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# Cytogenetic Effects of Grape Extracts (*Vitis vinifera*) and Polyphenols on Mitomycin C-Induced Sister Chromatid Exchanges (SCEs) in Human Blood Lymphocytes

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In the present study, the effects of extracts and polyphenol-rich fractions as well as monomer polyphenols identified in them, from both red and white grapes, on mitomycin C (MMC) induced sister chromatid exchanges (SCEs) in human peripheral blood lymphocytes were investigated. The grape extracts and two of the three polyphenol-rich fractions promoted MMC-induced SCEs at concentrations from 75 to 300 $\mu$g/mL. However, none of the extracts or fractions alone induced SCEs. Thus, these results suggest caution especially with regard to the use of grape extracts as dietary supplements. On the other hand, the fact that these extracts were not genotoxic alone may indicate a selective activity against genetically damaged cells. This is the first study regarding the clastogenic effects of grape extracts in human cells. Moreover, from the tested polyphenols, caffeic acid, gallic acid, and rutin hydrate enhanced MMC-induced clastogenicity, whereas ferulic acid, protocatechuic acid, (+)-catechin, (−)-epicatechin, and *trans*-resveratrol had no effect at concentrations between 5 and 100 $\mu$M. The differences in the chemical structures of the tested polyphenols may account for their differential effects on MMC clastogenicity.

**KEYWORDS: Grape extracts; *Vitis vinifera*; plant polyphenols; mitomycin C; human lymphocytes; sister chromatid exchanges**

## INTRODUCTION

In recent years, several studies have shown that grapes and wine possess ingredients with important biological properties that could be beneficial for human health (*1*). For example, it has been reported that grapes and wine may prevent cardiovascular diseases (*1*) and exhibit antimicrobial (*2*), antihypertensive (*3*), and antiulcer (*4*) activities. Moreover, a number of in vivo and in vitro studies have shown that grape extracts exhibited cytotoxicity toward cultured human cancer cells (e.g., breast and lung cancer cells) as well as inhibited human prostate tumor xenograft growth in mice (*5*, *6*). Some molecular mechanisms have been proposed for these chemopreventive activities of grapes, such as inhibition of oxidation of human low-density lipoprotein (LDL) (*7*), inhibition of enzymes playing an essential role in cell proliferation (*8*), inhibition of angiogenesis (*5*), and modulation of signal transduction biochemical pathways regulating the cell cycle (*5*). However, these protective mechanisms have not been elucidated, and their study is considered important.

DNA damage induced by reactive oxygen species (ROS) may result in a number of degenerative processes such as cancer, cardiovascular and neurodegenerative diseases, and premature aging (*9*, *10*). Moreover, several studies have shown that grape extracts are strong antioxidants and free radical scavengers (*11*). In a previous paper (*12*), we have shown that grape extracts inhibited mutagenicity induced by oxidative agents in bacterial *Salmonella typhimurium* TA102 cells. In the present study, we examined the effects of grape extracts from two Greek *Vitis vinifera* varieties, Mandilaria (red grapes) and Assyrtiko (white grapes), on the mitomycin C (MMC)-induced clastogenicity in human blood lymphocyte cultures. For this purpose, the sister chromatid exchanges (SCEs) assay was used. This method has been regarded as a sensitive indicator of DNA damage (*13*) and is used for the detection of both mutagens (*14*) and antimutagens (*15*). MMC, a bifunctional alkylating agent of DNA, is a very potent inducer of SCEs (*16*). In addition, it has also been proposed that ROS are involved in the induction of SCEs by

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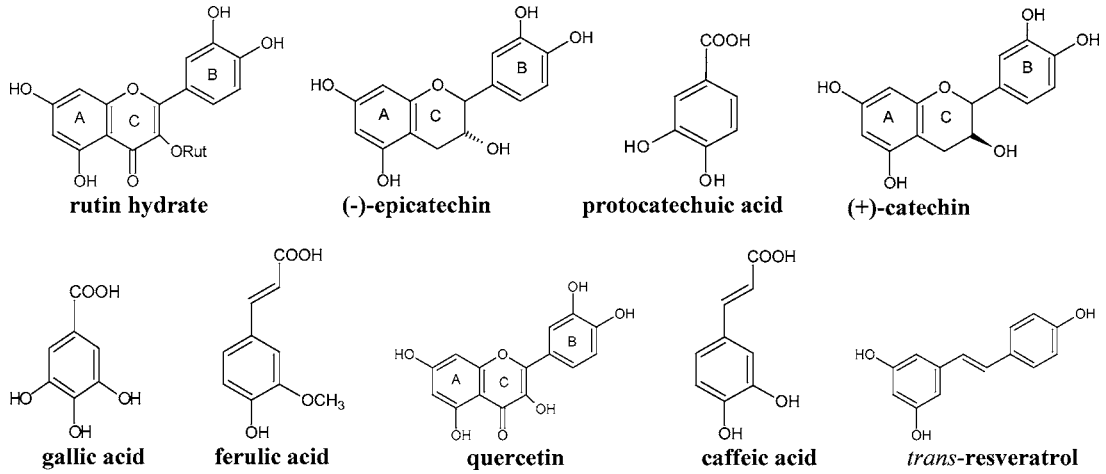


