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Chemotherapeutic potential of Grape Seed Extract (GSE) against experimentally induced precancerous stage in mice colon

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ABSTRACT

Colon cancer is a common cause of cancer-related mortality. 5-fluorouracil (5-FU) is the reference regimen alone or as a combined therapy in colon cancer treatment. Despite the excellent chemotherapeutic effect of 5-FU, its toxicity in normal cells remains a major problem. Recently, the use of natural substances as cancer preventative or therapeutic agents has become a worldwide trend. Grape seed extract (GSE) is a potent chemopreventive agent against colon cancer both in vitro and in vivo. In order to prepare for a human trial, we conducted a study with GSE as a chemotherapeutic agent in an O-Nitrotoluene (O-NT)-induced precancerous stage in mice colon. To compare the efficacy of either 5-FU or GSE in treatment of precancerous stage of colon, male albino mice were administrated O-NT (150mg/kg) orally three times a week for six consecutive weeks followed by orally administration of Low dose (150mg/kg, LGSE) or high dose (300mg/kg, HGSE) GSE daily for one, two and three consecutive months. Another group of mice was administrated O-NT (150mg/kg) orally three times a week for six consecutive weeks followed by i.p administered 5-FU (20mg/kg) for 14 consecutive days. We demonstrated that GSE is more effective than 5-FU in the treatment of (O-NT)-induced precancerous stage in mice colon as GSE reduced the dysplastic damage in colon tissue, COX-2 protein expression and DNA damage represented by Comet assay. Our results strongly suggest the colon cancer-chemotherapeutic potential of GSE in the early stage in dose- and time-dependent.

Key words: Colon precancerous stage; O-Nitrotoluene; Grape seed extract; 5-Fluorouracil; COX-2; Comet assay

Introduction

Colon cancer is a serious health problem in most developed countries and is the third leading cause of cancer mortality throughout the world (Wingo et al., 1995). There is evidence that colon cancer progresses from normal tissue to adenoma and carcinoma through an accumulation of genetic alterations (Boland et al., 1998; Chung, 2000; Baron and Sandler, 2000). In colon cancer, delineation of various stages during tumor progression offers a window of opportunity to intervene in the process by detecting stage-specific molecular changes (such as change in gene expression). An increase in cytoplasmic β-catenin levels and subsequent β-catenin/Tcf7 lymphoid enhancer factor complex formation are believed to be important events in the early stage of colonic carcinogenesis (Korinek et al., 1997; Morin et al., 1997; Tetsu and McCormick, 1999; Chung, 2000). This complex binds to the DNA and results in overexpression of prostaglandin endoperoxide H synthase-2 (COX-2), induced by nitric oxide. In the present study, immunohistochemical detection of COX-2 expression was used as a marker for early detection of colon cancer in mice treated by O-Nitrotoluene. Early detection markers indicate the existence of cancer or that cancer will occur with nearly a 100% certainty within a specified time interval (Srivastava et al., 2001).

5-Fluorouracil (5-FU) is a chemotherapeutic drug widely used in treating colorectal cancer. However, several studies have demonstrated that 5-FU has several side-effects including myelotoxicity, gastrointestinal disturbances, cardiotoxicity, hepatotoxicity, neurotoxicity, induction of chromosomal aberrations, infertility, and teratogenicity in experimental animals (Naya et al., 1990; Russell and Russell, 1991; Hrushesky et al., 1999; Paskulin et al., 2005). The toxicity of 5-FU towards normal cells and resistance to this drug are major barriers to successful cancer chemotherapy (Srimuangwong et al., 2012).

