Research Section

Safety evaluation of proanthocyanidin-rich extract from grape seeds

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Abstract
Proanthocyanidins, extracted from grape seeds, are widely used mainly as nutritional supplements. However, there has not been a systematic report to investigate toxicological studies on proanthocyanidins, especially in oral administration. In our studies, proanthocyanidin-rich extract from grape seeds was subjected to a series of toxicological tests to document its safety for use in various foods. The grape seed extract (GSE) was examined for acute and subchronic oral toxicity using Fischer 344 rats and for mutagenic potential by the reverse mutation test using Salmonella typhimurium, the chromosomal aberration test using CHL cells, and the micronucleus test using ddY mice. No evidence of acute oral toxicity at dosages of 2 and 4 g/kg, and no evidence of mutagenicity in the above tests was found. Administration of GSE as a dietary admixture at levels of 0.02, 0.2 and 2% (w/w) to the rats for 90 days did not induce noticeable signs of toxicity. The no-observed-adverse-effect level (NOAEL) of GSE in the sub-chronic toxicity study was 2% in the diet (equal to 1410 mg/kg body weight/day in males and 1501 mg/kg body weight/day in females). The results of our studies indicate a lack of toxicity and support the use of proanthocyanidin-rich extract from grape seeds for various foods. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Grape seed extract; Proanthocyanidin; Safety; Toxicology

1. Introduction
Proanthocyanidins are naturally occurring compounds widely available in fruits, vegetables, nuts, seeds, flowers and bark. They are a class of phenolic compounds which take the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (-)-epicatechin (Porter, 1986). Grape seeds are a particularly rich source of proanthocyanidins, and only the procyanidin-type of proanthocyanidins have been detected in the seeds (Santos-Buelga et al., 1995; Fuleki and Ricardo da Silva, 1997). A few monomeric flavanols have been also detected, but other flavonoid compounds such as anthocyanins and flavonols are not contain in the seeds (Waterhouse and Walzem, 1998). Prieur et al. (1994) found that 55% of the procyanidin extracted from grape seeds consisted of more than five monomer units and determined that their mean degree of polymerization ranged from 2.3 to 15.1 (by thiolysis) and from 2.4 to 16.7 (by gel permeation chromatography). Thus, the proanthocyanidins from grape seeds contain procyanidin oligomers and polymers.

A partially purified proanthocyanidin, consisting oligomers with polymerization from 2 to 7, has been used as a pharmaceutical for their purported activity in decreasing the fragility and permeability of the peripheral vasculature in Europe (Darteneuc et al., 1980; Brasseur, 1989; Bombardelli and Morazzoni, 1995). Recently, epidemiological data have shown that red wine may reduce the mortality rate from coronary heart disease, the so-called “French paradox” (St. Leger et al., 1979; Renaud and De Lorgeril, 1992). Proanthocyanidins are the major polyphenols in red wine as well as in grape seeds, and they have potent antioxidant activity (Ariga and Hamano, 1990; Ricardo de Silva et al., 1991), inhibit low density lipoprotein oxidation (Teissedre et al., 1996), as well as a variety of biological activities (Arii et al., 1998; Dauer et al., 1998; Saito et
al., 1998; Yamakoshi et al., 1999; Zhao et al., 1999). For these reasons, proanthocyanidin-rich extracts from grape seeds have appeared on the market as nutritional supplements mainly in the United States, Australia, Japan, Korea, as well as in other countries. The grape seed extract is also being used in Japan as an additive for various food applications.

However, there has not been a systematic report to investigate toxicological studies on proanthocyanidins, especially in oral administration. Bombardelli and Morazzoni (1995) reported a summary on the safety of the proanthocyanidin oligomers, described above, in which they stated that the acute oral LD50 values in rats were approximately 4 g/kg, and when were administered orally at a dose of 60 mg/kg daily for 6 months in rats and for 12 months in dogs, they were devoid of any toxic effects. The proanthocyanidins were also devoid of any mutagenic potential as well as of teratogenic effects. Although an extensive testing program had been conducted to confirm the safety of the proanthocyanidin oligomers, the data have not been published and the details are unknown.

