyclophosphamide did not prevent the toxic effect of cyclophosphamide, inflammatatory cellular infiltration were present throughout the lamina propria and the submucosa, signs of dysplasia, pyknotic and apoptosis nuclei were still present (Fig. 8 A & B). Similar results were observed in the group treated with grape seeds at low and high dose for 48 hrs (Fig. 8 C & D).

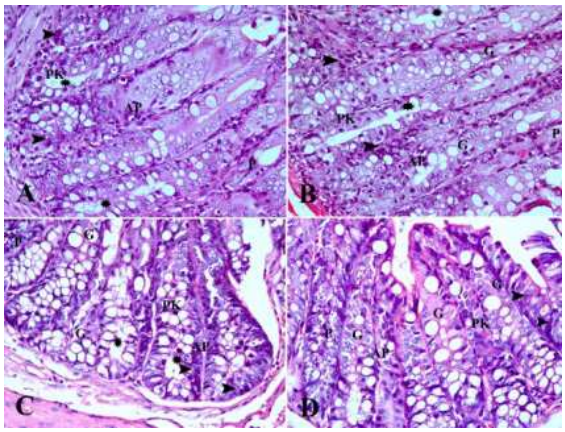


Figure 8. *A. Photomicrograph of colon section of mice received grape seeds low dose and cycophosphamide for 24 hrs showing crypt with dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P), apoptotic nuclei (AP) and dilated lumen of lamina propria. B. Photomicrograph of colon section of mice received grape seeds high dose and cycophosphamide for 24 hrs showing crypt with dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P), apoptotic nuclei (AP) and dilated lumen of lamina propria. Goblet cells are present (G). C. Photomicrograph of colon section of mice received grape seeds low dose and cycophosphamide for 48 hrs showing a moderate crypt with dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P), apoptotic nuclei (AP) and dilated lumen of lamina propria. Goblet cells are present (G). D. Photomicrograph of colon section of mice received grape seeds high dose and cycophosphamide for 48 hrs showing a mild crypt with dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P), apoptotic nuclei (AP) and dilated lumen of lamina propria. Goblet cells are present (G) (H & E x400).*

Histopathological examination of colon mice received low and high dose of grape seeds daily for one week and cyclophosphamide showed mild to moderate dysplasia and hyperchromatic were seen. Also, loss of the upper half of crypt epithelium with a mild inflammatory infiltrate was present in the lamina propria (Fig. 9 A & B).

Mice treated with grape seeds (low & high) dose and cyclophosphamide for two weeks displayed an improvement in colonic histopathology compared to cyclophosphamide-only mice, but there were still clear signs of inflammatory infiltrate. Also, mononuclear inflammation was still observed in the lamina propria and mucosal layers. Crypts were present, remained in contact with the basement membrane and restoration of a normal complement of goblet cells. The epithelium appeared generally intact (Fig. 9 C & D). Grape seeds stimulation produced ameliorative effect showed dose-dependent in the treatment the effect of cyclophosphamide.

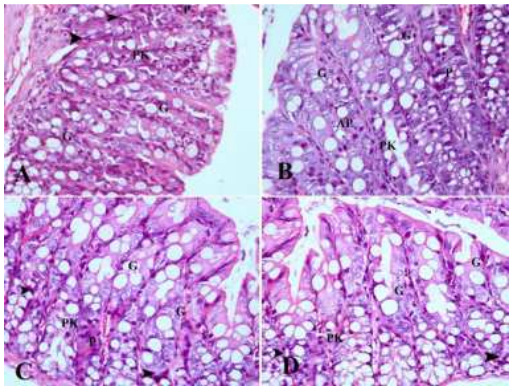


Figure 9. *A. Photomicrograph of colon section of mice received grape seeds low dose and cycophosphamide for one week showing a crypt with few dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P), apoptotic nuclei. Goblet cells are present (G). B. Photomicrograph of colon section of mice received grape seeds high dose and cycophosphamide for one week showing a crypt with few dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P), apoptotic nuclei. Goblet cells are present (G). C. Photomicrograph of colon section of mice received grape seeds low dose and cycophosphamide for two week showing a crypt with few dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P), apoptotic nuclei. Goblet cells are present (G). D. Photomicrograph of colon section of mice received grape seeds high dose and cycophosphamide for two week showing a crypt with few dysplasia features and hyperchromatic nucleus (arrow head), head), pyknotic (P), apoptotic nuclei. Goblet cells are present (G) (H & E x400).*

