Pro-oxidative Properties of Flavonoids in Human Lymphocytes

Gow-Chin Yen,† Pin-Der DuH, Hui-Ling Tsai, and Shih-Li Huang

1Department of Food Science, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan
2Department of Food Health, Chia Nan University of Pharmacy and Science, 60 Erh-jen Road, Section 1, Pao-An, Jen-te Hsiang, Tainan Hsien, Taiwan
3Department of Baking Management, National Kaoshiung Hospitality College, Kaoshiung, Taiwan

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The pro-oxidative properties of the four flavonoids, quercetin, morin, naringenin and hesperetin, in human lymphocyte system were investigated. Naringenin and hesperetin accelerated the oxidation of deoxyribose induced by Fe³⁺/H₂O₂ in a concentration range of 0–200 μM, but quercetin and morin decreased it when the concentration was greater than 100 μM. The generation of hydrogen peroxide and the superoxide anion and the production of TBARS in lymphocytes were increased with increasing concentration of a flavonoid. Cell membrane protein thiols of the lymphocytes decreased when treated with the four flavonoids. Quercetin and hesperetin had no significant effect (p > 0.05) on the activity of glutathione reductase, but morin and naringenin could inhibit the activity of the enzyme at a concentration of 200 μM, when compared to the control group. The glutathione S-transferase activity was slightly decreased by treatment with each of the four flavonoids only at a concentration of 200 μM. Therefore, the DNA damage in lymphocytes induced by the flavonoids only at a concentration of 200 μM. The DNA damage in lymphocytes induced by the flavonoids in the model system might have been due to their stimulation of oxidative stress in the lymphocytes, which resulted in the decrease of cell membrane protein thiols, increase of lipid peroxidation in cell membrane and in the influence of the antioxidative enzyme activities.

Key words: flavonoid; pro-oxidant; DNA damage; lymphocyte

Fruits, vegetables and beverages are important dietary sources of flavonoids. It has been reported that the human intake of flavonoids from the diet is about 20 mg to 1 g every day. Although flavonoids show biological such activities as antibacterial, antiviral, antimutagenicity, antioxidative activity and enzyme inhibition, there is evidence that shows that some flavonoids exhibit mutagenicity in vitro. The effect of toxicity might be due to the pro-oxidative activity of flavonoids. This pro-oxidative activity may accelerate free radical damage to such non-lipids as DNA, protein and carbohydrates. Some literature has reported that the pro-oxidation of phenolic compounds was due to the reduction of Fe³⁺ to Fe²⁺, and then Fe²⁺ reacted with H₂O₂ to form the hydroxyl radical. Pond et al. have found that some flavonoids could auto-oxidize in the chemical system and inhibit mitochondrial respiration action with concomitant production of the superoxide anion and hydrogen peroxide. This implicated that the DNA damage induced by flavonoids was due to the generation of free radicals or reactive oxygen species. In addition, quercetin, morin and naringenin induced single-strand DNA breakage in rat liver and opened plasmid DNA in calf thymus DNA. The flavonoids promote generation of the hydroxyl radical in the presence of metal ions and induced DNA damage. Moreover, quercetin, morin, naringenin and hesperetin have been shown to quench free radicals, whereas these four flavonoids have also been shown to induce DNA damage. The causes for these flavonoids inducing DNA damage are still unclear. On the basis of these considerations, the present work was carried out to explore whether the flavonoids, including quercetin, morin, naringenin and hesperetin, could accelerate the DNA damage in human lymphocytes. The effect and mechanism for the flavonoids tested on the pro-oxidative activity were also examined.