On the other hand, mutagenicity tests of some procyanidins have been performed. Several procyanidins with different degrees of polymerization (dimers, trimers and polymers) have been found to be non-mutagenic in the Salmonella mutagenesis assay system (Yu and Swaminathan, 1987). In chromosomal aberration tests, polyploidy was induced by the procyanidin trimer and tetramer such as procyanidin C1 and procyanidin D, respectively, in human lymphocyte cultures, and it was induced by the procyanidin dimer such as procyanidin B2 in a mammalian cell line (CHL cells). However no structural aberrations were induced by these procyanidins (Popp and Schimmer, 1991; Takahashi et al., 1999).

This paper describes studies conducted to confirm the safety of proanthocyanidin-rich extract from grape seeds, especially in oral administration. The proanthocyanidin-rich extract from grape seeds for acute and subchronic oral toxicity as well as mutagenic potential were studied. Furthermore, procyanidin dimers, trimers and tetrappers which were separated from the grape seed extract, for mutagenic potential by chromosomal aberration test using CHL cells were examined. The results of these studies can be used to assess the safety of proanthocyanidin-rich extracts from grape seeds when used in various foods.

2. Materials and methods

2.1. Proanthocyanidin-rich extract from grape seeds

Proanthocyanidin-rich extract (Gravinol Super™, Kikkoman Co., Japan) was prepared from grape seeds (Vitis vinifera L.). Briefly, the grape seeds were washed with water for 2 h and then extracted with water and ethanol under reflux for 2 h. The extract was condensed to remove solvents, then the concentrate was filtered through cellulose powder and Celite®. The filtrate was spray-dried to obtain powder of proanthocyanidin-rich extract. Total flavanols in the grape seed extract (GSE) were determined by the vanillin–HCl method of Broadhurst and Jones (1978) using (+)-catechin (Sigma Chemical Co., St. Louis, MO, USA) as standard. This method is widely used for analysis of proanthocyanidins (Lees et al., 1994; Agullo and Rodriguez, 1995; Ivancheva and Bourzeix, 1999). Monomeric flavanols in GSE was estimated by HPLC using catechins as standards (Dalluge et al., 1998). The content of proanthocyanidins in GSE was calculated by the equation (amounts of total flavonols – amounts of monomeric flavonols). The procyanidins in the extract consisted of the degree of polymerizing ranging from 2 to 15 that was detected by MALDI-TOFMS (unpublished data of Dr M. Kameyama, National Food Research Institute, Ibaraki, Japan). The mean degree of polymerization of procyanidins in GSE was determined as approximately 7 by 13C-NMR spectroscopy analysis using a BRUKER DIGITAL NMR AVANCE 500 operated at 125 MHz (Eberhardt and Young, 1994). The GSE was composed of 89.3% proanthocyanidins, which contained 6.6% dimers, 5.0% trimers, 2.9% tetrappers and 74.8% oligomers and polymers larger than pentamer. 66% monomeric flavanols (2.5% (+)-catechin, 2.2% (−)-epicatechin, 1.4% (−)-epigallocatechin and 0.5% (−)-epigallocatechin gallate), 2.24% moisture, 1.06% protein and 0.8% ash. The content of gallate ester in proanthocyanidins of GSE was analyzed by 13C-NMR; the proanthocyanidins of GSE contained 7.0% gallate ester.

2.2. Isolation of monomeric flavanols and proanthocyanidin oligomer

The GSE was chromatographed on Sephadex® LH-20 (Pharmacia Biotech Co., Uppsala, Sweden) with acetone and ethanol to separate four fractions corresponding to monomeric flavanols, procyanidin dimers, trimers and tetrappers. Each fraction was analyzed by thin-layer chromatography (TLC) (silica gel 60 TLC, 0.25 mm thickness; Merck, Darmstadt, Germany) developed with toluene/acetone/formic acid (3:6:1, by vol.), and showed single spot. Although each spot was a mixture of isomeric flavanols, each fraction contained 100% total flavanols. In the 13C-NMR analysis of the fractions, no signals were detected except for the signals arising from the carbon of flavanol. Integral ratios of their signals corresponding to C-3 in chain extender and terminal units in four fractions corresponding to monomeric flavanols, procyanidin dimers, trimers and tetrappers were 0:1, 1:1, 2:1 and 3:1, respectively. As a result of these analyses, it was confirmed that these
fractions were isomeric mixture of monomeric flavanols (catechins), procyonidin dimers, trimers and tetramers, respectively. They did not contain other flavonoid compounds. The content of gallate ester in the fractions were also analyzed by $^{13}$C-NMR analysis; procyonidin trimers and tetramers contained 5.0 and 7.0% gallate ester, respectively, but the dimers did not contain gallate ester.

