POTENTIAL PROTECTIVE EFFECT OF GRAPE SEED EXTRACT AGAINST ACRYLONITRILE-INDUCED MUTAGENICITY IN RATS

Ashraf M. Morgan and Osama S. El-Tawil

Toxicology and Forensic Medicine Department, Faculty of Veterinary Medicine, Cairo University, Egypt

ABSTRACT

Acrylonitrile (ACN), an environmental toxic pollutant, has been detected in drinking water, food products and occupational environment. ACN is reported as a potent in vivo and in vitro mutagen and carcinogen in human and experimental animals. Grape seed proanthocyanidine extract (GSE) is a highly bioavailable biologically active polyphenolic bioflavonoid. It is a potent antioxidant posses a broad spectrum of pharmacological and therapeutic activities against free radicals, DNA damage and oxidative stress. The objective of the present study was to investigate the possible in vivo protective effects of GSE against ACN-induced micronucleus and chromosomal aberrations in male rats. Animals were exposed to a single s/c dose of ACN (115 mg/kg body weight). Another two groups of animals were pretreated with GSE in a dose of 100 and 200 mg/kg body weight orally for seven consecutive days prior to ACN administration (single s/c dose of 115 mg/kg body weight). The animals were subjected to cyogenetic analysis in bone marrow by micronucleus induction and chromosomal aberrations assays. The present results indicate that ACN significantly induced micronuclei and chromosomal aberrations. Pretreatment with GSE significantly improved these mutagenic effects in a dose related manner.

Keywords: Acrylonitrile - grape seed - proanthocyanidine – rats – mutagenicity.

INTRODUCTION

Acrylonitrile (ACN) is an extensively produced aliphatic nitrile that is used in the synthesis of acrylic fibers, resins and plastics (IARC, 1979). It is also used in the manufacture of soft prosthesis material (Parker and Bradern, 1990), high permeable dialysis tubing (Ward et al., 1993) and medical gloves (Walsh et al., 2004). ACN has been detected in drinking water (Rubio et al., 1990), food products (Ventura et al., 2004), occupational environment (Ochiai et al., 2003) and cigarette smoke (Nazaroff and Singer, 2004).

Animal studies performed on male and female rats indicated that ACN is a carcinogen (Gallagher et al., 1988; Maltoni et al., 1988 and Quast and Friedman, 2002). Epidemiological studies conducted on workers exposed to ACN indicated increased incidence of DNA strand breakage and sex chromosome aneuploidy in human spermatozoa (Xu et al., 2003).

Genotoxicity of ACN has been previously reviewed (Whysner et al., 1998; Leonard et al., 1999 and IARC, 1999). ACN has been shown to have genotoxic potential, primarily based on its metabolism to cyanoethylene oxide (CEO) (Their et al., 2000 and Chanas et al., 2003). Specifically, ACN has been shown to be mutagenic with one or more strains of salmonella typhimurium (Lijinsky and Andrews, 1980 and Zeiger and Haworth, 1985); induce sister chromatid exchanges in Chinese hamster ovary cells (Chang et al., 1990). As well as ACN considered one of the micronucleus positive rat carcinogens (Wakata et al., 1998). Chromosomal aberrations and hemoglobin adducts are accurate and sensitive in vivo biomonitoring markers for ACN exposure (Borba et al., 1996).

Herbs have recently become attractive as health-beneficial foods (physiologically functional foods) as a source material for the development of drugs. Herbal medicines derived from plant extracts are being utilized increasingly to treat a wide variety of clinical diseases, with relatively little knowledge regarding their modes of action (Matthews et al., 1999). Grapes (Vitis vinifera) are considered as the world's largest fruit crops, with an approximate annual production of 58 million metric tones (FAO). 1997).

Grape seed proanthocyanidine extract (GSE) is highly bioavailable polyphenolic bioflavonoid, provides greater protection against free radicals and DNA damage (Bagchi et al., 2000). Its biological antioxidant activity is well known and possess broad spectrum of pharmacological and therapeutic activities against free radicals and oxidative stress (Bagchi et al., 2001). GSE has been recorded to have anti-inflammatory properties (Gabor, 1986), antiviral activity (Kakiuchi et al., 1991), inhibitory effects on skin tumor promotion (Gali et al., 1994), anticancer properties...
Although acute effects for ACN exposure and its protection are the primary concern, potential genotoxic and carcinogenic risks of ACN have been taken seriously in industries and the worldwide population using products containing and possibly liberating ACN. (Borba et al., 1996 and Leonard et al., 1999).