Since colon cancer has a long latency period before it is detected even in its pre-clinical stage (such as colon polyp), an opportunity exists to institute appropriate dietary preventive strategies to halt its progression (Grady and Carethers, 2008) and avoiding the toxic side effects of ordinary chemotherapy. Dietary bioactive phytochemicals that have chemopreventive properties, which occur in nature as complex mixtures in fruits, vegetables, grains and herbs, are particularly suited for this purpose (Liu, 2004).
Grape seed extract (GSE), a mixture containing about 95% standardized proanthocyanidins, is a popular dietary supplement due to its anti-cancer and anti-inflammatory properties (Agarwal et al., 2002). GSE showed anti-cancer activity both in vitro and in vivo (Radhakrishnan et al., 2011) and significantly inhibited cell viability and elevated apoptosis in cancer cells without altering the viability of the normal colon cell lines, thus selectively targeting cancer cells (Laurent et al., 2004).

The present study compares the therapeutic potential of GSE, 5-FU separately against early detected colon cancer induced by O-Nitrotoluene in mice as a possible strategy to prevent tumor progression. Moreover, the aim of this study was conducted to evaluate the genotoxicity of the widely used anticancer drug 5-FU.

Materials And Methods

Animals:

Male albino mice (8-10 weeks old; 26-30g, body weight) were used as experimental animals. Animals were purchased from the animal house of the National Research Center (Giza, Egypt). They were housed in plastic cages for 7 days to be accommodated with our laboratory conditions. Food and water were presented ad libitum. Animals received care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals”.

Tested drugs:

1) Grape Seed Extract (GSE)(China, Xiamen FGS) was dissolved in distilled water just before use and was administrated by an oral gavage at two different dose levels: 150mg/kg b.w. (Low GSE (LGSE), minimum therapeutic dose) and 300mg/kg b.w. (High GSE (HGSE), maximum therapeutic dose) (Exrexon, 2003; Clouatre et al., 2010; Mukherjee et al., 2010).

2) 5-Flurouracil (5-FU)(Egypt, ACDIMA) was dissolved in distilled water just before use and was intraperitonially (i.p.) injected at dose level of 20mg/kg (Chen et al., 2005).

3) O-Nitrotoluene (O-NT) was dissolved in corn oil just before use and was administrated by an oral gavage at dose of 150mg/kg (Slaga et al., 1985; Dunnick et al., 2003).

Treatment Schedule:

Animals were divided into 11 groups 5 mice per each. All mice were injected according to body weight and animals were sacrificed 24 hr after the last treatment except O-NT2 group.

Negative control group: injected i.p. with distilled water.

O-NT1 group: administrated O-NT (150mg/kg) orally three times a week for six consecutive weeks.

O-NT2 group: administrated O-NT (150mg/kg) orally three times a week for six consecutive weeks, and sacrificed after 1 month from the last treatment. This group was done to evaluate the normal cell repair mechanism after O-NT injury.

5-FU group: mice were injected i.p. with 5-FU (20mg/kg) for 14 consecutive days.

O-NT + 5-FU group: mice were administrated O-NT (150mg/kg) orally three times a week for six consecutive weeks, followed by i.p. injection of 5-FU (20mg/kg) for 14 consecutive days.

O-NT + LGSE groups: mice were administrated O-NT (150mg/kg) orally three times a week for six consecutive weeks, followed by oral administration of LGSE (150mg/kg) daily for one (LGSE1), two (LGSE2) and three (LGSE3) consecutive months.

O-NT + HGSE groups: mice were administrated O-NT (150mg/kg) orally three times a week for six consecutive weeks, followed by oral administration of HGSE (300mg/kg) daily for one (HGSE1), two (HGSE2) and three (HGSE3) consecutive months.