Materials and Methods

Materials. Quercetin, morin, naringenin, hesperetin, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), N-ethyl maleimide (NEM), glutathione (GSH), glutathione reductase, glutathione disulfide (GSSG), NADPH, trypan blue, triton X-100, ethidium bromide (EtBr), nitro blue tetrazolium (NBT), horseradish peroxidase (HRPase) and ferric chloride hexahydrate were from Sigma Co., Ltd. (St Louis, MO, USA). Histopaque 1077 was from Pharmacia Biotech (Uppsala, Sweden). Dutch modified RPMI 1640 medium, ultrapure low-melting-point agarose and normal-melting-point agarose (both electrophoretic grade) were from Gibco Life Co., Ltd. (Grand Island, NY, USA).
Dimethyl sulfoxide (DMSO) was from Merck Co., Ltd. (Darmstadt, Germany), and the protein assay kit and Tris were from Bio-Rad Lab. (Hercules, CA, USA). Blood samples were from healthy volunteers.

Preparation of the samples. Each flavonoid was dissolved in dimethyl sulfoxide (DMSO); the solvent concentration in the incubation medium never exceeded 1%.

Cell preparation. Human lymphocytes were isolated from fresh whole blood by adding blood to the RPMI 1640 medium, before underlaying it with Histopaque 1077 and centrifuging at 1600 rpm for 10–15 min. Lymphocytes were separated as a pink layer at the top of the Histopaque 1077. The lymphocytes were washed in the RPMI 1640 medium. The cell number and viability (Trypan blue exclusion) were determined with a Neubauer improved haemocytometer before treatment. Human lymphocytes were incubated at a density of $5 \times 10^5$ ml, the viability being over 90%.

Cytotoxicity. Cell suspensions were incubated with different concentrations of each flavonoid (up to a final concentration of $25-200 \mu M$) for 30 min at $37^\circ C$ in a dark incubator, together with untreated control samples. The samples were then centrifuged at 800–900 rpm, the lymphocytes were resuspended in RPMI 1640 and 0.4% trypan blue, and viable and dead cells scored.

Alkaline single-cell gel electrophoresis (comet assay). The comet assay was performed under alkaline conditions according to the method of Yen et al.\textsuperscript{10} The slides were observed with a fluorescence microscope attached to a CCD camera connected to a personal computer-based image analysis system (Komet 3.0; Kinetic Imaging). For each analysis, 50 individual cells were calculated, and in most cases, three separate experiments were conducted for each series. Single cells were analyzed under the fluorescence microscope as desired. The DNA damage is expressed as the percentage of Tail DNA = [tail DNA / (head DNA + tail DNA)] $\times 100$. A higher percentage of Tail DNA means a higher level of DNA damage.

Deoxyribose assay. This assay was carried out as described by Smith et al.\textsuperscript{12} Each reaction mixture contained, in a final volume of 3.5 ml, the following reagents at the final concentrations stated: deoxyribose (3 mm), hydrogen peroxide (3 mm), KH$_2$PO$_4$–KOH buffer at pH 7.4 (20 mm), FeCl$_3$ (50 $\mu M$), EDTA (100 $\mu M$) with or without additional ascorbic acid (100 $\mu M$) and different flavonoids. Test tubes were incubated at $37^\circ C$ for 1 h, 0.5 ml of 2.8% (w/v) trichloroacetic acid (TCA) and 0.5 ml of 1% (w/v) thiobarbituric acid (TBA) were then added, and the tubes were heated in a water-bath maintained at 100$^\circ C$ for 20 min. Measurement of the absorbance at 532 nm of the resulting chromogen gives a measure of the OH-dependent damage to deoxyribose. The concentration of TBARS was calculated by using the molar absorption coefficient of $1.56 \times 10^5$ M$^{-1}$ cm$^{-1}$.

Determination of hydrogen peroxide. The procedure used is a modification of that reported by Rinkus and Taylor.\textsuperscript{13} Phenol red (sodium salt) was prepared in 0.2 m potassium phosphate at pH 6.2, at a final concentration of 7.5 mM. HRPase was prepared in 0.2 m potassium phosphate at pH 6.2, at a final concentration of 0.5 mg/ml. Just before use, one volume of the HRPase solution and two volumes of the phenol red solution were combined. Each sample was incubated with 1 ml of the HRPase/phenol red solution. The tubes were vortexed and then allowed to sit for 30 min, this reaction being monitored at 610 nm.

