THE EFFECTS OF ORAL GRAPE SEED EXTRACT ON
CISPLATIN-INDUCED CYTOGENOTOXICITY IN MICE

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Running Title: The antimutagenic effects of oral grape seed extract.

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The present investigation was directed to study the possible chemoprotective activity of orally administered grape seed extract (GSE) against cisplatin-induced cytotoxicity and genotoxicity towards mouse somatic and germinal cells in vivo. Pretreatment of mice with GSE (100 mg/kg/day) for 7 days and simultaneously with a single dose of cisplatin (2.2 or 5.5 mg/kg, i.p.) for another day, significantly reduced the frequency of bone marrow micronucleated polychromatic erythrocytes by factors of 1.9 and 1.28, respectively. Furthermore, GSE caused a reduction in bone marrow suppression induced by cisplatin treatment, particularly before the lower dose. In male germline, orally administration of GSE (100 mg/kg/day) for 7 consecutive days before and 7 consecutive days after treatment with a single dose of cisplatin (2.2 or 5.5 mg/kg, i.p.), significantly elevated the levels of sperm motility reduced by cisplatin treatment. Furthermore, GSE significantly decreased the elevated levels of sperm head abnormality induced with cisplatin by factors of 1.6 and 1.2, respectively. Our results indicate that GSE plays a role in attenuating the genotoxicity induced by cisplatin and may provide decreases in the development of secondary malignancy and abnormal reproductive outcomes risks.

Key words: GSE; Antimutagene; Cisplatin; Somatic cell; Germ cell.
Introduction

Cisplatin is one of the anti-cancer agents that falls into the DNA damaging agent class (1). The current accepted paradigm about cisplatin mechanism of action is that the drug induces its cytotoxic properties through reacting with nucleophilic bases in DNA and form intra- and interstrand cross-links (cisplatin–DNA adducts) and subsequent interference with normal transcription, and/or DNA replication mechanisms (2). This has led to propose the involvement of multi-step and multi-level effects of cisplatin in the tumor cell/host during cisplatin-mediated cancer chemotherapy (3). However, full therapeutic efficacy of this drug is limited due to the development of acquired drug resistance by the cancer cells and various side effects in the host (2).

In fact, cisplatin has important side effects including reduction of antioxidant plasma levels and generation of free radicals in normal cells (4, 5). After application of cisplatin, damage to DNA may result in DNA fragmentation, chromosomal breaks, and micronucleus formation causing genomic instability, and may lead to mutagenesis, carcinogenesis, or finally to apoptotic cell death. It has been reported that cisplatin is highly mutagenic, causing sister-chromatid exchanges and chromosome aberrations in cultured mammalian cells, bone marrow cells, germ cells and in the peripheral blood lymphocytes of humans and animals (6-11). Moreover, cisplatin is known to be carcinogenic in mammalian cells and this feature is of a special interest due to the risk of inducing secondary malignancies (12). The generation of free radicals is believed to be an important mechanism in the development of cisplatin toxicity (4, 5). Much effort has been put into reducing the mutagenic side effects of cisplatin by administration of modulating agents, usually free radical scavenges.
GSE is a natural extract from the seeds of *Vitis vinifera*. It contains the most beneficial groups of plant flavonoids, proanthocyanidins oligomers. These flavonoids are potent antioxidants and exert many health-promoting effects (13). Their effects include the ability to increase intracellular vitamin C levels, decrease capillary permeability and fragility and scavenge oxidants and free radicals. Recently, there is great evidence that GSE prevents oxidative injury by modulating the expression of antioxidant enzyme systems (14). The oxidative DNA damage in the brain regions of aged rats was also modulated by GSE administration (15). GSE has been also shown to be protective against nitrosative/oxidative stress (16), and has exhibited superior antioxidant performance over vitamins C, E and beta-carotene in both in vivo and in vitro models (17). It has been demonstrated that the activity of proanthocyanidins oligomers is approximately fifty times greater than that of vitamin C and vitamin E, in term of antioxidant action (18). In addition, GSE has a significant cytotoxicity towards human breast, lung and gastric adenocarcinoma cells, while enhancing the growth and viability of normal cells (19). Moreover, GSE enhances anti-tumor effects of doxorubicin both in vitro and in vivo (20). These studies demonstrate that GSE is a potent scavenger of free radicals, bioavailable and provide significant protection towards multiple target organs against structurally diverse drug- and chemical-induced toxic manifestation (17). In view of the above findings, the present study was designed to evaluate the potential protective effects of orally administered GSE against cisplatin-induced cytotoxicity and genotoxicity towards mouse somatic and germinal cells as an in vivo model.
Materials and Methods

Animals. Inbred male Swiss albino mice (25 ± 2 g) were obtained from a closed bred colony at our university. The animals were maintained in an air-conditioned animal house at a temperature of 25-28 °C, relative humidity at ~50 % and photo-cycle of 12:12 h light and dark periods. The animals were provided with standard diet pellets (El-Nasr Co., Egypt) and water ad libitum. All experiments were carried out according to the Guidelines of the Animal Care and Use Committee of at our university.