**Figure 1.** Chemical structures of polyphenols under study.

**Table 1.** Quantities (Milligrams per Gram) of Monomer Polyphenols in the Grape Extracts and Fractions

| | extracts | | | | fractions | | |
|---|---|---|---|---|---|---|---|
| polyphenol | red grape methanolic | red grape water | white grape methanolic | white grape water | red grape water extract ethyl acetate | red grape water extract methanolic | red grape methanolic extract methanolic |
| *trans*-resveratrol | 0.22 | 0.04 | 0.01 | 0.02 | 20.11 | 0.1 | 3.14 |
| (+)-catechin | 0.9 | 0.35 | 2.25 | 4.09 | 55.38 | 0.9 | 17.60 |
| (–)-epicatechin | 1.1 | 0.32 | 1.08 | 2.10 | 87.35 | 0.9 | 18.19 |
| quercetin | | 0.13 | 0.04 | 0.03 | 21.45 | | 1.20 |
| gallic acid | 1.23 | 1.57 | 0.22 | 0.48 | 330.38 | 2.1 | 27.28 |
| ferulic acid | 0.1 | | | | | 0.01 | 0.24 |

MMC (*17*). Because one of the most distinct properties of grape extracts is their antioxidant activity, their potential protective activity against ROS-mediated DNA damage was investigated. Moreover, plant polyphenols are believed to be the most potent antioxidant compounds of grapes (*11*). In addition, several studies have shown that plant polyphenols may possess anticancer activities (*18*). Thus, polyphenol-rich fractions from the above extracts were tested, and the effects of monomer polyphenols [caffeic acid, ferulic acid, gallic acid, protocatechuic acid, (+)-catechin, (−)-epicatechin, quercetin, rutin hydrate, and *trans*-resveratrol] (**Figure 1**) quantified in the grape extracts and fractions on MMC-induced SCEs were investigated.

## MATERIALS AND METHODS

**Chemicals.** MMC was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). RPMI 1640, L-glutamine, penicillin, streptomycin, phytohemagllutinin, and fetal calf serum were from Biochrom (Berlin, Germany). Giemsa were purchased from Merck (Darmstadt, Germany). Caffeic acid, ferulic acid, gallic acid, protocatechuic acid, (+)-catechin, (−)-epicatechin, quercetin, rutin hydrate, *trans*-resveratrol, 5-bromo-2-deoxyuridine, Hoechst 33258, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

**Plant Material.** Methanolic and aqueous extracts from two Greek *V. vinifera* varieties cultivated in Santorini Island, Mandilaria (red grapes) and Assyrtiko (white grapes), were isolated. Some of the most common polyphenolics found in grapes were isolated and quantified by semipreparative HPLC [Supelcosil SPLC18, (5 $\mu$m, 250 × 10 mm), $H_2O$/AcCN gradient] with a Thermo Finnigan Spectra system and identified by NMR spectroscopy with a Bruker DRX400 by comparison with purchased standards (**Table 1**).

The isolation of extracts and their fractions was made as previously described (*8*). In summary, the juice was removed from the grapes during the winemaking process, and the solid residue was air-dried. A part of the residue (4 kg) was extracted three times with methanol (14 L) for 48 h and another part (4 kg) was extracted with water (14 L) at 45 °C, also for 48 h. The solvents were evaporated under reduced pressure to afford the dried residue of the methanol extract (300 g) and the water extract (90 g) from white grapes and the corresponding dried extracts (400 and 130 g, respectively) from red grapes. A part of the methanol extract from red grapes was submitted to medium-pressure liquid chromatography (MPLC) [RP-18 Si gel 60 Merck (20−40 mm)] at first with water as eluent to remove the sugars, and then there was a second elution with methanol to collect the methanol polyphenol-rich fraction. This fraction was evaporated to dryness under reduced pressure to afford the methanol fraction of the methanol extract. A part of the water extract was also submitted to MPLC [RP-18 Si gel 60 Merck (20−40 $\mu$m)], $H_2O/CH_3OH$ gradient] with water and methanol as eluents. After methanol elution, the fraction was collected and evaporated to dryness under reduced pressure to afford the methanol fraction of the water extract. A part of that fraction (methanol fraction of the water extract) was extracted twice with ethyl acetate/water (EtOAc/$H_2O$) to afford the EtOAc fraction of the water extract.