Therefore, the present work was designed to assess the potential mutagenic effects induced by ACN and the possible protection by GSE as a powerful antioxidant.

MATERIAL AND METHODS

Animals: Forty young male Sprague Dawley rats (75 – 85 gm) were used in this study. They were kept under good standard hygienic condition and supplied with food and water ad libitum.

Chemicals: Acrylonitrile was obtained from Sigma chemical Co. St. Louis, Mo. USA. Grape seed extract (GSE) "Noxylife" (95% proanthocyanidin) was obtained from Nulife international, company USA. All other used chemicals were of highest purity and analytical grade.

Experimental design: The animals were divided into 4 equal groups 10 rats each. The first group was kept as control. The 2nd group was injected subcutaneously with a single dose of 115 mg ACN / kg body weight (Nerland et al., 2001). The 3rd and 4th groups were administered orally with chemicals were of highest purity and analytical grade. Nulife international, company USA. All other used chemicals were of highest purity and analytical grade.

The micronuclei were prepared according to the method of Schmid (1975) with some modifications recorded by Adler (1984). Briefly, the femurs of each animal were dissected out, and the bone marrow was flushed gently from the channel into a tube with fetal bovine serum (FBS, approximately 1 ml/femur). The suspension was centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and the pellet was resuspended. Smears were drawn on the precleaned slides, using a drop of the resultant suspension in FBS. The slides were coded, air dried, fixed in absolute methanol for 20 min and stained by May–Grunwald stain followed by Giemsa stain (Sigma chemical Co. St. Louis, Mo. USA). Polychromatic erythrocytes (PCEs) and Normochromatic erythrocytes (NCEs) were counted in all stained slides. One thousand of each polychromatic erythrocytes (PCE) and Normochromatic erythrocytes (NCE) were examined for each animal for the presence of micronuclei. The in vivo micronucleus assay employed in the present study has been known to be one of the most widely used in vivo screening tests for genotoxicity and antimutagenecity testing (Hosseinimehr et al., 2003 and Kumar et al., 2003). An increase in the frequency of micronucleated PCE indicates chromosomal damage (Krishna and Hayashi, 2000). The unit of analysis is PCE, but not the number of micronuclei per PCE, because a PCE may contain more than one micronucleus. The ratio of PCE to NCE was determined for each experimental group to assess the ACN effects on bone-marrow proliferation with or without GSE pretreatment.

Chromosomal aberration assay:

The chromosomal aberrations were investigated according to the method of Preston et al (1987). Briefly, Bone marrow was flushed with a pre-warmed hypotonic solution (0.075 M KCl) and kept for 20 min then centrifuged at 1000 rpm for 5 min. After centrifugation, the cell pellet was washed 3 times and resuspended in the same fixative for 24 hour. The fixed cell suspensions were dropped on clean chilled wet slides from 2 – 2.5 m height. These slides were examined after air drying and staining with 10 % Giemsa stain. The possible chromosomal aberrations were recorded in all stained slides.

Statistical analysis:

Assessment of the results was performed using one-way analysis of variance (ANOVA) procedure followed by Tukey-Kramer multiple comparison post-tests. Statistical analyses were performed using Software GRAPHPAD INSTAT (Version 2). The 0.05 level of probability was used as the criterion for significance.

RESULTS

1 – Micronucleus assay:

Table (1) summarizes the frequency of polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) in addition to PCE/NCE ratio in bone marrow of control and treated rats. Animals exposed to ACN showed a significant increase in the incidence of micronucleated PCE (Figures 1, 3 and 4) and micronucleated NCE (Figures 2 and 5) and significant decrease in PCE/NCE ratio compared to control groups, indicating bone marrow cytotoxicity. However, pretreatment with GSPE showed significant improvement of ACN-induced effects in a dose related manner. GSE in a dose of 100 mg/kg body weight...
Morgan and El-Tawil (2006) Potential Protective Effect of Grape Seed Extract Against Acrylonitrile-Induced

significantly reduced the incidence of micronucleated PCE and NCE by 43.8 % and 56.3 % respectively, as compared with ACN- treated group. However, GSE in a dose of 200 mg/kg body weight significantly reduced the incidence of micronucleated PCE and NCE by 73.8 % and 82.5 % respectively, as compared with ACN - treated group (Figures 1 and 2).