Drugs. Cisplatin was supplied as vials (Platinol, Bristol Myers Squibb Co., USA). The vial contents thoroughly dissolved in saline in darkness 10-15 min before use and administered by intraperitoneal injection within 1 h following preparation. It was administered at the doses level of 5.5 and 2.2 mg/kg (1/2 and 1/5 of LD$_{50}$). LD$_{50}$ values were derived from literature data (21). The doses of cisplatin were selected on the basis of its effectiveness in inducing chromosome aberrations. GSE was provided by Kikkoman Corp. (Noda City, Japan) and stored at 4°C in light-tight containers until used. The composition of this preparation was previously described (22), to consist of 89.3% (w/w) proanthocyanidins, 6.6% of monomeric flavonols, 2.24% of moisture content, 1.06% of protein, and 0.8% of ash. The extract thoroughly dissolved in distilled water and administered through oral intubations at the dose levels of 100 mg/kg/day for 7-14 consecutive days. The antimutagenic dose for the GSE was chosen by reference to earlier studies (23, 24). All other chemicals and reagents used were of analytical grade.
Experimental protocol. Animals were divided into twelve groups: Group 1: mice served as a control group and treated daily with saline for 7 consecutive days. Group 2: mice were injected with a single dose of 2.2 mg/kg cisplatin. Group 3: mice were injected with a single dose of 5.5 mg/kg cisplatin. Group 4: mice were treated with GSE at a dose of 100 mg/kg/day for 7 consecutive days. Group 5: mice were treated with GSE at a dose of 100 mg/kg/day for 7 consecutive days (to cover the seven cell divisions of erythropoiesis (25) and 2.2 mg/kg of cisplatin was administrated on day 7, one hour after regular GSE exposure. Group 6: mice were treated with GSE at a dose of 100 mg/kg/day for 7 consecutive days and 5.5 mg/kg of cisplatin was administrated on day 7. To study the effect of GSE on the germ cell mutagenicity of cisplatin treatment, the remaining six groups were used in another set of experiment. Groups 7-12 were treated as described above but mice were treated with GSE for 7 consecutive days before and 7 consecutive days after cisplatin injection (to cover the 14 days of spermatocytes stages in mouse spermatogenesis (26).

Micronucleus assay. After 30 h of cisplatin treatment i.e., on 8th day of the experiment the animals in groups 1-6 were sacrificed by cervical dislocation. Bone marrow smears were done and the slides were stained with May-Gruenwald/Giemsa. Per animal, 1000 polychromatic erythrocytes (PCE) were blindly scored microscopically (1000× magnification) for the presence of micronuclei (MN) according to the criteria described earlier (25, Figure 1). The ratio PCE/Normochromatic erythrocytes (NCE) was calculated by counting a total of 1000 erythrocytes per slide. The values were expressed as % PCE of the total erythrocyte counts to determine a reduction of erythroblast proliferation.
Evaluation of spermatozoa motility, count and abnormalities. According to the timing of spermatogenesis (26), 35 days of after cisplatin treatment i.e., on 42nd day of the experiment the animals in groups 7-12 were sacrificed by cervical dislocation. The epididymal content of each mouse was collected to estimate the percentages of sperm motility, count and sperm abnormality percentage. Immediately after cervical dislocation, both caudae epididymes of each animal were dissected and incisions were made. Then they were placed individually into Eppendorf cups filled with 300 µl of fetal calf serum. The cups were placed on an Eppendorf incubator at 32°C for 30 min to allow the sperm to actively leave the epididymes. The tissue residuals were removed from the cups and a drop of the suspension was taken on a clean slide and a smear was made. The slides were stained and examined by bright field microscope with an oil immersion lens. Three thousand sperms per treatment were scored and the abnormalities were categorized as close as to those described by Wyrobek and Bruce (27). Abnormal sperms had forms readily recognizable as amorphous, beak, without hook, triangular, banana-shaped, tail abnormality, dwarf and giant (Figure 4). Sperm count and motility were determined under the microscope using a Neubauer hematocytometer according to the WHO manual for the examination of human semen (28), and two counts per animal were averaged. The coded slides of all experiments were scored by only one scorer (Attia SM).