**SCE Test.** Heparinized blood was obtained by venipuncture of an arm from one male and two female healthy blood donors, aged between 22 and 35 years, and informed consent was obtained from all participants. They did not smoke and were not taking drugs for medical or other reasons. Whole blood cultures were initiated by adding 10 drops of blood to each culture flask containing 5 mL of RPMI 1640 supplemented with 15% fetal calf serum, 4 mM L-glutamine, 100 units/mL penicillin, 100 $\mu$g/mL streptomycin, and 0.1 mL of phytohemagglutinin (Biochrom). 5-Bromo-2-deoxyuridine at a final concentration of 10 $\mu$g/mL was added to the cultures at the beginning of the 72 h incubation period in the dark at 37 °C. After 24 h in culture, MMC at a final concentration of 20 ng/mL and 100 $\mu$L of test compound solutions at different concentrations were added. The concentrations of grape extracts were 75, 150, and 300 $\mu$g/mL and those of plant polyphenols were 5, 50, and 100 $\mu$M. The metaphases were blocked during the last 2 h of the incubation period with colchicine at a final concentration of 0.5 $\mu$g/mL. After harvest, hypotonic treatment was conducted with prewarmed 0.075 M potassium chloride solution following 20 min of incubation at 37 °C. After centrifugation at 1500 rpm for 10 min, the cells were fixed at least three times with a fixative

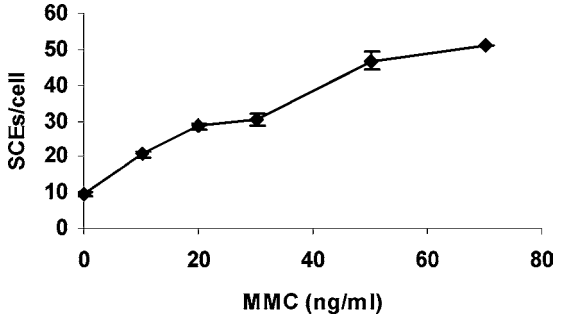


**Figure 2.** Dose–response curve of MMC-induced SCEs in cultures of human blood lypmpocytes. Each data point represents the mean ± SD number of SCEs from three independent experiments. In each experiment, the frequency of SCEs was scored in 30 s metaphase cells.

consisting of three parts methanol and one part glacial acetic acid, and then the cells were stored at −20 °C for overnight. Metaphase spreads were prepared by dropping the concentrated cell suspension onto glass slides and then stained by the fluorescence plus Giemsa (FPG) method (*19*) with slight modifications. The slides were stained with Hoechst 33258 (100 μg/mL) in distilled water for 12 min and exposed to UV light (Osram 300W UV lamp) for 75 min. After removal of the coverslips, the slides were stained with 3% Giemsa prepared in Sörensen phosphate buffer, pH 6.8, for 8 min, rinsed in tap water, and air-dried. The slides were coded and scored for SCEs. For each culture, the frequency of SCEs was determined randomly in 30 well-differentiated second-division metaphases with an optical microscope (Olympus BX41) and used to calculate the mean frequency of SCEs among all of the cells. Only metaphases with >42 chromosomes were scored, but the SCE frequency was normalized to 46 chromosomes and presented as SCEs per cell. The assay included both positive (MMC alone) and negative (without MMC or test compounds) cultures. All experiments were repeated three times. Also, each test compound was checked alone at the highest concentration used in combination with MMC for possible induction of SCEs.

Plant extracts and fractions were dissolved in methanol or distilled water. Stock solutions of polyphenols dissolved in methanol or dimethyl sulfoxide (DMSO) were prepared at concentrations of 100 mM and stored at −20 °C. The maximum concentrations of methanol and DMSO in cultures were 1 and 2%, respectively. The percentage of inhibition or induction of MMC-induced SCEs was calculated as follows:

$$\%\ \text{inhibition/induction} = \frac{(\text{mean SCE in MMC}) - (\text{mean SCE in MMC + test compound})}{(\text{mean SCE in MMC}) - (\text{mean SCE in negative control})} \times 100 \quad (1)$$

**Statistics.** For statistical analysis the one-way ANOVA was applied followed by Dunnett's test for post hoc analysis of group differences. Dose−response relationship was examined by Spearman's correlation analysis. Differences were considered to be significant at $p < 0.05$.

## RESULTS

**Dose−Response Curve of MMC-Induced SCEs in Human Lymphocyte Cultures.** A dose−response curve was designed from 0 to 70 ng/mL MMC to select the appropriate concentration of MMC for the experiments (**Figure 2**). As was expected, there was a significant dose−response effect of MMC on the SCEs frequency ($r = 0.98$, $p < 0.01$). From these data, the chosen concentration was 20 ng/mL because it was within the linear region of the dose−response curve and induced a sufficient increase in the number of SCEs without severe cytotoxic or cytostatic effects that could result in impractical SCEs scoring in human lymphocyte cultures.