2 – Chromosomal aberration assay:

The frequencies of chromosomal aberration in bone – marrow cells of control and experimental groups are presented in Table (2). ACN exposure induced a significant increase in the total number of aberrant cells and those with more than one aberration compared to those of control, indicating bone marrow cytotoxicity. Chromosomal and chromatid gaps, breaks and interchanges were the main aberrations observed (Figures 6- 10). Pretreatment with GSE significantly reduced the chromosomal aberration recorded by ACN exposure in a dose dependent manner. The number of aberrant cells which were found to be 10.20 ± 0.15 in ACN treated animals, was reduced to 6.44 ± 0.50 by 100 mg GSE and 4.32 ± 0.30 by 200 mg GSE (Table 2). All types of chromosomal aberrations induced by ACN, including breaks, exchanges, and other multiple damages were found to be reduced by GSE pretreatment.

Table (1) Protective effects of GSE on ACN-induced cytotoxicity in rat bone marrow cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>AN (115 mg/kg)</th>
<th>GSE (100 mg/kg)</th>
<th>GSE (200 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells analyzed</td>
<td>5000</td>
<td>5000</td>
<td>5000</td>
<td>5000</td>
</tr>
<tr>
<td>No. of PCEs analyzed</td>
<td>2567</td>
<td>1934</td>
<td>2240</td>
<td>2430</td>
</tr>
<tr>
<td>PCEs %</td>
<td>51.34</td>
<td>38.68</td>
<td>44.80</td>
<td>48.60</td>
</tr>
<tr>
<td>No. of NCEs analyzed</td>
<td>2433</td>
<td>3066</td>
<td>2760</td>
<td>2570</td>
</tr>
<tr>
<td>NCEs %</td>
<td>48.66</td>
<td>61.32</td>
<td>55.20</td>
<td>51.40</td>
</tr>
<tr>
<td>PCE / NCE ratio</td>
<td>1.06</td>
<td>0.63</td>
<td>0.81</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Each experimental group consisted of 5 animals.

Table (2): Protective effects of GSE on ACN-induced chromosomal aberrations in rat bone marrow cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>AN (115 mg/kg)</th>
<th>GSE (100 mg/kg)</th>
<th>GSE (200 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of aberrant cells</td>
<td>1.48 ± 0.02</td>
<td>10.20 ± 0.15 a</td>
<td>6.44 ± 0.50 ab</td>
<td>4.32 ± 0.30 ab</td>
</tr>
<tr>
<td>Cells with more than one aberration</td>
<td>0.00 ± 0.00</td>
<td>3.90 ± 0.57 a</td>
<td>0.88 ± 0.03 b</td>
<td>0.76 ± 0.04 b</td>
</tr>
<tr>
<td>Chromosome gap</td>
<td>0.25 ± 0.01</td>
<td>3.98 ± 0.76 a</td>
<td>2.12 ± 0.18 ab</td>
<td>1.02 ± 0.10 b</td>
</tr>
<tr>
<td>Chromosome break</td>
<td>0.00 ± 0.00</td>
<td>2.10 ± 0.01 a</td>
<td>0.83 ± 0.02 ab</td>
<td>0.35 ± 0.01 ab</td>
</tr>
<tr>
<td>Dicentric chromosome exchange</td>
<td>0.00 ± 0.00</td>
<td>0.80 ± 0.01 a</td>
<td>0.20 ± 0.01 ab</td>
<td>0.10 ± 0.01 ab</td>
</tr>
<tr>
<td>Tricentric chromosome exchange</td>
<td>0.00 ± 0.00</td>
<td>0.20 ± 0.01 a</td>
<td>0.00 ± 0.00 b</td>
<td>0.00 ± 0.00 b</td>
</tr>
<tr>
<td>Polycentric chromosome exchange</td>
<td>0.00 ± 0.00</td>
<td>0.15 ± 0.01 a</td>
<td>0.00 ± 0.00 b</td>
<td>0.00 ± 0.00 b</td>
</tr>
<tr>
<td>Chromatid gap</td>
<td>0.30 ± 0.01</td>
<td>3.60 ± 0.15 a</td>
<td>1.07 ± 0.09 ab</td>
<td>0.66 ± 0.03 ab</td>
</tr>
<tr>
<td>Chromatid break</td>
<td>0.20 ± 0.01</td>
<td>2.32 ± 0.14 a</td>
<td>0.95 ± 0.05 ab</td>
<td>0.65 ± 0.02 ab</td>
</tr>
<tr>
<td>Triradial chromatid exchange</td>
<td>0.00 ± 0.00</td>
<td>0.60 ± 0.03 a</td>
<td>0.02 ± 0.00 b</td>
<td>0.01 ± 0.00 b</td>
</tr>
<tr>
<td>Acenetic ring</td>
<td>0.00 ± 0.00</td>
<td>1.10 ± 0.12 a</td>
<td>0.34 ± 0.03 ab</td>
<td>0.12 ± 0.01 b</td>
</tr>
<tr>
<td>Centric ring</td>
<td>0.00 ± 0.00</td>
<td>0.15 ± 0.01 a</td>
<td>0.10 ± 0.02 a</td>
<td>0.08 ± 0.02 ab</td>
</tr>
<tr>
<td>Fragment</td>
<td>0.10 ± 0.01</td>
<td>0.78 ± 0.07 a</td>
<td>0.28 ± 0.04 b</td>
<td>0.16 ± 0.04 b</td>
</tr>
<tr>
<td>Double minute</td>
<td>0.00 ± 0.00</td>
<td>1.55 ± 0.12 a</td>
<td>0.45 ± 0.08 ab</td>
<td>0.32 ± 0.08 b</td>
</tr>
<tr>
<td>Minute</td>
<td>0.12 ± 0.01</td>
<td>1.95 ± 0.25 a</td>
<td>0.87 ± 0.01 ab</td>
<td>0.45 ± 0.01 b</td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E. of 5 animals.
(a) Significantly different from corresponding control group at $p < 0.05$.
(b) Significantly different from ACN alone-treated group at $p < 0.05$. 

