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Lack of in vivo clastogenic activity of grape seed and grape skin extracts in a mouse micronucleus assay

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Abstract

Meganatural™ brand grape seed extract (GSE) and grape skin extract (GSKE), containing proanthocyanidin polyphenolic compounds, are intended for use in food as functional ingredients exhibiting antioxidant activity. Proanthocyanidins, as well as the minor constituent phenolic compounds in GSE and GSKE, are present naturally in many foods such as fruits, vegetables, chocolate, tea, etc., and on average people consume 460–1000 mg/day of these combined substances. While some polyphenolic compounds, tested individually, have demonstrated antitumorigenic or antipromotional activity, at least one minor component of GSE and GSKE, quercitin, has exhibited positive activity in *Salmonella* and other in vitro mutagenicity assays. As part of a program to investigate the safety of GSE and GSKE, these products were tested for in vivo clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocyte (PCE) cells in Crl:CD-1®(ICR) BR mouse bone marrow. The appropriate test article was dissolved in 0.5% carboxymethylcellulose and dosed by oral gavage to five males/test article/dose level/harvest time point. Animals were dosed at 500, 1000 and 2000 mg/kg. Five animals dosed with either test article at 500, 1000 and 2000 mg/kg dose levels and five animals dosed with the cyclophosphamide (80 mg/kg) positive control were euthanized approximately 24 h after dosing for extraction of bone marrow. Five animals dosed with either test article at the 2000 mg/kg dose level and five animals dosed with the vehicle control article were euthanized approximately 24 and 48 h after dosing for extraction of bone marrow. At least 2000 PCEs per animal were analyzed for frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) in at least the first 500 erythrocytes for each animal. For both GSE and GSKE, no statistically significant increase in micronucleated PCEs was observed at any dose level or harvest time point. GSE produced indication of cytotoxicity (decreased PCE:NCE ratio) at the 2000 mg/kg dose level for the 48-h harvest time point, confirming that the test article reached the target bone marrow in significant amount. Meganatural™ GSE and Meganatural™ GSKE were evaluated as negative in the mouse bone marrow micronucleus assay under the conditions of this assay.

Keywords: Grape seed; Grape skin; Extract; Micronucleus; Polyphenol; Proanthocyanidin

1. Introduction

Grape seed extract (GSE) and grape skin extract (GSKE) contain oligomeric and polymeric proanthocyanidins (PACs), as well as lesser quantities of other natural phenolic compounds. PACs are based generally on (+)-catechin and (−)-epicatechin flavan-3-ol monomer units and exhibit antioxidant properties (Ricardo-DaSilva et al., 1991; Hammerstone et al., 2000). Published values indicate estimated dietary intake of flavanoids, catechins and proanthocyanidins by the average American consumer is in the range of 460–1000 mg/day (Santos-Buelga and Scalbert, 2000; Scalbert and Williamson, 2000). Intake arises from the common occurrence of these substances in fruits, juices, tea, coffee, vegetables, and in many other foods in beverages.

The antioxidant activity of GSE and GSKE makes them candidates for addition to foods and beverages to retard deterioration; it is possible that the antioxidant activity of GSE and GSKE ingested with these foods would also support physiologic defenses against in vivo-generated free radical species. However, GSE and GSKE contain minor amounts (0.1–1.0%) of quercetin and its glycosides, substances with suspected mutagenic activity. Isolation and multiple fractionation of grape polyphenols occurring in wine was reported to yield a

Abbreviations: GSKE; grape skin extract; GSE; grape seed extract; NCE; normochromatic erythrocyte; PACs; proanthocyanidins; PCE; polychromatic erythrocyte.

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subfraction that exhibited mutagenic activity in the *Salmonella* strain TA98 when tested in the presence of human fecal glycosidase with or without S9 microsomal fraction (Yu et al., 1986). Rutin (3-rhamnoglucosyl quercetin) was identified as the probable active component (Yu et al., 1986). In contrast, Stich (1991), for example, recently reviewed evidence demonstrating mutagenic as well as antimutagenic, anticlastogenic and antipromotional activity associated with (+) catechin and (−) epicatechin, their oligomers and other related phenolic compounds. From such evidence it was uncertain that potential activity of the GSE and GSKE complex polyphenolic mixtures towards genetic material in vivo could be assessed adequately on the basis of the possible activity of certain constituents demonstrated primarily in vitro.

Accordingly, as part of a program of safety evaluations, a mouse micronucleus study was conducted to evaluate GSE and GSKE for in vivo clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocyte cells in mouse bone marrow. The highest dose level was chosen to provide significant exposure to the test substance yet avoid potential nutritional interferences. The doses selected were 500, 1000 and 2000 mg/kg body weight for each test article.