**Effects of Grape Extracts and Polyphenol-Rich Fractions on MMC-Induced SCEs in Human Lymphocyte Cultures.**

**Table 2.** Effects of Grape Extracts and Polyphenol-Rich Fractions on MMC-Induced SCEs in Cultures of Human Lymphocytes

| treatment[a] | dose (μg/mL) | SCE[b] ($x$ + SD, $n = 3$) | % inhibition/ induction[c] |
|---|---|---|---|
| negative control | | 8.50 ± 0.74 | |
| ME (white grapes) | 300 | 8.58 ± 0.49 | 1[d] |
| MMC[e] | | 21.19 ± 0.27 | |
| MMC + ME (white grapes) | 75 | 19.84 ± 0.50 | +10 |
| | 150 | 23.50 ± 0.20[f] | +18 |
| | 300 | 24.24 ± 0.10[f] | +24 |
| negative control | | 8.85 ± 0.61 | |
| WE (white grapes) | 300 | 8.52 ± 0.28 | +4[d] |
| MMC | | 19.91 ± 0.16 | |
| MMC + WE (white grapes) | 75 | 20.44 ± 0.20 | +5 |
| | 150 | 22.91 ± 0.10[f] | +27 |
| | 300 | 23.42 ± 0.56[f] | +32 |
| negative control | | 7.51 ± 0.88 | |
| ME (red grapes) | 300 | 7.74 ± 0.42 | +3[d] |
| MMC | | 17.18 ± 0.16 | |
| MMC + ME (red grapes) | 75 | 16.75 ± 0.20 | 3 |
| | 150 | 19.87 ± 0.10[f] | +28 |
| | 300 | 22.63 ± 1.24[f] | +56 |
| negative control | | 7.51 ± 0.88 | |
| WE (red grapes) | 300 | 8.17 ± 0.32 | +9[d] |
| MMC | | 17.18 ± 0.16 | |
| MMC + WE (red grapes) | 75 | 17.32 ± 0.77 | +1 |
| | 150 | 18.01 ± 0.45 | +9 |
| | 300 | 21.50 ± 0.12[f] | +45 |
| negative control | | 7.65 ± 0.09 | |
| EAF from WE (red grapes) | 300 | 7.73 ± 0.38 | +1[d] |
| MMC | | 25.10 ± 0.52 | |
| MMC + EAF from WE | 75 | 28.76 ± 0.43[f] | +21 |
| (red grapes) | 150 | 32.60 ± 0.24[f] | +43 |
| | 300 | 37.66 ± 0.83[f] | +72 |
| negative control | | 7.65 ± 0.09 | |
| MF from WE (red grapes) | 300 | 8.15 ± 0.89 | +7[d] |
| MMC | | 25.10 ± 0.52 | |
| MMC + MF from WE | 75 | 28.04 ± 0.90 | +17 |
| (red grapes) | 150 | 29.22 ± 0.10[f] | +24 |
| | 300 | 33.57 ± 1.76[f] | +49 |
| negative control | | 8.12 ± 0.96 | |
| MF from ME (red grapes) | 300 | 7.31 ± 0.32 | 10[d] |
| MMC | | 21.15 ± 0.11 | |
| MMC + MF from ME | 75 | 22.26 ± 0.83 | +9 |
| (red grapes) | 150 | 20.60 ± 0.30 | 4 |
| | 300 | 22.53 ± 0.40 | +11 |

[a] ME, methanolic extract; WE, water extract; MF, methanolic fraction; EAF, ethyl acetate fraction. [b] Values are the mean ± SD number of SCEs in three independent experiments (the SCE frequency was scored in 30 s metaphases in each experiment). [c] The (+) denotes the percentage induction in the number of SCEs; otherwise, numbers denote inhibition. [d] Compared with negative controls (untreated cultures). [e] The concentration of MMC was 20 ng/mL in all cultures. Percent inhibition/induction of SCEs caused by test compounds was calculated as described under Materials and Methods. [f] $p < 0.05$ when compared with positive control (cultures with MMC alone) using Dunnett's multiple-comparison test.