Detection of early colon cancer induction:

Histological evaluation of colon tissue was performed on 5 μm thick paraffin sections stained by hematoxylin and eosin (H&E) for histopathological changes detection which may be associated with early stages of carcinogenesis. In addition, immunohistochemical evaluation for COX-2 protein expression counterstained with hematoxylin was performed as a marker for early detection of colon cancer (Ogino et al., 2006).
Comet assay:

The alkaline comet assay was performed as described by Singh et al. (1988). Conventional frosted microscopic slides were dipped into hot 1.0% normal melting point agarose to one-half of the frosted area and the underside of the slide wiped to remove agarose. A 65 µl of 0.5% low melting point agarose (LMPA) at 37°C was mixed with 10 µl of homogenized colon tissue in cold Hank’s Balanced Salt Solutions (HBSS), and a cover slip was applied to spread the samples. The slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10mMTris, NaOH to pH 10.0, 1% Triton-100 and 10% DMSO) at 4 °C for 2 hour to remove cell protein. They were then soaked in a coupling jar containing electrophoresis solution to unwind for 20 minutes and electrophoresed at a constant current of 300 mA, for 35 minutes. After electrophoresis, the slides were neutralized with Tris-HCl buffer at pH 7.5 by three washes for 5 minutes, followed with cold ethanol for 5 to 10 minutes and left to dry overnight. The slides were stained by placing 80 µl ethidium bromide (20 µg/mL) on each slide and covered with a coverslip for 20 minutes. Then slides were viewed under an epifluorescence microscope (Zeiss epifluorescent) with an attached CCD camera and computer. Images were saved as electronic files and for each sample, 50 isolated comets were randomly selected and measured for comet tail length, %DNA in tail and tail moment using COMETSCORE software based on the definition by Olive and Banath (1993).

Tail Moment = Tail length x %DNA in tail/ 100

Statistical data analysis:

Data were expressed as the mean ± standard deviation (M±SD). Statistical significances of differences between two groups were determined using Student's t-test. The difference between means at the level of p<0.05 was considered as significance. Two-way analysis of variance (two-way ANOVA) followed by Duncan’s multiple range test were used to assess the effect of GSE doses and post-treatment periods used in the present work and their interaction on the DNA damage estimated parameters represented by tail length, %DNA in tail and tail moment. Statistics were carried out using statistical analysis systems (SAS) program.

Results:

Histopathological evaluation:

Fig. 1A represents normal mice colon tissue showing regular colonic architecture, normal colonic mucosa (M), clear submucosa (S), lamina propria (P) and intact crypt architecture, large number of goblet cells (G).

The histopathological evaluation of the colon tissue of 5-FU group exhibited straight and parallel crypts, but the epithelium of the lower crypt regions are proliferative and epithelium in upper crypt regions is missing. Also, there is an intense mononuclear cell infiltration of the lamina propria (Fig. 1B).

As an indication of early signs of precancerous stages, different types of histologically altered colon crypts could be observed in the O-NT1 group (sacrificed 24h after last treatment). It showed colonic degeneration confined to the mucosa muscularis and submucosa, with loss of surface epithelium, also there were a leukocytic inflammatory cell infiltrations. In some sections necrotic tissue adjacent to surface cells could be observed. Crypts showed different criteria of epithelial hyperplasia and dysplasia. Some crypts showed dysplastic nuclear changes in their lining epithelium. Some nuclei showed variation in their shapes (pleomorphism), while some other nuclei were enlarged, round or ovoid, hyper-chromatic, mitotic activity and thickening of epithelium was seen. Other crypts showed hyperplastic criteria of epithelial lining. The upper segments of these crypts were characterized by few or completely absent goblet cells. In focal areas the crowded cells became arranged in many layers (Figs. 1C & 1D).

The histopathological evaluation of the colon tissue from the O-NT2 group (sacrificed one month after last treatment) exhibited severe dysplasia of the crypt cells with nuclei enlarged, round or ovoid and deeply stained. The number of mitotic cells is far more and the number of goblet cells markedly reduced. Severe mononuclear cellular infiltration was also observed in lamina propria of most sections of this group (Fig. 1E).

Colonic section of O-NT + 5-FU group revealed a mild hyperplasia in the colonic mucosal surface with all the crypt specific structures and other histoarchitectural structures still disturbed. The specimens displayed a moderate inflammatory infiltrate with a focal distribution (Fig. 1F).