Morgan and El-Tawil (2006) Potential Protective Effect of Grape Seed Extract Against Acrylonitrile-Induced

Figure (1): Protective effects of GSE on ACN-induced micronucleated PCEs

(a) Significantly different from corresponding control group at $p < 0.05$
(b) Significantly different from ACN alone-treated group at $p < 0.05$

Data expressed as mean ± S.E. of micronucleated PCE/1000 PCE

Figure (2): Protective effects of GSE on ACN-induced micronucleated NCEs

(a) Significantly different from corresponding control group at $p < 0.05$
(b) Significantly different from ACN alone-treated group at $p < 0.05$

Data expressed as mean ± S.E. of micronucleated NCE/1000 NCE

Figure (3): Bone marrow smear from ACN exposed rats showing micronucleated PCE (a) and micronucleated bone marrow cell (b).

Figure (4): Bone marrow smear from ACN exposed rats showing micronucleated PCE with two micronuclei.

Figure (5): Bone marrow smear from ACN exposed rats showing micronucleated NCE.

Figure (6): Normal bone marrow metaphase from control rats.
DISCUSSION

A considerable emphasis is being laid down on the use of dietary constituents as chemoprotective measure for control of genetic diseases (Mitscher et al., 1996). Bone marrow cytogenetics is a useful short-term technique, for elucidating the mechanism as well as to identify the substances for their clastogenic and anticlastogenic activity (Renner, 1990). Majority of the mutagenic/carcinogenic compounds acts by generating electrophilic intermediates by microsomal enzymatic reactions causing mutations (Klaassan, 1996 and Vainio et al., 1992). Antigenotoxic agents especially those present in natural substances acts through different cellular pathways involving endogenous sequestration of mutagens by various enzymes (Mitscher et al., 1996; Vainio et al., 1992 and Heddle et al., 1999).

The present investigation was undertaken to evaluate the antimutagenic effects of GSE against the mutagenic potential of ACN in rats using the micronucleus and the chromosomal aberration assays. The results of micronucleus assay revealed significant increase in the number of micronucleated PCEs and NCEs in rats exposed to ACN compared to control groups. In addition, the PCE / NCE ratio showed significant decrease. Similar findings were reported by Borba et al. (1996) and Wakata et al. (1998).

The micronucleus test was recommended as a rapid and convenient in vivo method for screening clastogenic effect in mammals (Heddle and Carano, 1977). The increase in the frequency of micronucleated erythrocytes indicated interference with nuclear division in the bone marrow erythroblasts. At anaphase the acentric fragments lag behind and fail to be incorporated into one of the daughter nuclei giving rise to micronuclei. Therefore, the presence of micronuclei in erythrocytes are the result of chromosomal breakage or interference with spindle assembly or function. Thus, an elevated frequency of micronucleated cells strongly suggests that one of these types of damage has occurred (Schmid, 1975; Gad and

Figure (7): Bone marrow metaphase from ACN exposed rats showing ring chromosome.

Figure (8): Bone marrow metaphase from ACN exposed rats showing minute (a), double minute (b) and chromatid break (terminal deletion) (c).

Figure (9): Bone marrow metaphase from ACN exposed rats showing chromatid deletion.