2.3. Oral toxicity studies

2.3.1. Oral acute toxicity study in rats

Four-week-old male and female F344/DuCrj rats were obtained from Charles River Japan (Kanagawa, Japan). The animals were housed in cages in a temperature-controlled animal room (23±1 °C) with a relative humidity of 55±5% and were fed a standard diet (MF, Oriental Yeast Co., Ltd, Kanagawa, Japan). The GSE was dissolved in purified water and administered at a rate of 10 ml/kg body weight. The animals were divided into three groups of five males and five females, and the GSE was administered by oral gavage at doses of 0 (control), 2 and 4 g/kg. The observations were continued for 14 days. On day 14, the rats were anesthetized with an ip injection of sodium pentobarbital, killed by exsanguinations, and examined by necropsy.

2.3.2. Subchronic 90-day oral toxicity study in rats

The GSE was added to a standard diet (CRF-1, Oriental Yeast Co. Ltd) at concentrations of 0 (control group), 0.02 (low-dose group), 0.2 (middle-dose group) and 2% (high-dose group). Food and water were available ad lib.

Four-week-old male and female F344/DuCrj rats were obtained as described above. After a 1-week quarantine period, the animals were divided into four groups of 10 males and 10 females. The animals were 5 weeks old at the start of dosing.

Routine clinical observations, body weight (Fig. 1), and food and water consumption were measured throughout the study period. Hematology and blood chemistry data were evaluated at the end of the study (Tables 1 and 2). Fresh urine was collected at necropsy and examined for protein, sugar, ketone bodies, pH, and occult blood (Ames, Labstick, Miles, Sankyo Co. Ltd, Tokyo, Japan). At the end of the treatment period, all animals were killed as described above. All organs mentioned bellow (40 organs/male rat and 37 organs/female rat) of animals in every group were examined grossly for pathological changes, and a portion of their organs was weighed, and the organs from all of the animals in the control group and high-dose group (2% GSE in the diet) were examined histopathologically. The following organs were weighed and organ/body
weight ratios were calculated: brain, pituitary, thyroid with parathyroid, thymus, lung, heart, liver, spleen, kidneys, adrenals, stomach, testes, ovaries, epididymides, seminal vesicles, prostate, uterus, urinary bladder and submandibular glands. In the high-dose and the control animals, all of the organs listed above plus the thoracic aorta, trachea, tongue, oesophagus, duodenum, ileum, jejunum, cecum, colon, rectum, pancreas, lymph nodes (mandibular and mesenteric), mammary glands, spinal cord, sciatic nerves, skin, eyes, optic nerves, Harderian glands, sternum, femur and skeletal muscles, were examined histopathologically.

2.4. Mutagenicity

2.4.1. Ames test

GSE was examined for its mutagenic potency in four histidine-requiring S. typhimurium mutant strains TA98, TA100, TA1535 and TA1537, using the treat and plate method (Ames et al., 1975; Maron and Ames, 1983). The different doses used between strains, because the GSE inhibited the growth of TA98 and TA100, but did not inhibit the growth of TA1535 and TA1537 at the highest dose of 5000 µg/plate in the preliminary test. Tester bacteria were exposed to seven concentrations ranging from 19 to 1250 µg/plate in TA98 and TA100, and were exposed to six concentrations ranging from 156 to 5000 µg/plate in TA1535 and TA1537, with and without S-9 mixture, respectively (Table 3). Negative and positive controls were run simultaneously with the test (Table 3).