The administration of GSE (LGSE and HGSE) for different treatment periods to O-NT group revealed more or less preservation of the histological structure of the colon tissue (Fig. 2). The histopathological examination of O-NT + LGSE1 showed much less dysplastic changes, necrosis although the crypt morphology still found (Fig. 2A). The microscopic features of O-NT + LGSE2 group showed a mild hyperplasia in the colonic mucosal surface with all the crypt specific structures and other histoarchitectural structures still disturbed (Fig. 2B). Fig.
2C represents O-NT + LGSE3 group that showed a lower tissue damage score than that assigned to the group treated with O-NT only.

Post O-NT treatment with GSE at high dose for 1 month (HGSE1) decreased severity of the cell damage. There were mild hyperplasia and dysplasia, less inflammatory cells in the lamina propria. In some areas, it was observed intact epithelium (Fig. 2D). O-NT + HGSE2 group revealed an improved tissue recovery with less of hyperplasia, dysplasia and colonic inflamed tissue when compared with O-NT group. Large numbers of goblet cells are also visible (Fig. 2E). O-NT + HGSE3 group showed apparently normal structures with reverting changes towards normal crypt structures with goblet cells and epithelial layer. Only focal epithelial lesions and a lower histological score were observed (Fig. 2F).

The consecutive post-treatment administration of GSE (low or high dose) for O-NT groups reduced features of dysplasia and hyperplasia. It seemed that the efficacy of high GSE dose was better than that of low dose. 

Fig. 1: Photomicrographs of mice colon sections representing A: negative control group showing normal intact histological structure of glands with straight crypts (†), B; 5-FU (20mg/kg) group showing proliferation of crypt’s lower region epithelium (†), C & D; O-NT1 (15mg/kg) group showing hyperchromasia in the anaplastic glandular cells (†) and mononuclear cellular infiltration in lamina propria, E; O-NT2 (150mg/kg) group showing necrosis, cellular infiltration and different shaped nuclei (††), F; O-NT (150mg/kg) + 5-FU (20mg/kg) group showing hyperplastic proliferation (††). In which: M, mucosal layer; G, goblet (glandular) cells; S, submucosal layer; P, lamina propria; I, infiltration; n, necrosis. Notice: Some crypts show dilated lumen (*) H&E X 400
Fig. 2: Photomicrographs of mice colon sections representing A: O-NT (150mg/kg) + LGSE1 (150mg/kg) group showing necrosis (→). B: O-NT (150mg/kg) + LGSE2 (150mg/kg) group showing mild infiltration. C: O-NT (150mg/kg) + LGSE3 (150mg/kg) group showing atrophy of goblet cells formation. D & E: O-NT (150mg/kg) + HGSE1 (300mg/kg) & O-NT (150mg/kg) + HGSE2 (300mg/kg) groups respectively showing atrophy of goblet cells formation and moderate infiltration. F: O-NT (150mg/kg) + HGSE3 (300mg/kg) group showing diffuse goblet cells formation in the hypertrophical lining epithelium and focal distribution of hyperchromatic nuclei. In which: G, goblet (glandula) cells; O, oedema; P, lamina propria. H&E X 400

Notice: Some crypts show dilated lumen (*); presence of hyperchromatic nuclei (▲); infiltration of inflammatory cells in lamina propria (→)
**Immunohistochemical evaluation:**

Immunohistochemical assay demonstrated that COX-2 protein was located in the cytoplasm and nuclear membrane. The immune COX-2 staining was weak yellow, dark yellow and brown at a low power field (Figs. 3A-D and Figs. 4A-F). Expression of COX-2 in colon tissues as a marker for early signs of colon cancer was classified according to the severity of the immune reaction staining as follows: (++++) very high, (+++) high, (+) moderate, (+) mild and (-) weak expression.

A weak staining of COX-2 was observed in negative control tissue group (-) (Fig. 3A). Fig. 3B represents 5-FU group showed moderate (++) cytoplasmic expression of COX-2 protein.