Statistical analysis. Significant differences of mean frequencies of MNPCE and % PCE [PCE/(PCE+NCE)x100] between individual treatment groups and corresponding solvent controls or between the solvent controls groups were calculated by Student's t-test and analysis of variance, ANOVA followed by Tukey-Kramer multiple comparisons test (29).
Results

Micronucleus assay. The results obtained from the micronucleus study are presented in Figure 2. Treatment of mice with GSE did not induce any significant variation in the incidence of MNPCE as compared to the control value. In additions, GSE was not cytotoxic to the bone marrow (i.e. no statistically significant decrease in the PCE:NCE ratio) at the tested dose level.

In comparison to the control group, the frequency of MNPCE was increased in cisplatin alone treated mice with the maximum MN being induced in PCEs at 5.5 mg/kg. The response was directly correlated with bone marrow cytotoxicity, as greater bone marrow suppression was noted in the group of mice treated with cisplatin 5.5 mg/kg body weight alone, indicating a reduction in erythroblast proliferation most likely by mitotic arrest. At a dose of 2.2 mg/kg, the frequency of MNPCE was 2.4 % compared to 0.32 % in the solvent control (p<0.01). Similarly, cisplatin at a dose of 5.5 mg/kg significantly increased the frequency of MNPCE to 5.14 % (p<0.01). In additions, cisplatin at a dose of 2.2 and 5.5 mg/kg was cytotoxic on the mouse bone marrow. The percent PCE was significantly decreased to 47.55 and 42.95 %, respectively, compared to 49.63 % in the control group (Figure 3).

Pretreatment of mice with GSE for 7 days and simultaneously with cisplatin for another day significantly reduced the frequency MNPCE induced by 2.2 mg/kg of cisplatin by a factor of 1.9, i.e. 1.26 % compared with the cisplatin alone value of 2.40 % (p<0.01). In animals treated with GSE plus 5.5 mg/kg of cisplatin combination, the frequencies of MNPCE decreased significantly by a factor of 1.28, i.e. 4.0 % compared with the cisplatin value of 5.14 % (p<0.01). However, the reductions were still significantly different from the control value. Furthermore, GSE caused a
reduction in bone marrow suppression induced by cisplatin; this is manifest as a reduction in the % PCE depression induced by cisplatin. The % PCE in the animals pretreated with GSE plus cisplatin 2.2 mg/kg was not significantly different from the control value, i.e. 48.10 compared with the control value of 49.63. However, the reduction was still significant when comparing with the control value in animals treated with GSE plus 5.5 mg/kg cisplatin.

_Evaluation of spermatozoa motility, count and abnormalities._ The results of the germ cells experiment are shown in Figures 5-7. Treatment of mice with GSE did not affect the parameters studied as compared to the control value. The percentage sperm motility was significantly decreased at the two cisplatin doses, compared to the value observed in the control group. At a dose of 2.2 mg/kg, the percentage sperm motility was 63.0% compared to 84.0% in the solvent control (_p_ <0.05). Similarly, cisplatin at a dose of 5.5 mg/kg significantly decreased the percentage sperm motility to 47.0% (_p_ <0.01). Treatment with cisplatin caused a significant decrease in the sperm count only at the high dose (5.5 mg/kg, _p_ <0.05). Moreover, cisplatin caused a significant increase in the percentage of abnormal sperm at the two doses tested. At a dose of 2.2 mg/kg, the percentage of abnormal sperm was 6.0 compared to 2.8 in the solvent control (_p_ <0.01). Similarly, 5.5 mg/kg of cisplatin significantly increased the percentage of abnormal sperm to 7.0% (_p_ <0.01).

Pretreatment of mice with GSE for 7 consecutive days before and 7 consecutive days after cisplatin treatment significantly increase the decreased percentage sperm motility and the percentage of abnormal sperm only at the low dose tested by cisplatin (i.e. 2.2 mg/kg). The percentage sperm motility, sperm count and the percentage of abnormal sperm were also restored with GSE pretreatment;
however, these ameliorations were not significant when compared to the values observed in the group treated with 5.5 mg/kg of cisplatin alone.