Determination of the superoxide anion. The generated superoxide anion was determined according to the method of Ramanathan et al.\textsuperscript{14} A cell suspension (1 $\times 10^6$ ml) was incubated with a flavonoid (0–200 $\mu M$) at $37^\circ C$ for 30 min, and then centrifuged at 1000 rpm. The cell pellet was washed twice with phosphate-buffered saline and then incubated with 400 $\mu l$ of NBT (0.1%) at $37^\circ C$ for 15 min, before a spectrophotometric determination was carried out at 560 nm to assess the amount of formazan produced. The absorbance values were converted to nmols of NBT reduced by using the extinction coefficient of 15,000 M$^{-1}$ cm$^{-1}$.

Determination of TBARS. The TBARS generated were determined according to the method of Ramanathan et al.\textsuperscript{14} A cell suspension (1 $\times 10^5$ ml) was incubated with a flavonoid (0–200 $\mu M$) at $37^\circ C$ for 30 min and then centrifuged at 1000 rpm. The cell pellet was washed twice with phosphate-buffered saline and suspended in 0.1 N NaOH. This cell suspension (1.4 ml) was incubated with 10% TCA and 0.6 M TBA in a boiling water bath for 10 min. Upon cooling, the absorbance was read at 532 nm with a spectrophotometer. The protein content of the cells was determined by Bio-Rad protein assay kit. TBARS are expressed as the malonaldehyde equivalent, calculated by using the molar extinction coefficient for MDA of $\Sigma = 1.56 \times 10^5$ M$^{-1}$ cm$^{-1}$.

Assays for enzymes. The analysis of glutathione reductase activity was carried out as described by Racker.\textsuperscript{15} Lymphocytes were incubated with 10 $\mu l$ of a sample at $37^\circ C$ for 30 min, before 0.5% Triton X-100 was added. The reaction mixture was centrifuged at 12,000 rpm for 10 min and the cell suspension was used. After a 100 mm potassium phosphate
buffer (pH 7.0) which contained 1.1 mM MgCl₂ · 6H₂O, 5.0 mM GSSG and 0.1 mM NADPH had been added to the cell suspension, the change in absorbance value at 340 nm was recorded for 5 min.

The analysis of glutathione S-transferase activity was carried out as described by Habig.¹⁶ Lymphocytes were incubated with 10 μl of a sample at 37°C for 30 min, before 0.5% Triton X-100 was added. The reaction mixture was centrifuged at 12,000 rpm for 10 min, and the cell suspension was then used. After a 100 mM potassium phosphate buffer (pH 7.0) which contained 1 mM GSH and 50 mM 1-chloro-2,4-dinitrobenzene had been added to the cell suspension, the change in absorbance value at 340 nm was recorded for 3 min.

**Determination of cell membrane protein thiols.** The cell membrane protein thiols were determined by the method of Boyne and Ellman.⁷⁷ After incubating, the cell pellet was washed twice with phosphate-buffered saline, a 20 mM potassium phosphate buffer (pH 7.0) was added, and the solution centrifuged at 4000×g. The cell pellet was washed with a 20 mM potassium phosphate buffer (pH 7.0) and a 50 mM sodium phosphate buffer added. After vortexing, the cells were incubated with 23 μl of 100 mM DTNB for 30 min and then centrifuged at 10,000×g for 10 min. The absorbance of the suspension was measured at 412 nm (a), and then 0.8 M NEM was added to the suspension for 20 min and the absorbance determined again at 412 nm (b). Calculation of SH is based on an E molar value at 412 nm of 13,600 M⁻¹ cm⁻¹, the amount of SH being (a)–(b)/13,600 (M⁻¹ cm⁻¹).

**Statistical analysis.** All analyses were run in triplicate and the results averaged. Statistical analyses were performed according to the SAS Institute User’s Guide. The analysis of variance was performed by the ANOVA procedure. Significant differences (P<0.05) between the means were determined by Duncan’s multiple-range test.