3.3.1. Cox2 Liver

The livers of control mice did not show COX-2 immunopositivity reaction (Fig. 10A). The administration of cyclophosphamide for five consecutive days showed COX-2 immunoreactivity was strongly expressed in the cytoplasm of hepatocytes as compared to the control group. Also, observed mainly in necrotic areas, showed predominantly pericentral staining (Fig. 10B).

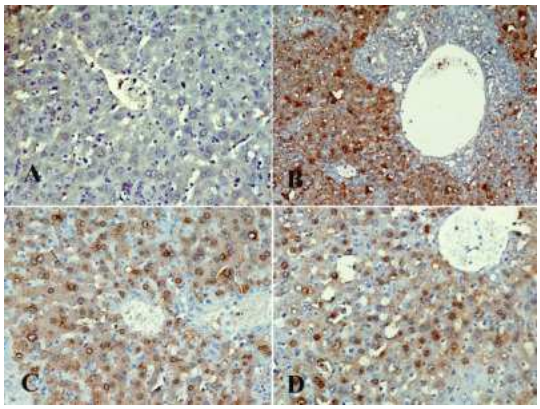


Figure 10. *COX-2 immunohistochemistry counterstained with Hematoxylin. A. COX-2 -immunohistochemistr cells were rarely present in liver of control mice. B. Mice treated with cycophosphamide showing increase intenisty of COX-2. C. Mice treated with grape seeds low dose for 24 hrs and cycophosphamide showing increase number of COX-2. D. Mice treated with grape seeds high dose for 24 hrs and cycophosphamide showing moderate increase number of COX-2. (COX-2 immunohistochemistry, haematoxylin counterstain, x 400).*

Immunohistochemistry analysis of COX-2 expression in mice livers treated with grape seeds low and high dose and cyclophosphamide for 24 & 48 hrs revealed increased of COX2 immunopositivity (Fig. 10 C& D). However, grape seeds low and high dose and cyclophosphamide for one week treated mice showed mild decreased in the expression of COX-2 in the liver tissue (Fig. 11 A & B). Grape seeds (low & high) dose and cyclophosphamide for two week treated rats decreased COX-2 immunopositivity in the livers, which was attenuated by the low high dose of grape seeds extract (Fig. 11 C & D). The high dose of grape seeds ethanoloic extract markedly reduced COX-2 expression when compared to cyclophosphamide group.