Discussion

The developments of micronuclei and sperm head abnormality have been commonly used as sensitive biological indicator in the mutagenic bioassays of a drug (25). In our study the development of these mutagenic parameters were seen after cisplatin treatment which supports earlier findings of its genotoxic properties in mice (8-10). The present study showed that cisplatin significantly increased the MN frequencies and decreased the number of PCE close to the previous results by Nersesyan et al. (10), indicating the genotoxicity and cytotoxicity for bone marrow cells. The results obtained in the present study showed that GSE did not increase the incidence of micronucleated PCEs and did not reduce the % PCE. These findings clearly indicated that GSE did not have clastogenic or cytotoxic effect at the doses tested (100 mg/kg/day for 7 consecutive days). Our observations are in harmony with that of the cytogenetic studies with GSE in mouse bone marrow by Erexsom, (30). Who reported no statistically significant increase in micronucleated PCEs was observed in animals orally treated with 500, 1000 and 2000 mg/kg of GSE at 24 harvest time after dosing with GSE.

The comparative analysis of the frequency of MN in the mice treated with cisplatin alone and GSE plus cisplatin showed significant reductions in the frequencies of micronucleated cells in the later groups by factors of 1.9 and 1.28 compared with that induced by 2.2 and 5.5 mg/kg cisplatin, respectively. However, the reductions were still significantly different from the control value, indicating that no complete protections occurred. Furthermore, GSE caused a reduction in the
cytotoxicity induced by cisplatin, this is manifest as a reduction in the NCE:PCE ratio
depression induced by the low dose of cisplatin. Hence, we showed that GSE intake
significantly decreased the genotoxic and cytotoxic effects of cisplatin on somatic
cells however; this reduction does not reach normal levels after treatment.

In male germline, administration of cisplatin to mice decreased sperm motility
and sperm concentration (p < 0.05), increased total abnormal sperm mice (p < 0.01) as
compared with the control group. This suggests that cisplatin could reach the germ
line cells and indicates its potentiality as germ cell mutagen too. These findings agree
with those of Misra and Choudhury (8) and Nersesyan, et al. (9). Whilst GSE did not
affect the parameters studied as compared to the control value. Pretreatment of mice
with GSE significantly elevated the level of sperm motility reduced by the low dose
of cisplatin. At higher dose of cisplatin the effect of GSE was not statistically
significant comparing with the cisplatin group alone. Moreover, the level of sperm
count was also restored with GSE pretreatment to simulate the value observed in the
control group. Analysis of sperm head abnormality in mice treated with 2.2 or 5.5
mg/kg of cisplatin as a single agent and in combination with GSE showed a
significant decrease in sperm head abnormality in the combined treated groups at both
dose levels by 37% and 17 %, respectively. However, this reduction was not
statistically significant when compared to the value observed in the animals treated
with higher dose of cisplatin alone.

Hence we showed that GSE intake decreased significantly the genotoxic
effects of cisplatin on somatic and germ cells. Sugisawa et al., (31) found that GSE
showed dose-dependent prevention of H₂O₂ chromosomal damage evaluated by
cytokinesis block micronucleus assay *in vitro*. Furthermore, GSE prevented DNA
oxidative damage in various tissues and DNA fragmentation induced by many agents
Ray et al. (23) suggested that in vivo protection of DNA by GSE might be due to detoxification of cytotoxic radicals and presumed contribution to DNA repair. The cause of the antigenotoxic effect of the GSE 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 that, because of its 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 (34).

Many authors found that genotoxicity and chromosomal instability induced by many agents are straightly correlated with the parameters of oxidative stress (35, 36). In addition, the reaction of cisplatin with DNA leading to perturb its secondary structure that results in interstrand and intrastrand cross-linkings and in DNA-protein cross-linkings that could result in clastogenic and aneugenic effects (2). It has been shown that most antioxidants can decrease the genotoxic effects of cisplatin, such as glutamine, curcumin, cedron-leaf infusion, saffron, beta-glucan, vitamins C and seabuckthorn juice (7-9, 37-40). It is well known that cisplatin upon hydrolysis in aqueous solution forms various reactive hydroxyl species (41), which can be destroyed by the previous mentioned antioxidants.

Based on the published literatures and the pharmacological properties of GSE, which are mostly described as antioxidant, might explain the way by which it protects the bone marrow chromosomes against cisplatin-induced genotoxicity. However, the protective action of GSE on normal somatic and germinal cells raises the question whether the effect exerted by the GSE would affect the anti-tumor efficacy of cisplatin in cancer cells. This problem cannot be solved without performing
experiments on cancer cells and their results may depend on the nature of a particular cancer. The only indirect evidence in the opposite direction is the publications describing that a pre-treatment with some antioxidant extracts as *Phellinus rimosus* (Berk) Pilat, provided protection to cisplatin’s side effects in animals without interfering with the cisplatin’s anti-tumor efficacy (42). In additions, anti-cancer and chemopreventive efficacy of GSE against various malignancies via its anti-proliferative, pro-apoptotic and anti-angiogenic activities in both cell culture and animal models may enhances the cisplatin’s anti-tumor efficacy (43-45). Overall, these studies suggest the potential of GSE in the cancer chemoprevention; however, future studies need to be conducted in order to determine if the GSE can effectively inhibit the power of cisplatin to induce genetic damage in normal cells without interfering with its capacity to reduce tumor growth in cells treated with cisplatin.