**Results**

**Cytotoxicity of the flavonoids**

The cytotoxicity of the flavonoids toward human lymphocytes, induced at 37°C for 30 min, is shown in Table 1. All the flavonoids tested by the model system generally showed cytotoxicity toward lymphocytes. The cell viability was greater than 90% for quercetin and morin at the concentration of 10–100 μM, indicating no cytotoxicity toward lymphocytes; however, at the concentration of 100–200 μM, the cell viability decreased with increasing concentration of quercetin and morin. The cell viability was 78%, 71%, 90% and 84% for quercetin, morin, naringenin and hesperetin, respectively, at a concentration of 200 μM. Of the four compounds tested, naringenin showed the least cytotoxicity toward lymphocytes.

**Effect of the flavonoids on DNA damage**

Figure 1 shows the DNA damage in human blood lymphocytes. Human lymphocytes were incubated for 30 min with a flavonoid. DNA strand breakage was measured by the comet assay. Each result is the mean ± SD for n=4.
The concentration of deoxyribose decreased with increasing concentration.

**Values in a column with different superscript letters are significantly different (P<0.05).**

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Quercetin</th>
<th>Morin</th>
<th>Naringenin</th>
<th>Hesperetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.62 ± 0.00f</td>
<td>0.62 ± 0.00f</td>
<td>0.62 ± 0.00f</td>
<td>0.62 ± 0.00f</td>
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<tr>
<td>10</td>
<td>2.14 ± 0.01b</td>
<td>2.12 ± 0.02b</td>
<td>2.00 ± 0.02b</td>
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<td>25</td>
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<td>2.14 ± 0.01b</td>
<td>2.03 ± 0.01b</td>
<td>2.11 ± 0.01c</td>
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<tr>
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<td>2.13 ± 0.01b</td>
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<td>2.11 ± 0.02c</td>
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<td>2.02 ± 0.03c</td>
<td>2.14 ± 0.02b</td>
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<td>2.12 ± 0.01c</td>
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<tr>
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<td>2.22 ± 0.01c</td>
<td>2.17 ± 0.01d</td>
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</tbody>
</table>

* Each value is the mean ± SD of triplicate determinations of TBARS.

**Values in a column with different superscript letters are significantly different (P<0.05).**

**Effect of the flavonoids on deoxyribose damage**

Table 2 shows that deoxyribose was substantially damaged by the four flavonoids. Naringenin and hesperetin in the concentration range of 0–200 μM accelerated the oxidation of deoxyribose induced by Fe(III)/H₂O₂. The oxidation of deoxyribose accelerated by quercetin and morin increased with increasing concentration up to 100 μM, and then the oxidation of deoxyribose decreased with increasing concentration.

**Generation of hydrogen peroxide by the flavonoids**

The generation of hydrogen peroxide by the flavonoids in the peroxidase-phenol red model system is shown in Table 3. Quercetin and morin accelerated the generation of hydrogen peroxide, whereas the concentration of hydrogen peroxide could not be detected when naringenin or hesperetin were added in the concentration range of 0–100 μM. The generation of hydrogen peroxide increased with increasing concentration of quercetin in the concentration range of 25–200 μM. No concentration of hydrogen peroxide could be detected when morin was in the concentration range of 0–100 μM, whereas the concentration of hydrogen peroxide increased with increasing concentration of each flavonoid. At a concentration of 200 μM, the generation of hydrogen peroxide by the flavonoids could be detected when naringenin or hesperetin were added. The concentration of hydrogen peroxide increased with increasing concentration of each flavonoid. The four flavonoids at 200 μM showed 8.87, 4.53, 3.09 and 3.02 nmol of superoxide anion for quercetin, morin, naringenin and hesperetin, respectively.

**Effect of the flavonoids on the production of lipid hydroperoxide**

The effect of the flavonoids on the production of lipid hydroperoxide is shown in Table 4. The production of lipid hydroperoxide increased with increasing concentration of each flavonoid. A concentration of 200 μM showed 8.87, 4.53, 3.09 and 3.02 nmol of superoxide anion for quercetin, morin, naringenin and hesperetin, respectively.