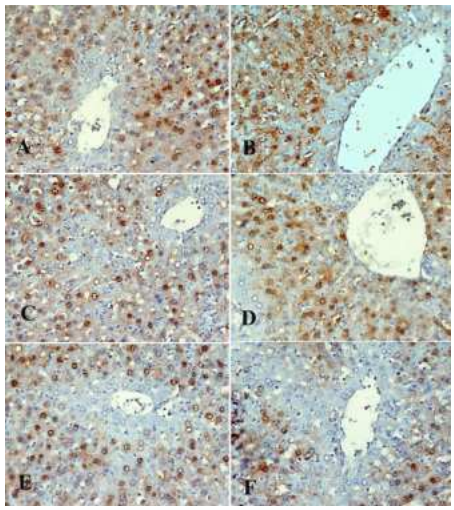


Figure 11. *A. Mice treated with grape seeds low dose for 48 hrs and cycophosphamide showing moderate increase number of COX-2. B. Mice treated with grape seeds high dose for 48 hrs and cycophosphamide showing moderate increase number of COX-2. C. Mice treated with grape seeds low dose for 1 week and cycophosphamide showing mild increase number of COX-2. D. Mice treated with grape seeds high dose for 1 week and cycophosphamide showing mild increase number of COX-2. E. Mice treated with grape seeds low dose for 2 weeks and cycophosphamide showing mild increase number of COX-2. F. Mice treated with grape seeds high dose for 2 weeks and cycophosphamide showing mild increase number of COX-2. (COX-2 immunohistochemistry, haematoxylin counterstain, x 400).*

3.3.2. Cox2 Colon

Immunohistochemical analysis of COX-2 in the colonic tissue revealed the absence of COX-2 in the control (Fig 12 A). The administration of cyclophosphamide for five consecutive days showed COX-2 immunoreactivity was strongly expressed in the colon epithelia (Fig. 12 B). The colon epithelia from mice treated with grape seeds and cyclophosphamide for 24 & 48 hrs showed still increase of COX-2 immunoreactivity (Fig. 12 C & D) The expression of COX-2 was mild decreased in the group treated with grape seeds and cyclophosphamide for one week (Fig.13 A & B). However, after tow weeks showed marked decreased of expression of COX-2(Fig. 13 C & D)

However, expression of COX-2 immunohistochemistry revealed in the nuclei located in the lower third of the crypts. They attain brown color.

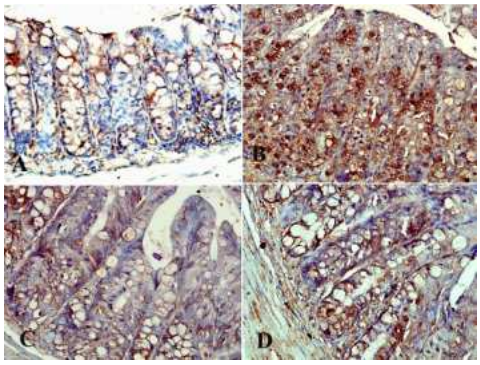


Figure 12. *COX-2 immunohistochemistry counterstained with Hematoxylin. A. COX-2 -immunohistochemistr cells were rarely present in colon of control mice. B. Mice treated with cycophosphamide showing increase intensity of COX-2. C. Mice treated with grape seeds low dose for 24 hrs and cycophosphamide showing increase number of COX-2. D. Mice treated with grape seeds high dose for 24 hrs and cycophosphamide showing moderate increase number of COX-2. (COX-2 immunohistochemistry, haematoxylin counterstain, x 400).*

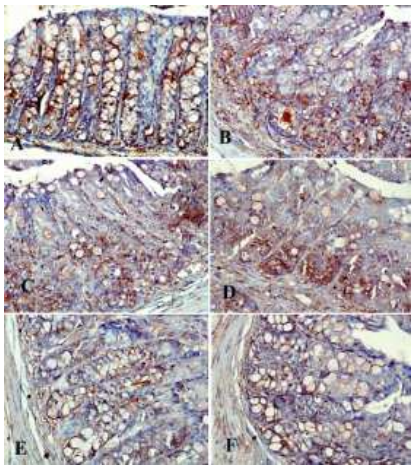


Figure 13. *A. Mice treated with grape seeds low dose for 48 hrs and cycophosphamide showing moderate increase number of COX-2. B. Mice treated with grape seeds high dose for 48 hrs and cycophosphamide showing moderate increase number of COX-2. C. Mice treated with grape seeds low dose for 1 week and cycophosphamide showing mild increase number of COX-2D. Mice treated with grape seeds high dose for 1 week and cycophosphamide showing mild increase number of COX-2E. Mice treated with grape seeds low dose for 2 weeks and cycophosphamide showing mild increase number of COX-2F. Mice treated with grape seeds high dose for 2 weeks and cycophosphamide showing mild increase number of COX-2. However, expression of COX-2 immunohistochemistry revealed in the nuclei located in the lower third of the crypts. They attain brown color. (COX-2 immunohistochemistry, haematoxylin counterstain, x 400).*