2. Materials and methods

2.1. Study design

Young adult male mice of the Crl:CD-1®(ICR) BR strain were purchased from Charles River Laboratories (Raleigh, NC, USA) and acclimated for at least 7 days before being placed on study. Mice were randomly assigned by computer program and housed in polycarbonate cages in groups of six animals for each of the vehicle control, positive control and test article treatment and bone marrow harvest time points; the sixth animal serving as a replacement in case one of the first five was lost during treatment. Test articles were dissolved in 0.5% aqueous carboxymethylcellulose, which also served as vehicle control. GSE and GSKE were administered by single oral gavage in a volume of 20 ml/kg at doses of 0 (control), 500, 1000 and 2000 mg/kg to groups scheduled for the 24-h harvest time point; additional vehicle control and high-dose test article groups were dosed for the 48-h harvest time-point. Cyclophosphamide dissolved in sterile deionized water served as positive control and was administered in a volume of 10 ml/kg at a dose of 80 mg/kg to a single group for marrow harvest at the 24-h time point. The assay was conducted at Covance Laboratories, Inc. (Vienna, VA, USA) and in accordance with US FDA Good Laboratory Practice Recommendation. The design was based on OECD Guideline 474, updated and adopted July 21, 1997. The study design is summarized in Table 1.

2.2. Test articles

The test articles were commercial Meganatural™ GSE (lot 2501-040157) and Meganatural™ GSKE (lot 2511-040060), supplied by the manufacturer, Polyphenolics Inc., a Canandaigua Wine Company subsidiary. Each test article contained proanthocyanidin polyphenolic compounds and related phenolic substances derived from grapes. The representative composition of each test article is detailed in Table 2.

2.3. Bone marrow extraction and preparation

At the appropriate harvest time points, the animals were euthanized by CO_2 inhalation followed by incision of the diaphragm. The hind limb bones (tibias) were removed for marrow extraction from five surviving animals in each treatment and control group. For each animal, the marrow flushed from the bones was combined in an individual centrifuge tube containing 3–5 m fetal bovine serum (one tube per animal). Following centrifugation to pellet the tissue, the supernatant was removed by aspiration and portions of the pellet were spread on slides and air-dried. The slides were fixed in methanol, stained in May–Grünwald solution followed by Giemsa, and protected by permanently mounted coverslips. For control of bias, all slides were coded prior to analysis.

2.4. Slide analysis

Slides prepared from the bone marrow collected from five animals per group at the designated harvest time points were scored for micronuclei and the PCE:NCE cell ratio. The micronucleus frequency (expressed as percent micronucleated cells) was determined by analyzing the number of micronucleated PCEs from at least 2000 PCEs per animal. The PCE:NCE ratio was determined by scoring the number of PCEs and NCEs observed in at least the first 500 erythrocytes per animal.

The historical background frequency of micronucleated cells was expressed as percent micronucleated cells based on the number of PCEs analyzed. The historical background frequency of micronuclei in the Crl:CD-1®(ICR) BR strain at the performing laboratory is about 0.0–0.4%, which is within the range reported in the published data (Salamone and Mavournin, 1994).

The criteria for the identification of micronuclei were those of Schmid (1976). Micronuclei were darkly stained and generally round, although almond- and ring-shaped micronuclei occasionally occurred. Micronuclei were sharp bordered and generally between one-twentieth

Table 1
Dosing scheme for the micronucleus assay with Meganatural™ GSE and Meganatural™ GSKE

Target treatment (mg/kg)	Stock concentration (mg/ml)	Route of administration	Dosing volume (ml/kg)	Males/harvest 24-h	Time point* 48-h
Vehicle control, 0.5% carboxymethylcellulose	0	Oral gavage	20	6	6
Positive control, Cyclophosphamide, 80	8	Oral gavage	10	6	–
Meganatural™ GSE					
500	25.0	Oral gavage	20	6	–
1000	50.0	Oral gavage	20	6	–
2000	100	Oral gavage	20	6	6
Meganatural™ GSKE					
500	25.0	Oral gavage	20	6	–
1000	50.0	Oral gavage	20	6	–
2000	100	Oral gavage	20	6	6

Table 2
Meganatural^{bht} grape skin extract (GSKE) and grape seed extract (GSE) composition

	GSKE profile	GSE profile
	Average	Average
Catechin % by wt.	3.4	4.8
Epicatechin % by wt.	4.6	4.4
Gallic acid % by wt.	2.4	1
Total phenols % by wt.	87.3	90.5
Total anthocyanins % by wt.	2.6	NA
HPLC relative profile of monomers-%	16.7	10.4
HPLC relative profile of oligomers-%	67.4	74.9
HPLC relative profile of polymers-%	15.9	14.7
Moisture % by wt.	3.7	3.6

and one-fifth the size of the PCEs. The unit of scoring was the micronucleated cell, not the micronucleus; thus, the occasional cell with more than one micronucleus was counted as one micronucleated PCE, not two (or more) micronuclei.