**Fig. 2. Production of the Superoxide Anion in Human Lymphocytes by Flavonoids.**

The concentration of the superoxide anion was calculated by using a molar absorption coefficient of 15,000 M⁻¹ cm⁻¹. Each result is the mean ± SD for n ≥ 3.

**Table 4. Production of Lipid Hydroperoxide in Human Lymphocytes Treated with Flavonoids**

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Quercetin</th>
<th>Morin</th>
<th>Naringenin</th>
<th>Hesperetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.24 ± 0.05e</td>
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</tr>
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<td>0.30 ± 0.03b</td>
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<td>0.32 ± 0.03b</td>
<td>0.33 ± 0.01bc</td>
<td>0.39 ± 0.02c</td>
</tr>
<tr>
<td>50</td>
<td>0.42 ± 0.04c</td>
<td>0.35 ± 0.03bc</td>
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<td>0.42 ± 0.03c</td>
</tr>
<tr>
<td>100</td>
<td>0.44 ± 0.05d</td>
<td>0.39 ± 0.01c</td>
<td>0.34 ± 0.02b</td>
<td>0.40 ± 0.02c</td>
</tr>
<tr>
<td>125</td>
<td>0.53 ± 0.03a</td>
<td>0.44 ± 0.03b</td>
<td>0.35 ± 0.03a</td>
<td>0.44 ± 0.02a</td>
</tr>
<tr>
<td>150</td>
<td>0.69 ± 0.02f</td>
<td>0.53 ± 0.02f</td>
<td>0.49 ± 0.03f</td>
<td>0.59 ± 0.02f</td>
</tr>
<tr>
<td>200</td>
<td>0.81 ± 0.02g</td>
<td>0.69 ± 0.02g</td>
<td>0.59 ± 0.02g</td>
<td>0.71 ± 0.02g</td>
</tr>
</tbody>
</table>

* Each value is the mean ± SD of triplicate determinations of TBARS.

**Values in a column with different superscript letters are significantly different (P<0.05).**
Pro-oxidative Properties of Flavonoids

of 200 μM, the concentration of lipid hydroperoxide was 0.53, 0.44, 0.44 and 0.35 nmol/mg of protein for quercetin, morin, hesperetin and naringenin, respectively.

**Effect of the flavonoids on the enzyme activity**

Figure 3 shows the effect of the flavonoids on the GR activity in human lymphocytes. Among the four flavonoids tested, quercetin and hesperetin showed no significant \( P > 0.05 \) effect on the GR activity. In contrast, morin and naringenin significantly decreased the GR activity at concentrations greater than 25 and 150 μM, respectively.

Figure 4 shows that quercetin at 200 μM significantly \( P < 0.05 \) decreased the GST activity in human lymphocytes. The GST activity decreased with increasing concentration of morin. Naringenin in the range of 25–50 μM enhanced the GST activity and at greater than this concentration, naringenin significantly \( P < 0.05 \) reduced the GST activity. Hesperetin at 50 μM increased the GST activity and at greater than this concentration, the GST activity decreased significantly \( P < 0.05 \). The data in Fig. 4 clearly demonstrate that the flavonoids tested in this study could affect the GST activity and cause DNA damage.

**Effect of the flavonoids on the cell membrane protein thiols**

The effect of the flavonoids on the cell membrane protein thiols in human lymphocytes is shown in Fig. 5. After adding a flavonoid, the cell membrane protein thiols decreased with increasing concentration of each flavonoid. The respective amounts of cell membrane protein thiols were 8.99, 6.78, 5.91 and 5.71 μmol for quercetin, morin, naringenin and hesperetin at 200 μM.

**Discussion**

The present work has shown that the viability of the lymphocyte cells was higher than 90% when the concentration of each of the four flavonoids was less than 100 μM, indicating no toxicity toward the lymphocyte cells. This finding reflects that all the flavonoids above 100 μM were highly cytotoxic toward the lymphocyte cells.

The comet assay is a sensitive method for detecting DNA strand breakage at the level of an individual cell. Hence, an increasing number of laboratories have begun to use this effective and rapid method for detecting DNA damage. There have been many studies on the antioxidative activity of flavonoids, whereas few studies have been focused on the pro-
oxidative effect of flavonoids on human cells. According to the data in Fig. 1, each of the four flavonoids damaged DNA, suggesting that quercetin, morin, naringenin and hesperetin are capable of inducing strand breakage in human DNA.