4. Discussion

Most of the chemotherapeutic agents available today are immunosuppressants, cytotoxic and exert a variety of side effects that are particularly evident in cancer chemotherapy. Cyclophosphamide is an alkylating agent and a powerful immunosuppressant acting as it cross links DNA in actively multiplying cells (Gonsette, 1986). Modulation of the immune response through stimulation may help in maintaining a disease-free state.

Agents that activate host defense mechanisms during an impaired immune responsive condition can provide supportive therapy to conventional chemotherapy (Wagner, 1984). Plant extracts used in traditional therapy are being reviewed for their chemoprotective and immunomodulatory activities.

It has been shown that immunomodulatory compounds used with chemotherapy may reduce enhance the immune response (Praveen et al., 1996). In the present study we evaluate the effect of *grape seeds extract* on toxic effect of cyclophosphamide on liver and colon

Cyclophosphamide as a colon and hepatic cancer inducer had induced micronucleated polychromatic erythrocytes (PEs), in a high frequency, meaning that the drug has a clastogenic effect on somatic mice cells. A high peak was observed after 30 day of treatment. Grape seed extracts (GSE) had a decreasing effect of the cytogenetic effect of the cancer cells.

Our results were agreed with Erexson (2003) who reported that, the results of his assay demonstrate that meganatural grape seed extract (GSE) and grape skin extract (GSKE) are devoid of clastogenic activity when administered orally to mice at doses as high as 2000 mg/kg. These results additionally support the view that consumer exposure to a few mg/kg/day of GSE and GSKE through their anticipated use in foods and beverages is expected to be safe, represents a small increment in consumer intake and provides a wide margin of safety compared to the doses administered in this assay.

Grape seed extract (GSE) caused a protective effect on mice treated with the anti-cancer agent "cisplatin", where Attia et al. (2008) reported that GSE caused a reduction in the cytotoxocity induced by cisplatin, this is manifest as a reduction in the PEs%, and it did not increase the incidence of micronucleated PEs at the tested doses (100 mg/kg/day for 7 consecutive days).

GSE with the two high and low doses was act as a recovery factors to the frequency of chromosomal aberrations induced with cancer cells, where the high dose of GSE was more effective than the low dose. However, the lowest frequency of metaphases with chromosomal aberrations was that recorded one day after GSE treatment, then it increased and reached its maximum values 30 days of treatments.

Ahna et al. (2002) demonstrated that grape seed extract (GSE) has a strong radical scavenging activity. They concluded that strong radical scavenging activity of GSE *in vitro* may be resulted from high total tannin concentrations at least in part and the decrease of lipid peroxidation in GSE-administered rats was due to the increase of antioxidant enzyme activity *in vivo*.

It is obviously mentioned that the frequency of chromosomal aberrations was more than the micronucleated PE's percentage, along the treatments. That phenomenon was recorded by Savage (1988) who postulated that the occurrence of comparatively lower frequency of MN than CA could be due to the fact that the fate of chromosomal fragments are uncertain so far their

segregation in MN is concerned. Apart from that all fragments do not necessarily form visible MN.

Ray *et al*. (2000) 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 (Bagchi et al., 2001).

Administration of intermittent doses of cyclophosphamide has been found to be advantageous in chemotherapy (Skrablin, et al, 2007). Cellular mechanism of toxicity is mediated by an increase in free radicals through intracellular phosphoramide mustard and acrolein, the principle alkylating metabolites of cyclophosphamide (Tripathi & Jena, 2008). In the present study, the administration of cyclophosphamide damages the liver and colon, and this observation is consistent with (Aliesa, 2007, Elkiran, et al, 2007). Tissue damage due to cyclophosphamide might be alleviated due to the antioxidant property and membrane stabilizing property of grape seeds.