COX-2 expression was relatively much stronger in the O-NT1 group tissue cells (++++) with dark yellow and brown staining with occasional granular distributions (Fig. 3C), which indicates pre-cancerous stage of colon induced by O-NT administration. Estimation of COX-2 protein expression was not performed for the O-NT2 group because the previous histopathological evaluation was sufficient to show the absence of repair for the damage tissue caused by caused by O-NT administration. O-NT + 5-FU group showed high cytoplasmic expression of COX-2 (+++).

O-NT group administrated low dose of GSE for one month (LGSE1) or two months (LGSE2) showed a higher expression of COX-2 protein compared to the negative control group (++) but less severe than the O-NT group (++++) (Figs. 4A & 4B). On the other hand, O-NT + LGSE3 group showed a remarkable reduction in cytoplasmic expression of COX-2, but still higher than normal (+) (Fig. 4C).

A much less intense presence of the expression of COX-2 in the daily post-treatment with high dose GSE of O-NT group was observed. The expression of COX-2 ranged from moderate in O-NT + HGSE1 group (++) to mild in O-NT + HGSE2 group (+) and totally showed minimum expression in O-NT + HGSE3 group (-) which seems nearly normal with cells positively stained for COX-2 protein remain at the lower and middle parts of the crypts (Figs. 4D, E & F, respectively).

![Figure 3: Photomicrographs of mice colon sections showing immunohistochemical localization of COX-2 protein, identified by brown staining: A; negative control group showing rare expression of COX-2 (-), B; 5-FU (20mg/kg) group showing intracellular increased expression of COX-2 in the mucosal glandular cells (+++), C; O-NT1 (150mg/kg) showing highly increased expression of COX-2 (+++), D; O-NT (150mg/kg) + 5-FU (20mg/kg) group showing increased expression of COX-2 (+++) (COX-2 immunohistochemistry, hematoxylin counter stain, X 400)]
Fig. 4: Photomicrographs of mice colon sections showing immunohistochemical localization of COX-2 protein, identified by brown staining: A; O-NT (150mg/kg) + LGSE1 (150mg/kg) group showing moderate expression of COX-2 (++), B; O-NT (150mg/kg) + LGSE2 (150mg/kg) group moderate expression of COX-2 (++), C; O-NT (150mg/kg) + LGSE3 (150mg/kg) group showing low expression of COX-2 (+), D; O-NT (150mg/kg) + HGSE1 (150mg/kg) group showing moderate expression of COX-2 located in the lower and middle part of the crypts (++), E; O-NT (150mg/kg) + HGSE2 (150mg/kg) group showing low expression of COX-2 in the lower part of the crypts (+), F; O-NT (150mg/kg) + HGSE3 (150mg/kg) group showing only few COX-2 expression, almost similar to the control sections (-) (COX-2 immunohistochemistry, haematoxylin counterstain, X 400)
Comet assay:

Table 1 shows statistically significant increase (P < 0.05) in all DNA damage parameters (tail length, %DNA in tail and tail moment) after different treatments in comparison with the negative control group, except for groups treated with LGSE and HGSE for 3 consecutive months.

In addition, Table 1 shows statistically significant decrease in DNA damage parameters in O-NT+5-FU group when compared with O-NT groups but still significantly higher than the negative control levels.

The two way ANOVA (analysis of variance) and Duncan’s multiple test were used to determine statistical difference in the same parameters between different variables at the same time (Table 2).

Two way ANOVA showed that period explains more than 50% of the variation in tail length and %DNA in tail, while treatment was responsible for more than 50% of variation in tail moment only. Interaction between GSE dose and treatment period indicated non-significant effect on the studied parameters.

The DNA damage caused by the two variables (dose and treatment periods) was evaluated for each parameter using Duncan’s multiple range test to determine which variable had the most impact on the different DNA parameters. The observed significant difference in DNA damage parameters indicated that HGSE is more effective than LGSE in reducing DNA damage induced by O-NT. As shown in Table 2, 3 months of treatment with LGSE and HGSE indicated significant difference regarding the studied parameters when compared with one and two months of treatment. The observed exceptions were in tail length after two months and tail moment after one month of treatment. It was clear that the effect of GSE on O-NT groups was dose- and time-dependent.