The extracts, from both red and white grape varieties, exhibited pro-oxidant activity by enhancing MMC-induced SCEs in cultures of human lymphocytes. In particular, the methanolic extract from red grapes enhanced the number of SCEs in a dose-dependent manner ($r = 0.77$, $p < 0.01$) (**Table 2**). The aqueous extract of the same variety gave statistically significant enhancement of the MMC-induced SCEs only at a concentration of 300 μg/mL (**Table 2**). Moreover, the methanolic and aqueous extracts from white grapes enhanced in a dose-dependent manner ($r = 0.77$, $p < 0.01$; $r = 0.92$, $p < 0.01$, respectively) the clastogenic activity of MMC (**Table 2**). However, it was noteworthy that none of the extracts alone affected the spontaneous number of SCEs (**Table 2**), indicating that the extracts acted synergistically and not additively with MMC.

Like the red grape aqueous extract, its polyphenol-rich methanolic and ethyl acetate fractions enhanced dose-dependently ($r = 0.92$, $p < 0.01$; and $r = 0.97$, $p < 0.01$, respectively) the number of SCEs induced by MMC (**Table 2**). On the other hand, the methanolic fraction from the methanolic red grape extract did not affect the frequency of MMC-induced SCEs, although as has been mentioned previously the extract itself enhanced the activity of MMC (**Table 2**).

**Effects of Plant Polyphenols on MMC-Induced SCEs in Human Lymphocyte Cultures.** From the tested hydroxybenzoic acids, gallic acid exerted pro-oxidant activity by enhancing dose-depedently ($r = 0.97$, $p < 0.01$) the frequency of SCEs induced by MMC (**Table 3**). In contrast, the other hydroxybenzoic acid, protocatechuic acid, did not affect at any concentration the MMC-induced SCEs (**Table 3**). Neither gallic acid nor protocatechuic acid alone had an effect on spontaneous number of SCEs (**Table 3**).

From the tested hydroxycinnamic acids, caffeic acid enhanced by 55% the acivity of MMC at a concentration of 100 $\mu$M (**Table 3**). Furthermore, caffeic acid alone increased the spontaneous number of SCEs by 26% at 100 $\mu$M (**Table 3**). In contrast to caffeic acid, ferulic acid did not affect the number of SCEs either in the presence or in the absence of MMC (**Table 3**).

The flavonoid quercetin was the only one among the tested polyphenols that inhibited the clastogenicity of MMC. The inhibition was dose-dependent ($r = -0.97$, $p < 0.01$), and its statististically significant values were 70 and 78% at concentrations of 50 and 100 $\mu$M, respectively (**Table 3**). However, quercetin alone increased the spontaneous number of SCEs by 32% at 100 $\mu$M, exhibiting genotoxic activity (**Table 3**). However, its glycosylated form, rutin hydrate, enhanced MMC-induced SCEs at concentrations of 50 and 100 $\mu$M, whereas rutin hydrate alone had no effect on the spontaneous number of SCEs (**Table 3**). The other two tested flavonoids, (+)-catechin and (−)-epicatechin, did not affect the frequency of SCEs at any concentration either in the presence or in the absence of MMC (**Table 3**).

Finally, *trans*-resveratrol did not affect the activity of MMC at 5 and 50 $\mu$M, whereas it showed high cytostatic activity, both alone and in the presence of MMC, at 100 $\mu$M. The fact that there were only cells of the first mitotic division made the scoring of SCEs untenable at this concentration (**Table 3**).