The deoxyribose assay is a simple method which allows determination of the rate constant for reaction between antioxidants and hydroxyl radicals. This assay has also been adapted to assess prooxidative action. In this model system, the hydroxyl radical attacks the deoxyribose to form products that, on heating with thiobarbituric acid (TBA) at a low pH value, yield a pink chromogen. Hence, the deoxyribose assay can be used to evaluate hydroxyl radical formation. The data in Table 2 show that the oxidation of deoxyribose that was accelerated by the four flavonoids might be attributable to hydroxyl radical formation. The reason for this might have been that all the four flavonoids enhanced the generation of the hydroxyl radical, causing DNA damage. Hiramoto et al. have reported that 4-hydroxy-2-(hydroxy-methyl)-5-methyl-3(2H)-furanone (HHMF) derived from the glucose/glycine Maillard reaction could produce hydroxyl radicals and induce DNA strand breakage.

The data in Table 3 show that the generation of hydrogen peroxide was enhanced by quercetin and morin which resulted in DNA damage. Duthie et al. have noted that quercetin and myricetin could directly modulate hydrogen peroxide-induced DNA damage in human lymphocytes. Hydrogen peroxide causes DNA strand breakage by generating of hydroxyl radicals close to the DNA molecule, by the Fenton reaction. This may result in DNA instability, mutagenesis and ultimately carcinogenesis. Aruoma et al. have noted that hydrogen peroxide at a low level seems to be poorly reactive, although a higher level of hydrogen peroxide can attack several cellular energy-producing systems.

When the superoxide anion accepts a proton, it forms the hydroperoxy radical (HO₂•) which is able to cross a membrane and then conceivably create damage. In addition, the superoxide anion is converted to H₂O₂ and O₂ by superoxide dismutase and then, in the presence of transition metal ions, H₂O₂ forms OH•. It is well known that superoxide anions produced in a significant quantity can directly or indirectly damage biomacromolecules by forming hydrogen peroxide, hydroxyl radicals, peroxynitrite or singlet oxygen during pathophysiological events such as ischemia-reperfusion injury. According to the data in Fig. 2, the four flavonoids promoted the generation of superoxide anions in human lymphocytes. The samples at 200 ppm showed that the generation of superoxide anions was approximately 4.87-, 2.49-, 1.70-, and 1.66-fold for quercetin, morin, naringenin, and hesperetin, respectively, that of the control. In other words, the four flavonoids could directly or indirectly damage the lymphocytes owing to their significant generation of superoxide anions. According to the data in Table 3 and Fig. 2, the flavonoids in the model systems tested generated hydrogen peroxide and superoxide anions, which may have accelerated the generation of lipid peroxides (Table 4) and then attacked the DNA molecule. Maridonneva-parini et al. have reported that the generation of oxygen-free radicals in red cells induced a K⁺ loss which probably resulted from membrane lipid peroxidation. Moreover, flavonoids are able to modify membrane-dependent processes such as the free-radical-induced membrane ability to interact with and penetrate lipid bilayers. Based on the data in Tables 3 and 4 and Fig. 2, the cytotoxicity of the flavonoids toward human lymphocytes may have been due to the generation of reactive oxygen species and lipid peroxides.

Zhou et al. have noted that morin enhanced the generation of •OH and H₂O₂ as a result of photosensitization, stimulating DNA damage and causing cell apoptosis. Sahu et al. have revealed that naringenin and morin induced the peroxidation of nuclear membrane lipids concurrently with DNA strand breakage. On the basis of this information, lipid peroxidation, and the generation of free radical and reactive oxygen species by the flavonoids tested may be responsible for the DNA damage in human lymphocytes.