The staining procedure permitted the differentiation by color of PCEs and NCEs (bluish-grey and red, respectively).

3. Results (Table 3)

For Meganatural™ GSE, one high-dose (2000 mg/kg) animal was found dead 1 h after dosing; however, no further signs of clinical toxicity were observed in any of the remaining animals at any dose level. Meganatural GSE was cytotoxic to the bone marrow (i.e. a statistically significant decrease in the PCE:NCE ratio) at the 2000 mg/kg dose level for the 48-h harvest time point. No statistically significant increase in micronucleated PCEs was observed at any dose level or harvest time point.

Meganatural GSKE induced no signs of clinical toxicity in any of the treated animals and was not cytotoxic to the bone marrow (i.e. no statistically significant decrease in the PCE:NCE ratio). No statistically significant increase in micronucleated PCEs was observed at any dose level or harvest time point for either test article.

The positive control, cyclophosphamide, induced statistically significant increases in micronucleated PCEs as compared with that of the vehicle controls, with a mean and standard error of 2.19±0.20%.

Table 3
Micronucleus data summary table

Treatment	Dose	Harvest time (HR)	% Micronucleated PCEs mean of 2000 per animal±S.E. Males	Ratio PCE:NCE mean±S.E. Males
Controls				
Vehicle	0.5% CMC	24 h	0.04±0.02	0.53±0.03
		48 h	0.05±0.02	0.79±0.09
Positive	CP 80.0 mg/kg	24 h	2.19±0.50*	0.41±0.03**
Test articles				
GSE	500 mg/kg	24 h	0.02±0.01	0.61±0.06
	1000 mg/kg	24 h	0.02±0.01	0.76±0.14
	2000 mg/kg	24 h	0.04±0.01	0.55±0.06
		48 h	0.06±0.02	0.44±0.04**
GSKE	500 mg/kg	24 h	0.04±0.03	0.84±0.06
	1000 mg/kg	24 h	0.01±0.01	0.75±0.07
	2000 mg/kg	24 h	0.05±0.03	0.50±0.05
		48 h	0.03±0.02	0.68±0.08

0.5% CMC = 0.5% aqueous carboxymethylcellulose solution. CP = cyclophosphamide; PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte.

* Significantly greater than the corresponding vehicle control, $P < 0.01$.

** Significantly less than the corresponding vehicle control, $P \leq 0.05$.

4. Discussion

The test articles, Meganatural GSE and Meganatural GSKE, were evaluated as negative in the mouse bone marrow micronucleus assay under the conditions of this assay. The statistically significant decrease in the PCE:NCE ratio observed in mice treated with 2000 mg/kg GSE is interpreted as evidence that the test article reached the target bone marrow cells in significant amount and supports the validity of these test results. The one high-dose GSE animal that was found dead 1 h after dosing is considered to have succumbed due to the dosing procedure; the death is not considered to be test article related.

North American consumers are estimated to ingest on average approximately 460 (Santos-Buelga and Scalbert, 2000) to 1000 mg/day (Scalbert and Williamson, 2000) (9–20 mg/kg, based on a 50-kg body weight person) of proanthocyanidins and related phenolics as a result of the natural occurrence of these substances in foods and beverages. The anticipated uses of Meganatural GSE and GSKE as antioxidant substances added to foods and beverages would increase consumer proanthocyanidin intake by perhaps 20–40%. Similarly, consumer background exposure to quercitin, a minor component of GSE and GSKE, has been estimated at approximately 20 mg/day (NTP, 1992). Anticipated uses of Meganatural GSE and GSKE in foods and beverages would result in a small, 1–3 mg/day, increment in quercitin exposure.

The results of this assay demonstrate that Meganatural GSE and GSKE are devoid of clastogenic activity when administered orally to mice at doses as high as 2000 mg/kg. These results additionally support the view that consumer exposure to a few mg/kg/day of GSE and GSKE through their anticipated use in foods and beverages is expected to be safe, represents a small increment in consumer intake and provides a wide margin of safety compared to the doses administered in this assay.

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