Table 1: The effect of grape seed extract (low dose, LGSE and high dose, HGSE) post-treatment on the DNA damage (Comet assay) induced by O-Nitrotoluene (O-NT) treatment in mice colon cells.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Period of treatment</th>
<th>Tail Length Mean ± SD</th>
<th>%DNA in Tail Mean ± SD</th>
<th>Tail Moment Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>24hr</td>
<td>2.17 ±0.68</td>
<td>1.93 ±1.09</td>
<td>0.06 ±0.06</td>
</tr>
<tr>
<td>5-FU (20mg/kg)</td>
<td>14d</td>
<td>4.54 ±1.05*</td>
<td>8.71 ±2.97*</td>
<td>0.87 ±0.26*</td>
</tr>
<tr>
<td>O-NT1 (150mg/kg)</td>
<td>6 wks</td>
<td>9.75 ±3.52*</td>
<td>21.78 ±6.71*</td>
<td>3.03 ±1.78*</td>
</tr>
<tr>
<td>O-NT2 (150mg/kg)</td>
<td>6 wks</td>
<td>23.25 ±3.63*</td>
<td>36.81 ±2.11*</td>
<td>9.05 ±1.53*</td>
</tr>
<tr>
<td>O-NT + 5-FU (150mg/kg)</td>
<td>6 wks + 14d</td>
<td>5.61 ±1.32*</td>
<td>10.34 ±0.52*</td>
<td>1.20 ±0.22*</td>
</tr>
<tr>
<td>O-NT + LGSE1</td>
<td>6 wks + 1mo</td>
<td>5.78 ±0.69*</td>
<td>10.65 ±1.52*</td>
<td>0.96 ±0.09*</td>
</tr>
<tr>
<td>O-NT + LGSE2</td>
<td>6 wks + 2mo</td>
<td>4.96 ±0.53*</td>
<td>9.92 ±1.36*</td>
<td>1.04 ±0.43*</td>
</tr>
<tr>
<td>O-NT + LGSE3</td>
<td>6 wks + 3mo</td>
<td>3.37 ±2.33*</td>
<td>6.73 ±4.22*</td>
<td>0.52 ±0.52*</td>
</tr>
<tr>
<td>O-NT + HGSE1</td>
<td>6 wks + 1mo</td>
<td>3.70 ±0.82*</td>
<td>6.97 ±2.44*</td>
<td>0.40 ±0.10*</td>
</tr>
<tr>
<td>O-NT + HGSE2</td>
<td>6 wks + 2mo</td>
<td>3.83 ±0.45*</td>
<td>7.87 ±0.92*</td>
<td>0.53 ±0.13*</td>
</tr>
<tr>
<td>O-NT + HGSE3</td>
<td>6 wks + 3mo</td>
<td>2.50 ±0.23*</td>
<td>3.14 ±1.19*</td>
<td>0.16 ±0.11*</td>
</tr>
</tbody>
</table>

Notice: All groups are sacrificed after 24hr except O-NT2 group sacrificed after 1 month (mo) from last treatment. Results are expressed as mean ± standard deviation (SD)

*: Significant difference at P ≤ 0.05 using student t-test.
a: Statistically compared with negative control group.
b: Statistically compared with O-NT+5-FU group.
c: Statistically compared with O-NT+LGSE1 group.
d: Statistically compared with O-NT+LGSE2 group.
e: Statistically compared with O-NT+LGSE3 group.
f: Statistically compared with O-NT+HGSE1 group.
g: Statistically compared with O-NT+HGSE2 group.
h: Statistically compared with O-NT+HGSE3 group.