Figure (10): Bone marrow metaphase from ACN exposed rats showing acentric fragment (a) and minute (b).
Concerning the chromosomal aberrations, our results revealed that ACN exposure induced various chromosomal aberrations mainly chromatid and chromosomal gaps, breaks and interchanges, minute and double minute. This finding is in accordance with the early report indicating that the chromosomal aberrations and hemoglobin adducts considered as an accurate sensitive in vivo markers for ACN exposure (Borba et al., 1996). ACN was confirmed to induce clastogenic effects in human (Czeizel et al., 2000). In addition, ACN recorded to have mutagenic effects in human lymphoblasts (Recio et al., 1990) and in Drosophila (Lijniski and Andrews, 1980).

Chromosomal aberrations occurred due to lesions in the DNA that lead to discontinuities in the DNA double strand helix. The breakage–reunion hypothesis implied that discontinuity in the DNA might be stabilized to appear as a break at metaphase. Alternatively, the discontinuity might be restituted by repaired processes to the original state, whereby the chromosome did not exhibit structural changes. Two DNA discontinuities in temporal and spatial proximity might interact in the reunion of the broken ends, thus forming exchange configurations. The exchange hypothesis postulated that all aberrations were resulted from the exchange processes (WHO, 1985).

Although the exact mechanism of ACN induced cytotoxicity has not yet been fully explored, the action of cyanide which is liberated inside the organism and ACN molecule itself was considered to play some roles (Hashimoto, 1980). The metabolic activation of ACN appeared to be a prerequisite for its mutagenic activity (Leonard et al., 1999). ACN is metabolized in humans and experimental animals via two competing pathways, the gluthathione–dependant pathway was considered to represent an avenue of detoxication whilst the oxidative pathway leads to a genotoxic epoxide cyanoethylene oxide via CYPE1 and to its subsequent metabolism via epoxide hydrolases to yield cyanide (Their et al., 2000 and Chanas et al., 2003). The mutagenic activity present in urine of animals treated with ACN was tentatively related to the excretion of three ACN urine metabolites: thiocyanate (SCN–), hydroxyl ethyl mercapturic acid (CN-MA) and cyanohydrine mercaptouric acid (CN - MA) (Lambotte – Vandepoe et al., 1985).

Concerning the effects of GSE on the ACN induced bone marrow cytotoxicity, our results revealed that GSE pretreatment induced in a dose dependent manner a significant decrease in the incidence of micronucleated PCEs and NCEs and significant decrease in the frequencies of chromosomal aberrations compared to ACN exposed animals. The doses of GSE (100 and 200 mg/kg) have been chosen in the present study because it is safe and non mutagenic. Yamakoshi et al. (2002) stated that GSE itself has been confirmed to be safe and non mutagenic to rats at oral doses of 2 and 4 g/kg. The exact protective mechanism of GSE is not clear at present. The recorded protective effects of GSE have been attributed to both inhibitions of metabolism and/or detoxification of cytotoxic radicals produced during chemicals biotransformation (Ray et al., 2000). GSE has been shown to protect most rodents specially mice from the toxic effects of ACN when it was ingested before ACN (Darmon et al., 1990).

Grape seeds are rich sources of monomeric phenolic compounds, such as (+)-catechins, (-)-epicatechin and dimeric, trimeric and tetrameric procyanidins. These compounds act as antimutagenic and antiviral agents (Sito et al., 1998 and Skrede and Wrolstad, 2002). The multiple mechanisms of their antioxidative activity are expressed in its ability of free radical scavenging, metal chelation, and synergism with other antioxidants (Lu and Foo, 1999). Antioxidant activity of grape seed extract has been confirmed by β-carotene linolate and linolicate acid peroxidation methods (Jayaprakasha et al., 2001) as well as by DPPH method (Jayaprakasha et al., 2003). Grape seed extract has been evaluated for its antioxidative effects on a few meat types and has been reported to improve the oxidative stability of cooked beef (Ahn et al., 2002) and turkey patties (Lau and King, 2003).

In conclusion, the result of the present study reveals that grape seeds extract exerts antimutagenic effects of varied potency, dependent on dose against ACN-induced bone marrow cell cytotoxicity. Hence the oral administration of GSE is found to be capable of preventing micronucleus induction and chromosomal aberrations caused by ACN. The protective effect of GSE towards ACN induced cytotoxicity and cytogenetic damage implies as a good marker of its antimutagenic and antineoplastic activity. Further investigations are needed to elucidate the interactions of GSE constituents with genotoxic compounds at genetic level.

REFERENCES


Morgan and El-Tawil (2006) Potential Protective Effect of Grape Seed Extract Against Acrylonitrile-Induced