Antigenotoxic activity of GSE is not sudden; it is well known that consumption of fresh fruits and vegetables is associated with decline in cancer incidence (46). It is due 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 (47). Many vegetables and fruits are known to prevent chromosomal and DNA damage in animals (9, 48). Usually antimutagens acting in rodents are active in humans too (49). Our results may have a practical use in decline of genotoxic effects of cisplatin in cancer patients, some health care workers as nurses and pharmaceutical plant workers handle this drug which may attenuate the higher risks for development of secondary malignancy and for abnormal reproductive outcomes due to its antimitagenic and antioxidant activity.
of GSE. Radicals, which can induce damage in biologically important molecules can be trapped by antioxidants and hence preventing genotoxicity (46).

Acknowledgement

This work was funded by the Egyptian Government-Al-Azhar University.

References


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Figure (1). Microscopic image of a bone marrow preparation after May-Gruenwald/Giemsa staining showing nucleated as well as enucleated cells. One polychromatic erythrocyte (PCE) contains a micronuclei (MN).
Figure (2). Frequencies of MNPCE in bone marrow of mice 30h after treatment with the indicated doses of cisplatin (CP) and/or grape seed extract (GSE).

<table>
<thead>
<tr>
<th>Doses (mg/kg)</th>
<th>%MNPCE (mean ± SD)</th>
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<tbody>
<tr>
<td>Control</td>
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<td>CP (2.2)</td>
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<td>CP (5.5)</td>
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<td>GSE</td>
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<td>CP (2.2) + GSE</td>
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<td>CP (5.5) + GSE</td>
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MNPCE = micronucleated polychromatic erythrocytes. **P< 0.01, compared with the control, *P< 0.05, compared with cisplatin 2.2 and 5.5 mg/kg, respectively. (Student's t-test and analysis of variance, ANOVA followed by Tukey-Kramer multiple comparisons test).

Each value represents the mean ± SD of five animals.
Figure 3. Frequencies of PCE in bone marrow of mice 30h after treatment with the indicated doses of cisplatin (CP) and/or grape seed extract (GSE).

Doses (mg/kg)

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<th>CP (2,2)</th>
<th>CP (5,5)</th>
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<th>CP (2,2) + GSE</th>
<th>CP (5,5) + GSE</th>
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<td>PCE (%)</td>
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<td>Control</td>
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<td>CP (5,5)</td>
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<td>CP (2,2) + GSE</td>
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<td>CP (5,5) + GSE</td>
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PCE = polychromatic erythrocytes. *P< 0.05, **P< 0.01, compared with the control,
*aP< 0.05, compared with cisplatin 5.5 mg/kg (Student's t-test and analysis of variance, ANOVA followed by Tukey-Kramer multiple comparisons test).

Each value represents the mean ± SD of five animals.

Figure (4). Illustration of the various abnormal shapes in epididymal sperm of mice. Triangular (t), without hook (w-h) and coiled tail (c-t).
Figure (5). Frequencies of sperm motility 35 days after treatment with indicated doses of cisplatin (CP) and/or grape seed extract (GSE).

Doses (mg/kg)
- Control
- CP (2.2)
- CP (5.5)
- GSE
- CP (2.2) + GSE
- CP (5.5) + GSE

Sperm motility (%)
- 0
- 20
- 40
- 60
- 80
- 100
- 120

*P<0.05, **P<0.01, compared with the control. aP<0.05, compared with cisplatin 2.2 mg/kg (Student's t-test and analysis of variance, ANOVA followed by Tukey-Kramer multiple comparisons test). Each value represents the mean ± SD of five animals.
Figure (6). Frequencies of sperm count 35 days after treatment with indicated doses of cisplatin (CP) and/or grape seed extract (GSE).

*P<0.05, compared with the control (Student's t-test and analysis of variance, ANOVA followed by Tukey-Kramer multiple comparisons test).

Each value represents the mean ± SD of five animals.
Figure (7). Frequencies of abnormal sperm 35 days after treatment with indicated doses of cisplatin (CP) and/or grape seed extract (GSE).

**P< 0.01, compared with the control, *P< 0.05, compared with cisplatin 2.2 mg/kg (Student’s t-test and analysis of variance, ANOVA followed by Tukey-Kramer multiple comparisons test). Each value represents the mean ± SD of five animals.