2.4.2. In vitro chromosomal aberration test with CHL cells

GSE was examined for its potential to induce structural chromosome aberrations and aneuploidy or polyploidy in cultured CHL cells. The test was conducted with and without S-9 mixture. In the absence of S-9 mix, the cells were exposed continuously for 24 and 48 h to GSE at doses of 9.4, 18.8 and 37.5 µg/ml, because 83% of the cells died at the higher dose of 78.1 µg/ml, when the cells were exposed for 48 h to GSE. The cells were exposed for 6 h to GSE at doses of 18.8, 37.5, 75.0, 150.0 and 300.0 µg/ml in the presence of S-9 mix, and were exposed also to GSE at dose of 18.8, 37.5 and 75.0 µg/ml in the absence of S-9 mix, and washed and cultured for an additional 18 h. In the preliminary test, IC₅₀ values of GSE with and without S-9 mix were 300.0 and 75.0 µg/ml, respectively. Procyanidin dimers, trimers and tetramers, which were separated from GSE, were also examined for their potentials by the chromosomal aberration test. The tests of their procyanidins were conducted at seven to nine dose levels including the highest dose of 1250 to 5000 µg/ml. Three to seven dose levels were finally chosen due to their cytotoxicities. Namely, in the absence of S-9 mix, the cells were exposed for 24 h to procyanidin dimers at four dose levels from 39.1 to 312.5 µg/ml, trimers at six dose levels from 4.9 to 156.3 µg/ml and tetramers at four dose levels from 9.8 to 78.1 µg/ml. The cells were also exposed for 48 h to procyanidin dimers at three dose levels from 39.1 to 156.3 µg/ml, trimers at six dose levels from 4.9 to 156.3 µg/ml and tetramers at four dose levels from 9.8 to 78.1 µg/ml.

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### Table 1

#### Hematological values of rats fed GSE for 90 days

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GSE in the diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
</tr>
<tr>
<td>WBC (10⁶/µl)</td>
<td>21.3±4.1</td>
</tr>
<tr>
<td>RBC (10⁶/µl)</td>
<td>850.9±41.9</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>15.7±0.6</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>49.4±2.9</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
</tr>
<tr>
<td>WBC (10⁶/µl)</td>
<td>14.1±4.5</td>
</tr>
<tr>
<td>RBC (10⁶/µl)</td>
<td>818.4±27.4</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>15.6±0.6</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>48.2±2.3</td>
</tr>
</tbody>
</table>

Parameters: WBC (white blood cell count), RBC (red blood cell count), Hb (hemoglobin), HCT (hematocrit). Data not shown from hematological parameters: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), platelet count (PLT), mean corpuscular hemoglobin concentration (MCHC) and reticulocyte count (Ret) and differential leukocyte count.

### Table 2

#### Clinical chemistry values of rats fed GSE for 90 days

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GSE in the diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
</tr>
<tr>
<td>Tot.Prot (g/dl)</td>
<td>5.99±0.32</td>
</tr>
<tr>
<td>Ca (mg/dl)</td>
<td>9.53±0.19</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>23.13±1.64</td>
</tr>
<tr>
<td>AST (IU/litre)</td>
<td>110.2±75.1</td>
</tr>
<tr>
<td>ALT (IU/litre)</td>
<td>54.3±38.0</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
</tr>
<tr>
<td>Tot.Prot (g/dl)</td>
<td>6.01±0.15</td>
</tr>
<tr>
<td>Ca (mg/dl)</td>
<td>9.51±0.21</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>24.69±1.75</td>
</tr>
<tr>
<td>AST (IU/litre)</td>
<td>99.6±26.7</td>
</tr>
<tr>
<td>ALT (IU/litre)</td>
<td>42.1±11.5</td>
</tr>
</tbody>
</table>

Parameters: Tot. Prot (total protein), BUN (blood urea nitrogen), Ca (calcium), BUN (blood urea nitrogen), AST (aspartate aminotransferase), ALT (alanine aminotransferase), ALP (alkaline phosphatase), ALP (alkaline phosphatase), Na (sodium), K (potassium), Cl (chloride), P (phosphate), ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), LDH (lactic dehydrogenase).
levels from 9.8 to 78.1 μg/ml. In the presence of S-9 mix, the cells were exposed for 6 h to procyanidin dimers at seven dose levels from 78.1 to 5000 μg/ml, trimers at six dose levels from 39.1 to 1250 μg/ml, and tetramers at six dose levels from 19.5 to 625 μg/ml. In the absence of S-9 mix, the cells were also exposed for 6 h to procyanidin dimers at four dose levels from 78.1 to 625 μg/ml, trimers at five dose levels from 39.1 to 625 μg/ml and tetramers at five dose levels from 19.5 to 312.5 μg/ml. The cells were exposed for 6 h with or without S-9 mix, washed and cultured for an additional 18 h. Mitomycin C (MMC) at a dose of 0.04 μg/ml was treated to the cells for 24 or 48 h as positive control. Dimethylnitrosamine (DMN) at dose of 1000 μg/ml and MMC at dose of 0.4 μg/ml were also treated to the cells for 6 h as positive control with and without S-9 mix, respectively. At the each sampling time, 100 or 200 metaphases were analyzed.