Elliott et al. have reported that the cell cytotoxicity might be due to inhibition of the redox reaction by flavonoids. In addition, flavonoids have been shown to autooxidize with the concomitant production of the superoxide anion radical, hydrogen peroxide, and hydroxyl radical to cause the metabolism disorder which results in cell cytotoxicity. In the present work, the generation of free radicals and reactive oxygen from the autooxidation by flavonoids might have been responsible for the cytotoxicity and DNA damage to the lymphocytes.

Several natural and synthetic antioxidants have been compared for their biological activities based on their structure. Comparing the relationship between the structure of the flavonoids tested and the cytotoxicity, two hydroxyl groups in the orthodiphenolic arrangement of quercetin were more effective for antioxidative activity than those in the meta-diphenolic arrangement of morin; hence, quercetin caused less DNA damage than morin. In addition, the unsaturation (2,3-double bound) in the C ring of both quercetin and morin allows electron delocalization across the molecule for stabilization of the aryloxyl radical; however, naringenin and hesperetin lack such a double bond. These structural features of quercetin and morin might have been responsible for the fact that the DNA damage by quercetin and morin was less than that by hesperetin and naringenin.
Although several scientists have shown that neither selenium glutathione peroxidase nor GSH transferase were capable of acting on peroxidized lipids in an organized membrane structure, it is well-known that these antioxidative enzymes play an important role in stabilizing lipid peroxidation in animal tissues, i.e., the removal of hydrogen peroxide by the selenium enzyme in vivo decreases the formation of hydroxyl radicals by the Fenton reaction and prevents one route for the initiation of peroxidation.22) Although the four flavonoids tested have been shown to be cytotoxic to lymphocytes, the question arises as to whether these four flavonoids are able to affect the activity of antioxidative enzymes. Thus, it is necessary to evaluate the actions of the four flavonoids on the activity of antioxidative enzymes such as glutathione reductase (GR) and glutathione transferase (GST). Elliott et al.27) have reported that polyphenolic compounds, especially flavonoids, formed a complex with proteins through hydrogen and covalent bonds, causing precipitation and/or enzyme inhibition. In addition, the ability of producing \( \ddot{O}^{2-} \), \( \text{H}_2\text{O}_2 \) and \( \cdot\text{OH} \) could have greater toxicological implications by inhibiting key antioxidative enzymes and producing toxic oxygen species, thereby promoting extensive oxidative stress in vivo. In the present study, the data in Fig. 3 show that morin and naringenin decreased the GR activity. This result suggests that morin and naringenin may directly react with GR to form an intermediate which, in turn, inhibited GR.27) Consequently, the DNA damage by morin and naringenin may have been due to the reduction of GR activity.

GST might detoxify and repair free radical damage to membrane lipids. In addition, GST has been shown to inhibit microsomal lipid peroxidation by reducing the free fatty acid hydroperoxides released from the peroxidized membrane by phospholipase A.28) GST has been proven to play an important role in detoxification, and to repair free radical damage to DNA; hence, it is essential to evaluate whether the GST activity was affected by the flavonoids. The data in Fig. 4 show that quercetin, morin, naringenin and hesperetin decreased the GST activity, suggesting that the ability of GST to detoxify and repair free radical damage to cell membranes was reduced which, in turn, caused the DNA damage.

Cell membrane protein thiols play a key part in the ability of cells to quench singlet oxygen.29) The decrease in the amount of protein thiols in the cells indicates a decrease in the antioxidative defense system. The data in Fig. 5 show that all the four flavonoids tested reduced the cell membrane protein thiols. This might have lowered the ability for quenching reactive oxygen species and free radicals, thus causing the DNA damage. In addition, the decrease in cell membrane protein thiols might have been due to the fact that flavonoids form a complex with proteins and change the cell membrane structure, thus affecting the biological system and causing DNA damage.

In conclusion, the pro-oxidative properties of quercetin, morin, naringenin and hesperetin appear to be dictated by the superoxide anion and hydrogen peroxides produced by autoxidation, causing DNA damage to human lymphocytes. In addition, the decrease in cell membrane protein thiols and the change of antioxidative enzyme activities might have resulted in DNA damage to human lymphocytes.

Acknowledgments

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