Table 2: Two-way ANOVA and multicomparisons by treatment and period with their interaction. Significance level * P ≤ 0.05, ns not significant. % SS = % of sums of squares (variance). Mean values in the same column in the same multicomparison with different superscript letter is differ significantly. (Duncan's multiple range test at P ≤ 0.05).

<table>
<thead>
<tr>
<th>Item</th>
<th>Tail Length</th>
<th>DNA % in the tail</th>
<th>Tail Moment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of variation</td>
<td>Sign</td>
<td>% SS</td>
<td>Sign</td>
</tr>
<tr>
<td>Dose</td>
<td>*</td>
<td>53.8</td>
<td>*</td>
</tr>
<tr>
<td>Period</td>
<td></td>
<td>ns</td>
<td>5.9</td>
</tr>
<tr>
<td>Dose x Period</td>
<td></td>
<td>ns</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Mean multicomparison by Dose

| LGSE          | 4.70 *    | 9.1 *    | 0.838 *  |
| HGSE          | 3.34 *    | 5.99 *   | 0.364 *  |

Mean multicomparison by Period

| One month     | 4.7 *     | 8.808 *  | 0.68 *   |
| Two month     | 4.4 20    | 8.89 *   | 0.788 *  |
| Three month   | 2.9 a     | 4.91 b   | 0.33 b   |
Discussion:

The present results showed that O-NT (150mg/kg) administration for 6 consecutive weeks induced precancerous stage in mice colon. This induction was declared by the distortion of different histoarchitectural structures, the high expression of COX-2 protein and induction of DNA damage. Expression of COX-2 protein was evaluated for O-NT1 group only to detect early stage of colon cancer. Because the histopathological evaluation for O-NT2 group was clearly demonstrated, that there is no sign of repair for the damage colon tissue cause by O-NT, therefore we didn’t evaluate COX-2 protein expression in this group.

Results of the present study are in agreement with previous studies, in which O-NT exposed mice developed carcinomas of the large intestine. Mice exposed to 1250, 2500 or 5000 p.p.m. O-NT in feed for 2 years induced large intestinal tumors in B6C3F1 mice “as a model of human colon cancer in their molecular pathogenesis” that shows a positive immunohistochemical staining of cytokeratins 20 (CK20) and a negative CK7. CK20 and CK7 have been used successfully in studies determining primary location of adenomas in mice from a chronic study, in which most of the colon tumors had increased protein levels of p53, β-catenin and cyclin D1 but no difference in protein expression was found for adenomatous polyposis coli (APC) protein between the colon tumors and normal colon tissue. Also, mutations in all three genes (p53, β-catenin and K-ras) were identified in most of the O-Nitrotoluene-induced colon tumors. In addition, male and female mice receiving O-NT developed tumors of the circulatory system, including hemangiomas and hemangiosarcomas. These hemangiosarcomas were found in the skeletal muscle, subcutaneous tissue, and/or mesentery (National Toxicology Program, 1993a, 1993b).

In agreement with the results of the present work, cumulative studies have demonstrated that COX-2 up-regulation is directly involved in carcinogenesis and development of colorectal cancer (Eberhart et al., 1994; Williams et al., 1996; Dannenberg and Zakim, 1999; Janssen et al., 2006). In addition, it has been reported that COX-2 plays an important role in the carcinogenesis of other kinds of human cancers such as gastric cancer (Ohno et al., 2001; Saukkonen et al., 2001), lung cancer (Wolff et al., 1998) and esophageal cancer (Shamma et al., 2000).

Fluorouracil (5-FU) has been the focus of considerable research as a chemotherapeutic agent for colorectal cancer (Milano et al., 1999). In the present study 5-FU group showed slight change in the histological architecture of the colon tissue, moderate cytoplasmic expression of COX-2 protein and significant increase in DNA damage parameters compared with the negative control group. In O-NT + 5-FU group, 5-FU failed to reduce the dysplastic damage and COX-2 protein expression in the colon tissue. In addition, DNA damage represented by the Comet assay in 5-FU group didn’t show difference from that occurred in O-NT + 5-FU group. These toxicity results are in agreement with Sloan et al. (2002) who confirms an earlier finding that women receiving 5-FU-based colorectal cancer chemotherapy in 5-day bolus schedule experience toxicity more frequently and with more severity than men.