### 2.4.3. Mouse micronucleus test

Male 8-week-old ddY mice (Japan SLC, Inc., Hamamatsu, Japan) were used in this test. The micronucleus test was carried out according to the method of Hayashi et al. (1990). GSE was dissolved in distilled water and administered orally, twice, 24 h apart, at doses of 0, 0.5, 1 and 2 g/kg to five or six mice each. The highest dose in this test was settled to 2 g/kg, because the dose was 240 times of the estimated daily ingestion of proanthocyanidin by human (about 0.5 g/60 kg body weight) (Deprez and Scalbert, 1999). Peripheral blood (5 μl) was collected by piercing the ventral tail 24 h after the final dose and placed on an acridine orange-coated glass slide (Toyobo Co., Ltd, Japan). The frequency of micro-nucleated peripheral reticulocytes (MNRETs) was counted based on an examination of 1000 reticulocytes per mouse. MMC was given to mice as a positive control.

### 2.5. Statistical analysis

In the acute study, a one-way parametric ANOVA with Dunnett’s test was used to body weights. In the subchronic study, the same test was used for statistical analysis to body weights, food and water consumptions, hematology and blood chemistry data, and organ weights. Fisher’s exact test was used to urinalysis values, and data on histopathological examination. The data obtained from each mutagenicity test were statistically analysed with Student’s t-test.

### 3. Results

#### 3.1. Oral acute toxicity study in rats

No deaths occurred in either the control or the GSE groups. The general condition of all rats was normal. The weight of the male and female rats in the high-dose 4 g/kg group decreased slightly on day 1 and from days 1 to 3, respectively, but increased thereafter almost the same as in the control rats. No abnormalities were found in all rats at necropsy on day 14.

#### 3.2. Subchronic 90-day oral toxicity study in rats

GSE was administered to rats as a dietary admixture at levels of 0.02% (low-dose group), 0.2% (middle-dose group) and 1% (high-dose group) for 90 days. Two groups of male and female rats were used in the acute and subchronic studies, respectively.
group) and 2% (high-dose group). The rats in the control group were given only standard diet.

Survival was 100% in all groups. There were no abnormal clinical signs related to feeding of GSE in any of the groups. There were no statistically significant differences in body weight (Fig. 1) or water consumption between any of the groups at any time during the study. The mean total food consumption and mean body weights of the rats given GSE did not differ significantly from the values in the controls (data not shown). The calculated average GSE consumption of males on a daily basis were 13.3±0.4 mg/kg/day in the low-dose group, 129.1±3.5 mg/kg/day in the middle-dose group, and 1409.8±49.8 mg/kg/day in the high-dose group.

The GSE consumption of females were 14.8±0.5 mg/kg/day in the low-dose group, 154.0±4.7 mg/kg/day in the middle-dose group, and 1501.1±55.1 mg/kg/day in the high-dose group. The main hematological and clinical chemistry values are shown in Tables 1 and 2, respectively. There were no differences between groups in hematological values (Table 1), leukocyte differentials, clinical chemistry values (Table 2) or urinalysis values. The only statistically significant differences were higher epididymis weights in the middle-dose group (0.2% in the diet) of males (863.4±44.2 mg in the control vs 920.7±37.0 mg in the middle-dose group), a lower thymus weight (161.7±37.7 mg in the control vs 127.8±16.9 mg in the middle-dose group), a lower thymus/body weight ratio in the middle-dose group of females (92.9±15.4 mg/100 g body weight in the control vs 75.8±9.9 mg/100 g body weight in the middle-dose group), and a lower stomach/body weight ratio in the low-dose group of females (502.2±44.3 mg/100 g body weight in the control vs 474.6±15.6 mg/100 g body weight in the low-dose group).

Gross examination at necropsy did not reveal any treatment-related changes. Some microscopic changes were observed in some organs of the males and females both in the control group and in the high-dose group; however, in incidence and severity of the changes, there were no differences noted between the control group and high-dose group. Therefore, histopathological examination did not reveal any treatment-related changes.