Histopathological studies revealed that cyclophosphamide causes damage to the liver and colon, and this was evidenced by vacuolation in some hepatocytes and bridging necrosis with early fibrosis between portal tract and central veins, inflammatory cell infiltration, dysplastic hepatocytes, basophilic cells with large polymorphic and hyperchromatic with deeply stained shrunken pyknosis and apoptosis nuclei of liver tissues. Histopathological examination of colon mice also, showed massive mononuclear cellular infiltration, signs of dysplasia, nuclei were enlarged, and thickening of epithelium cells with hyper-chromatic and showed increased mitotic activity was seen. This might be due to membrane damaging potential of the cyclophosphamide's metabolites. These pathological changes correlated well with the altered enzyme activities, these findings are compatible with (Subramanian, et al, 2006).

All of the available evidence indicates that cyclophosphamide exerts its carcinogenic activity via a genotoxic mechanism (McCarroll *et al.*, 2008). The metabolite widely thought to be responsible for the antitumour activity of cyclophosphamide is the phosphoramide mustard (Povirk & Shuker, 1994). This metabolite is also generally considered to be the most genotoxic, but the contribution of acrolein, which is highly toxic,

After treatment with grape seeds these abnormal pathological findings of liver and colon tissue injury were reduced and tissues were protected from oxidative damage. The histopathological observations suggested the possibility of the grape seeds being able to protect the tissues and thus decreasing the damage tissue, This might be due to scavenging activity of grape seeds against the toxic metabolite that was produced during the activation of the cyclophosphamide by liver microsomal enzymes.

COX-2 is an inducible form of the prostaglandin synthase enzymes, which catalyse the committed step in the prostaglandin production pathway (Dubois et al., 1998). COX-2 expression is increased in inflammatory conditions as a result of induction by several different stimuli, including proinflammatory cytokines (Akarasereenont et al., 1995). The inhibition of COX-2 has been shown to exert the hepatoprotective effect in CCl4-induced liver damage (Vadiraja et al., 1998). Also, the decrease in COX-2 expression by berberine indicates suppression of prostaglandin synthesis and amelioration of the inflammatory response in CCl4-intoxicated mice, as reported previously (Kuo et al., 2004).

In the present study, grape seeds extract down regulated the COX-2 expression in cyclophosphamide, showing its anti-inflmmatory potential. Ahn *et al*. (2002) reported that grape seeds are rich in antioxidant compounds, including phenolic compound (predominantly tannins), and it has been demonstrated that these compounds reduce the risk of chronic disease by protecting against free radical mediated damage. Tannins have been described to have antimutagenic, anticarcinogenic and antioxidant activities. Lipid peroxidation has been and remains one of the most widely used indicators of free radical formation. Thiobarbituric acid reactive substances primarily reflect production of lipid peroxides, which are broken down during the assay to yield malondialdehyde. Li *et al*., (2001) reported that treatment with grape seed extract reduced the increase in thiobarbituric acid reactive substances. Also, Devi et al. (2006) found that intake of proanthocyanidin which is a naturally occurring antioxidant from grape seed extract in moderately low quantity is effective in up-regulating the antioxidant defense mechanism by attenuating LPO. Moreover, Sehirli et al. (2008), reported that GSE could reduce organ injury through its ability to balance the oxidant-antioxidant status, and to regulate the release of inflammatory mediators.

5. Conclusion

From our observations, it's possible to conclude that cyclophosphamide administration result in the pronounced oxidative stress and tissue damage due to its toxic metabolite and grape seeds protect the liver and colon tissue by scavenging the toxic metabolite.

In conclusion, the results suggest the chemoprotective effect of the methanolic Grape seeds extract and this may be due to the stimulation of the antioxidant effect.

Conflict of interest

The authors declare no conflicts of interest.

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