**Table 3.** Effects of Plant Polyphenols on MMC-Induced SCEs in Cultures of Human Lymphocytes

| treatment | dose ($\mu$g/mL) | SCEs/cell + SD[a] | % inhibition/ induction of SCEs[b] |
|---|---|---|---|
| negative control | | 9.17 ± 0.44 | |
| gallic acid | 100 | 9.02 ± 0.11 | 2[c] |
| MMC[d] | | 23.45 ± 0.36 | |
| MMC + gallic acid | 5 | 27.92 ± 0.47[e] | +31 |
| | 50 | 33.05 ± 1.17[e] | +67 |
| | 100 | 38.25 ± 0.18[e] | +104 |
| negative control | | 9.17 ± 0.44 | |
| protocatechuic acid | 100 | 8.86 ± 0.11 | 3[c] |
| MMC | | 23.45 ± 0.36 | |
| MMC + protocatechuic acid | 5 | 23.12 ± 0.57 | 2 |
| | 50 | 22.55 ± 0.58 | 6 |
| | 100 | 22.43 ± 0.42 | 7 |
| negative control | | 8.07 ± 0.33 | – |
| caffeic acid | 100 | 10.15 ± 0.03[f] | +26[c] |
| MMC | | 23.48 ± 0.62 | |
| MMC + caffeic acid | 5 | 24.26 ± 0.74 | +5 |
| | 50 | 24.37 ± 0.16 | +6 |
| | 100 | 32.01 ± 1.25[e] | +55 |
| negative control | | 8.07 ± 0.33 | |
| ferulic acid | 100 | 8.17 ± 0.72 | +1[c] |
| MMC | | 23.48 ± 0.62 | |
| MMC + ferulic acid | 5 | 23.84 ± 0.07 | +2 |
| | 50 | 24.79 ± 0.25 | +9 |
| | 100 | 23.28 ± 0.07 | 1 |
| negative control | – | 8.81 ± 0.40 | |
| (+)-catechin | 100 | 9.26 ± 0.39 | +5[c] |
| MMC | | 19.90 ± 0.71 | |
| MMC + (+)-catechin | 5 | 21.09 ± 0.24 | +11 |
| | 50 | 21.39 ± 0.35 | +13 |
| | 100 | 21.75 ± 0.42 | +17 |
| negative control | | 8.81 ± 0.40 | |
| (–)-epicatechin | 100 | 8.71 ± 0.90 | 1[c] |
| MMC | | 19.90 ± 0.71 | |
| MMC + (–)-epicatechin | 5 | 19.26 ± 1.06 | 6 |
| | 50 | 19.38 ± 0.34 | 5 |
| | 100 | 20.77 ± 1.33 | +8 |
| negative control | | 9.06 ± 0.10 | |
| quercetin | 100 | 12.00 ± 0.12[f] | +32[c] |
| MMC | | 22.58 ± 0.08 | |
| MMC + quercetin | 5 | 20.39 ± 0.84 | 16 |
| | 50 | 13.11 ± 0.28[e] | 70 |
| | 100 | 12.04 ± 0.48[e] | 78 |
| negative control | | 9.06 ± 0.10 | |
| rutin | 100 | 9.97 ± 0.66 | |
| MMC | | 22.58 ± 0.08 | +10[c] |
| MMC + rutin | 5 | 22.86 ± 1.62 | +2 |
| | 50 | 27.36 ± 0.85[e] | +35 |
| | 100 | 27.17 ± 0.28[e] | +34 |
| negative control | | 7.65 ± 0.09 | |
| *trans*-resveratrol | 100 | cytotoxicity | |
| MMC | | 25.10 ± 0.52 | |
| MMC + *trans*-resveratrol | 5 | 25.84 ± 0.70 | +4 |
| | 50 | 24.86 ± 0.31 | 1 |
| | 100 | cytotoxicity | |

[a] Values are the mean ± SD number of SCEs in three independent experiments. [b] The (+) in front of numbers denotes the percentage induction in the frequency of SCEs; otherwise, numbers denote inhibition. [c] Compared with negative controls (untreated cultures). [d] The concentration of MMC was 20 ng/mL in all cultures. [e] $p < 0.05$ when compared with positive control (cultures with MMC alone) using Dunnett's multiple-comparison test. [f] $p < 0.05$ when compared with negative control.

## DISCUSSION

**Effects of Grape Extracts and Polyphenol-Rich Fractions on MMC-Induced SCEs in Human Lymphocyte Cultures.** Grape extracts have been shown to exert anticarcinogenic activity (*5*, *6*). The mechanism of this activity is not completely understood but may be attributed to their free radical scavenging activity (*11*). We have reported previously (*12*) that the grape extracts used in the present study inhibited mutagenicity of bleomycin and hydrogen peroxide, two oxidative agents, in bacterial *Salmonella* cells. Therefore, in the present study, we focused on the potential protective activity of these extracts against clastogenicity induced by MMC, another oxidative agent, in human blood lymphocyte cells. In contrast to the results from bacterial cells, the extracts (methanolic and aqueous) from both grape varieties enhanced MMC-induced SCEs, indicating a prooxidant activity (**Table 2**). However, it is worth mentioning that none of the extracts alone affected the spontaneous number of SCEs (these extracts had also no mutagenicity in Ames *Salmonella*/reversion assay). This observation can be explained by a synergistic action of extracts with MMC. Moreover, in other studies grape extracts did not show mutagenicity in Ames *Salmonella*/reversion, comet, and micronucleus assays (*20*, *21*). The polyphenol-rich fractions, like the extracts, either did not affect or enhanced MMC-induced SCEs and, also, alone had no effect on spontaneous number of SCEs (**Table 2**). In general, a co-genotoxic effect, such as that observed in our study, is considered to be "unbeneficial" to humans, because it may lead to carcinogenesis. Thus, these results initially suggest caution

regarding especially the use of grape extracts as dietary supplements. On the other hand, the fact that the genotoxic activity of grape extracts was shown only in the presence and not in the absence of MMC may indicate a selective activity. Namely, the grape extracts exhibited prooxidant activity not in normal cells but only in those being under the genotoxic pressure of an oxidant agent. Furthermore, it has been reported that grape extracts exerted cytotoxicity against cancer cells, whereas they had no effect on normal cells (*11*). The selective prooxidant activity is thought to be an important chemopreventive mechanism, because it may lead to apoptosis, a programmed cell death, which eliminates cells showing a genomic instability (*22*).