3.3. Ames test

No increase in the number of revertant colonies occurred in the four test strains at any concentrations of GSE, either in the presence or absence of S-9 mix (Table 3). In the preliminary test, GSE inhibited the growth of TA98 and TA100 at dose of 5000 μg/ml (data not shown), but no precipitate was noted on the plates at the highest dose levels after the incubation period, so the bacterial growth inhibition was not caused by precipitation.

3.4. In vitro chromosomal aberration test with CHL cells

No biologically or statistically significant increases in the frequency of metaphases with aberrant chromosomes were seen in the cultures treated with GSE at either sampling times, either in the presence or absence of S-9 mix (data not shown). Aneuploidy or polyploidy was also not observed in the test (data not shown). Procyanidin dimers, trimers and tetramers did not cause aneuploidy or polyploidy in both the activated or non-activated system, with or without S-9 mix. In only a few highest dose levels of the dimers and tetramers, the frequencies of chromosomal aberrations (chromatid type; chromatid exchange and chromatid break) increased in the test, and the frequencies of aberrations tended to increase in a few highest dose levels of the trimers. When the cells were exposed for 6 h to the dimers with S-9 mix, the frequencies were 14% at a dose of 2500 μg/ml and 31% at a dose of 5000 μg/ml. When the cells were exposed for 6 and 48 h to the dimers without S-9 mix, the frequency was 9%, which was a false positive test result, at a dose of 625 μg/ml and 15% at a dose of 156.3 μg/ml, respectively.

Although the test results of the chromosomal aberrations were not positive, when the cells were exposed for 6 and 48 h to the dimers without S-9 mix, the frequencies were 9%, which was a false positive test result, at a dose of 625 and 156.3 μg/ml, respectively. On the other hand, MMC or DMN induced strong chromosomal aberrations and elevated the frequency of the aberrations. When the cells were exposed for 6 h to DMN with S-9 mix, the frequency was 92% at a dose of 1000 μg/ml, and when the cells were exposed for 48 h to MMC without S-9 mix, the frequency was 45% at a dose of 0.04 μg/ml. So the procyanidin dimers and tetramers displayed only weak activities.

3.5. Mouse micronucleus test

The frequencies of MNRETs in the micronucleus test in mice were not significantly different between the GSE groups and the control group (Table 4).

<table>
<thead>
<tr>
<th>Treatment (P.O.)</th>
<th>MNRETs(^a) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.13±0.10</td>
</tr>
<tr>
<td>0.5/g/kg×2 times</td>
<td>0.10±0.06</td>
</tr>
<tr>
<td>1.0/g/kg×2 times</td>
<td>0.22±0.10</td>
</tr>
<tr>
<td>2.0/g/kg×2 times</td>
<td>0.36±0.21</td>
</tr>
<tr>
<td>MMC(^b)</td>
<td>8.28±0.67</td>
</tr>
</tbody>
</table>

\(^a\) MNRETs = Micronucleated peripheral reticulocytes. The data showed frequencies of MNRETs from mouse peripheral blood after oral administration of two times GSE.

\(^b\) Mitomycin C (MMC) was treated to mice as positive control at dose of 2 mg/kg by single ip administration.
4. Discussion

Proanthocyanidin-rich extracts from grape seeds are used as nutritional supplements in the United States, Australia, Japan, Korea and other countries, and GSE is also used in Japan as a food additive.

In the past, proanthocyanidins were considered to be non-toxic because they are not absorbed. However, dimeric procyanidins have been found to be absorbed into the bloodstream (Harmand and Blanquet, 1978; Laparra et al., 1978; Jimenez-Ramsey et al., 1994), and some of the products of hydrolyses of the higher oligomers and polymers were presumed to be absorbed through the intestinal membrane (Levrat et al., 1993; Deprez and Scalbert, 1999; Koga et al., 1999; Scalbert et al., 1999), and then the absorbed procyanidins and/or hydrolytes of procyanidins might display antioxidative activity in vivo (Koga et al., 1999; Yamakoshi et al., 1999).

On the other hand, information on the safety of GSE has been limited, especially by oral administration. Although an extensive testing program had been conducted to confirm the safety of the partially purified proanthocyanidin oligomers (Bombardelli and Morazzoni, 1995), their data have not been published and the details are unknown.