The genotoxicity of 5-FU is attributed to the incorporation of uracil of 5-FU into DNA which results from the use of deoxyuridine monophosphate (dUMP) instead of deoxycytidine monophosphate (dTMP) by DNA polymerases which is the most common type of endogenous DNA damage (Guillet and Boiteux, 2003). This toxicity and the resistance to 5-FU have been major obstacles in successful colorectal cancer chemotherapy (Srimuangwong et al., 2012). Therefore, an alternative post-treatment with non-toxic agents that can inhibit COX-2 activity, decrease DNA damage might be useful for stopping colon carcinogenesis.

In the current study, we evaluated the chemotherapeutic effect of administration of grape seed extract (GSE) at low dose (LGSE) and high dose (HGSE) for 1, 2 and 3 consecutive months on early signs of colon cancer induced in mice by O-NT (150mg/kg) administration. Generally, LGSE and HGSE post-treatment reduced the dysplastic damage, COX-2 protein expression and DNA damage in the colon tissue. However, longer treatment with the high dose of GSE (HGSE3 group) renders the histological architecture which appeared intact, highly reduced expression of COX-2 that appears more or less as the negative control group.

Watanabe et al. (2010) indicated the oxidative DNA damage caused by O-nitrotoluene (O-NT metabolite), in which the nitroso metabolite of O-Nitrotoluene can be reduced to hydronitroxide radicals by an endogenous reductant, NADH. When the radicals autoxidize to O-Nitrosotoluene, the generation of O2- would then occur from molecular oxygen. Thereafter, O2- is dismutated to generate H2O2. NADH reduces the nitroso form to hydronitroxide radicals, and the radicals autoxidize again to the nitroso form, resulting in the generation of reactive oxygen species and DNA damage through the redox cycle. NADH itself is oxidized to NAD+, and then NAD+ with the formation of O2-.

The amelioration effect by the post-administration of GSE is probably due to its renowned anti-inflammatory and antioxidant potency. GSE is also reported to possess significant multi-organ histological protection against various toxic assaults (Bagchi et al., 2002; El-Ashmawy et al., 2006; Hemmati et al., 2008; Sehirli et al., 2008; El-Beshbishy et al., 2010; El-Ashmawy et al., 2010; Safa et al., 2010; Yalcin et al., 2010; Khalifa et al., 2011). In addition, Veluri et al. (2006) reported that gallic acid (one of the major active
constituents of GSE) showed a very strong dose- and time-dependent growth inhibition and apoptotic death of human prostate cancer DU145 cells. GSE showed potent antioxidant activity by trapping free radicals (hydroxyl, lipid free radicals, free iron molecules and lipid peroxides), delaying fat oxidation, inhibiting the major substance responsible for generating oxygen derived free radicals (xanthin oxidase) and reducing the concentration of H2O2 (Sugisawa et al. 2004) that produced by the oxidative stress resulted from O-Nitrotoluene treatment.

In accordance with the findings of the present study, the antioxidant activity of grape extract was reported to increase when the extract concentration increased (Baydar et al. 2007). In addition to the antioxidant/antiradical activity of grape seed extract, it was shown to possess many biological properties including the inhibition of DNA damage (Balu et al., 2006) and COX-2 gene expression (Lala et al., 2006).

In summary, the current results showed the therapeutic activity of GSE as indicated by its ameliorating effect of the signs of precancerous stage in colon tissues induced in mice by O-NT administration in dose- and time-dependent. While these preliminary results appear promising, further studies are required to elucidate the modulatory effect of GSE on early and late stages of colon cancer. Moreover, the present study confirmed the toxicity of the chemotherapeutic drug 5-FU.

References


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