**Effects of Plant Polyphenols on MMC-Induced SCEs in Cultures of Human Lymphocytes.** The effects of monomer polyphenols identified in grape extracts and polyphenol-rich fractions on MMC-induced SCEs in human lymphocytes were examined. The tested hydroxycinnamic acids, caffeic acid and ferulic acid, showed different effects, although the only difference in their chemical structure is that the former has two hydroxyl groups in the 3- and 4-positions, whereas the latter has one hydroxyl group at the 4-position and one methoxy group at the 3-position (**Figure 1**). In particular, caffeic acid enhanced MMC clastogenicity by 55% at 100 $\mu$M, showing prooxidant activity, whereas ferulic acid had no effect at any concentration (**Table 3**). Also, caffeic acid alone increased the spontaneous number of SCEs but by only 26% at 100 $\mu$M, and so it could be inferred that caffeic acid acted synergistically with MMC. It has also been reported (*23*) that caffeic and ferulic acid at concentrations of 33 and 100 $\mu$M enhanced MMC-induced SCEs in Chinese hamster ovary (CHO K-1) cells. Moreover, in another paper (*8*), we showed that both caffeic and ferulic acid enhanced the MMC-induced strand breakage of plasmid DNA at concentrations of 50 and 100 $\mu$M, but the former was more potent than the latter. The mechanism by which caffeic acid increased the SCE frequency both alone and in combination with MMC may be related to its inhibitory activity against topoisomerase I as shown by our previous study (*8*). It has been reported that topoisomerase inhibitors alone induced SCEs in human lymphocytes by a mechanism not totally independent from that one of MMC (*24*).

Between the two hydroxybenzoic acids, which differ in that gallic acid has one more hydroxyl group than protocatechuic acid (**Figure 1**), gallic acid potentiated MMC-induced clastogenicity, whereas protocatechuic acid did not show any effect (**Table 3**). The incapability of gallic acid alone to induce SCEs indicates that it acted synergistically with MMC, in contrast to previous papers showing (*25*) that gallic acid inhibited MMC-induced SCEs in CHO cells. The difference between previous results and our study could be attributed to the use of different cell types from different organisms. In addition, both of these hydroxybenzoic acids had no effect on mutagenicity induced by oxidative agents in the Ames *Salmonella*/reversion assay (*12*), although antimutagenic activity of gallic acid in this assay has been reported (*26*) but against nonoxidant mutagens.

The flavonoid quercetin was the only from the tested polyphenols that inhibited at 50 and 100 $\mu$M the SCEs frequency induced by MMC (**Table 3**). Additionally, in another paper (*27*), quercetin protected against MMC-induced DNA damage in human lymphocytes as shown by comet assay. However, quercetin alone increased the spontaneous number of SCEs by 32% at 100 $\mu$M. These results were in agreement with several other studies using both mammalian and bacterial cells in which quercetin alone showed mutagenicity while at the same time it exerted antimutagenic activity (*28*, *29*). It has been proposed that its antimutagenic activity is attributed to its strong antioxidant and metal-chelating properties (*30*). Interestingly, it has been reported that metal-chelating agents inhibited MMC-induced DNA strand breaks (*31*). On the other hand, the genotoxicity induced by quercetin alone may be due to its ability to form complexes with metal ions (e.g., $Fe^{3+}$ and $Cu^{2+}$) resulting in its autoxidation (*30*). Another explanation for the induction of SCEs by quercetin alone may be its strong topoisomerase inhibitory effects, as mentioned for caffeic acid (*32*). Although the contradiction between the mutagenic and the antimutagenic capabilities of quercetin has not been elucidated, it is believed that its activity depends on the redox state of its biological environment (*30*).

By contrast with the activity of quercetin, its glycosylated form, rutin hydrate (**Figure 1**) showed a completely different action. It enhanced the MMC-induced SCEs at 50 and 100 $\mu$M, whereas it did not affect the spontaneous number of SCEs (**Table 3**). Subsequently, rutin hydrate seems to act in synergism with MMC for the increase in SCE frequency. Therefore, the present results suggest that the substitution of the 3-hydroxyl group of quercetin by the sugar moiety rutinose not only abolishes its antigenotoxic activity but also results in potentiation of MMC-induced clastogenicity. On the contrary, the comet assay showed that rutin hydrate protected against MMC-induced DNA damage in human lymphocytes (*27*), whereas rutin hydrate had no effect on bleomycin- and hydrogen peroxide-induced mutagenicity in *S. typhimurium* TA102 cells in our previous study (*12*).