A series of safety studies were performed to investigate the safety of proanthocyanidin-rich extracts from grape seeds containing procyanidin oligomers and polymers in detail.

GSE was found to be non-mutagenic in the reverse mutation test, the chromosomal aberration test, and the micronucleus test when orally administered to mice. In the chromosomal aberration test, polyploidy has been observed only in the highest doses of their procyanidins (156.3 μg/ml with S-9 mix and 5000 μg/ml without S-9 mix). This phenomenon might be due to the rapid autoxidation of phenolics under alkaline conditions, which led to the generation of H2O2 and free radicals. Guo et al. (1999) also reported the free radical generated from the oxidation of proanthocyanidin-rich pine bark extract, which contained 80% oligomeric procyanidins such as procyanidin B1, B2, B3, C1 and C2, under only alkaline conditions, and the formation of the radicals was dependent on the phenolics concentration and pH levels. Therefore, we examined the change of pH in culture medium with CHL cells with and without procyanidins. In the absence of the procyanidins, pH in culture medium with CHL cells was 7.46. When 156.3 μg/ml of procyanidin dimers, 156.3 μg/ml of procyanidin trimers and 78.1 μg/ml of procyanidin tetramers, which were doses that induced chromatid type aberrations, were added to the culture medium with CHL cells, the pH changed 7.78, 7.76 and 7.65, respectively. These changes of pH were small, but chromatid type aberrations induced by procyanidin dimers, trimers or tetramers in our study might be due to the rapid autoxidation of the procyanidins under the alkaline conditions. The chromatid type aberrations observed only in the highest doses of their procyanidins. This phenomenon might be due to only formation of radicals of their procyanidins in highest doses. In our study, procyanidin trimers and tetramers contained 5.0 and 7.0% gallate ester, respectively, but the dimers did not contained gallate ester. Ohshima et al. (1998) reported that DNA strand breaks induced stronger epigallocatechin gallate than epigallocatechin in combination with an NO-releasing compound. This result supported that gallate ester related to DNA strand breaks under this condition. The gallate molecule might contribute to chromatid type aberrations induced by procyanidin tetramers or trimers in our study. However, the procyanidin dimers and tetramers, not to mention trimers, displayed only weak activities in an in vitro chromosomal aberration test. In vivo, GSE is ingested, and is absorbed at an acid or neutral pH conditions. It seems that GSE is stable under these conditions. In fact,
though 2 g/kg of GSE, which was 240 times of the estimated daily ingestion of proanthocyanidin by human, was administered orally, twice, to mice, GSE did not induce micronuclei (Table 4). Recently, the International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) have been issued ‘Genotoxicity: A standard battery for genotoxicity testing of pharmaceuticals’ (Muller et al., 1999). A major change in testing philosophy is the acceptance of the interchangeability of testing for chromosomal aberrations in mammalian cells and the mouse lymphoma tk assay. We secondly examined GSE for mutagenic potential by L5178Y tk mouse lymphoma assay (MLA). GSE at five dose levels from 93.8 to 1500 µg/ml did not induce mutations in the MLA (data not shown). This result strongly supported that GSE was found to be non-mutagenic.

The LD₅₀ (median lethal dose) value of the GSE was found to be greater than 4 g/kg in the male and female rats combined in the oral acute toxicity study. Bombardelli and Morazzoni (1995) reported that acute oral LD₅₀ value of proanthocyanidin oligomers was approximately 4 g/kg. Therefore, GSE was not toxic in this study, as well as the proanthocyanidin oligomers.

In the 90-day subchronic oral toxicity study, the statistically significant differences were found in the increased epididymides weight in the middle-dose group (0.2% in the diet) of males, the lower thymus weight and thymus/body weight ratio in the middle-dose group of females, and the lower stomach/body weight ratio in the low-dose group (0.02% in the diet) of females. These were slight changes and no dose dependency, and no adverse effects were seen histopathologically. Therefore, they were not considered to be treatment-related effects.

The highest dose, 2% GSE in the diet, did not induce noticeable signs of toxicity. The no-observed-adverse-effect level (NOAEL) of the GSE in the subchronic toxicity study was 2% in the diet. Thus, the NOAEL was equal to 1410 mg/kg body weight/day in males and 1501 mg/kg body weight/day in females.

These results indicated a lack of toxicity and supported use of proanthocyanidin-rich extracts from grape seeds in various foods.

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References