The other two tested flavonoids, (+)-catechin and (−)-epicatechin, which are stereoisomeres (**Figure 1**), did not show any effect on MMC-induced SCEs (**Table 3**). We have also shown previously that these flavonoids had no effect on mutagenicity induced by oxidant agents in the Ames *Salmonella*/reversion assay (*12*). The difference in the chemical structure between these flavonoids and quercetin is that the latter has a 4-keto group and a 2,3 double bond (**Figure 1**). It is believed that flavonoids with a 4-keto group and a 3- or 5-hydroxyl group, such as quercetin, exhibit strong metal-chelating properties (*33*). Moreover, the presence of a 4-keto group has been assigned responsibility for the stronger free radical scavenging activity of quercetin in comparison with (+)-catechin (*33*).

*trans*-Resveratrol, one of the most studied plant polyphenols exhibiting anticarcinogenic activity (*34*), did not affect MMC-induced SCEs (**Table 3**). Moreover, it showed high cytostatic activity at 100 $\mu$M either alone or in the presence of MMC, possibly due to its inhibitory activity against enzymes that are important for cell proliferation such as ribonucleotide reductase (*35*). Additionally, in a study using a Chinese hamster cell line (*36*), *trans*-resveratrol showed high cytostatic activity at 80 $\mu$M. Also, in the same study, *trans*-resveratrol induced SCEs at concentrations between 10 and 40 $\mu$M and was positive in the micronucleus test, although it did not cause abnormalities in chromosome number. A different study using the comet assay (*37*) showed that *trans*-resveratrol induced DNA damage at 50 $\mu$M in human lymphocytes but only in the presence of $Cu^{2+}$ and not alone.

Consequently, the results showed that the tested polyphenols (apart from quercetin), like the grape extracts and polyphenol-rich fractions, did not affect or enhance MMC-induced SCEs in human lymphocyte cultures. However, the potentiation of extracts could not be attributed to any individual polyphenol, because polyphenols enhanced MMC clastogenicity at much higher concentrations than their concentrations in the extracts

(**Table 1**). It seems that there was a synergism between polyphenols in the extracts for the enhancement of MMC activity. On the other side, it cannot be excluded that structures other than the tested polyphenols such as oligomeric and polymeric forms of polyphenols (i.e., procyanidins) may account for the observed activity of extracts. Furthermore, the fact that the methanolic polyphenol-rich fraction from the methanolic extract of red grapes was less potent than the extract itself indicates that there may be a synergism between polyphenols and other compounds present in the extracts. The enhancement of MMC clastogenicity by the tested polyphenols is rather intriguing, because these polyphenols are strong antioxidants as has been both shown by several studies (*38*, *39*) and assessed by us using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (unpublished data). In addition, the results from similar studies are conflicting. For example, two other antioxidants, vitamin C and $\beta$-carotene, potentiated and inhibited MMC-induced SCEs in human lymphocytes, respectively (*40*, *41*). Nevertheless, it should be noted that, in an in vitro assay, a compound maybe exhibit prooxidant activity because the environment is highly oxygenated, unlike the in vivo situation (*40*). Furthermore, the concentrations used were in part similar to those found in human plasma, because the maximum plasma polyphenol concentrations attained after a polyphenol-rich meal are in the range of 0.1−10 $\mu$M (*42*). On the other hand, recent studies have shown that the prooxidant activity of polyphenols may be a chemopreventive mechanism (*22*), as long as it is both selective with genetically damaged cells responding differently to the normal cells and exerted at concentrations achievable in the human organism through the diet. The present results indicate that the above preconditions may stand for gallic acid. Moreover, some polyphenols (i.e., quercetin and caffeic acid) alone induced SCEs, but this occurred at a high concentration (100 $\mu$M) that cannot be reached in the human organism through the diet (*42*). Finally, the potentiation of MMC activity, a chemotherapeutic drug (*43*), by some polyphenols suggests that they could possibly be used in chemotherapy. This hypothesis is supported by a recent study (*44*), which showed that tannic acid, a polyphenol present in some grape varieties, exerted a synergistic cytototoxic effect with MMC against malignant human cholangiocytes.

In conclusion, to the best of our knowledge, this is the first report of clastogenic effects of grape extracts in human cells. The grape extracts and their polyphenol-rich fractions enhanced the MMC-induced clastogenicity. However, this genotoxic activity may be selective, because it was exerted only in genetically damaged cells and not in normal ones. Further studies, mainly in vivo, are needed to clarify the biological importance of the observed effects.

## ABBREVIATIONS USED

FPG, fluorescence plus Giemsa; CHO, Chinese hamster ovary cells; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMSO, dimethyl sulfoxide; MPLC, medium-pressure liquid chromatography; MMC, mitomycin C; ROS, reactive oxygen species; SCEs, sister chromatid exchanges.

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