Protective Effects of Quercetin and Its Metabolites on H2O2-Induced Chromosomal Damage to WIL2-NS Cells

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We investigated chromosomal damage caused by a typical flavonoid, quercetin, and its two conjugates, quercetin-3-O-sulfate and isorhamnetin, and their protective effects against chromosomal damage induced by H2O2. The chromosomal damage was detected by the cytokinesis-block micronucleus (CBMN) assay using a lymphoblastoid cell line, WIL2-NS. We found that quercetin itself induced chromosomal damage at 10 μM, but quercetin-3-O-sulfate and isorhamnetin did not induce damage up to 30 μM. In the medium used for the CBMN assay, quercetin (at 100 μM) generated a high concentration of H2O2, but the two conjugates did not at the same concentration. On the other hand, pretreatment with quercetin (at 1 μM), quercetin-3-O-sulfate (at 10 μM), and isorhamnetin (at 5 μM) prevented H2O2-induced chromosomal damage to WIL2-NS cells. These findings suggest that the induction and prevention of H2O2-induced chromosomal damage are different between quercetin and its metabolites.

Key words: quercetin; quercetin-3-O-sulfate; isorhamnetin; WIL2-NS; cytokinesis-block micronucleus assay

Flavonoids are a large group of polyphenolic compounds providing much of the color and flavor of plant foods. Daily dietary intake of flavonoids was estimated to be 25 mg in the Netherlands by Hertog et al.1) Moreover, the mean intake of quercetin was estimated to be 12 mg/day in Denmark.2) The consumption of herbal extracts as supplemental foods has been increasing, then, there is a possibility of excess intake of flavonoid extract. Thus, it is important to confirm the benefit and protective effects against oxidative stress.3) However, some flavonoids such as quercetin, myricetin, and kaempferol have been shown to be cytotoxic and have DNA damaging ability in vitro.4–10) Furthermore, quercetin has been reported to be carcinogenic.11–13) Damage to DNA or chromosomes is important because the damage influences cell functions, resulting in various diseases, and aging.

Because many of these interesting observations about flavonoids were obtained in vitro, it is important to consider the physiological forms of the chemicals. Ueno et al. reported that the major flavonoid quercetin mainly metabolized to glucuronide and sulfate conjugate form in plasma after oral administration.14) It would be interesting to clarify whether the flavonoid metabolites have genotoxicity, carcinogenicity, and protective effects against reactive oxygen species (ROS). In the Ames assay, while quercetin had mutagenic activity with and without the S9 fraction, its metabolite 3′-O-methylquercetin did not show any mutagenicity.15) Shirai et al. used quercetin-3-O-β-D-glucuronide to clarify the antioxidant role of quercetin metabolites in cellular oxidative stress.16) But very few attempts have been made to measure the effects of the glucuronide or sulfate conjugate forms of flavonoids in vitro, because it is difficult to prepare these metabolites for experiments. Therefore, we first tried to examine quercetin, quercetin-3-O-sulfate, and isorhamnetin, which we could obtain commercially as reagents.

To study the chromosomal responses of these chemicals and ROS, it is necessary to use a system that is able to detect chromosomal aberrations very sensitively. Umegaki and Fenech developed the CBMN assay using WIL2-NS which is a human B lymphoblastoid cell line isolated from the spleen of a Caucasian male (American Type Culture Collection, 1992).17) This assay system is very sensitive to ROS-induced chromosomal damage such as that caused by activated neutrophils.

This study aimed to investigate whether quercetin aglycon and quercetin-3-O-sulfate and isorhamnetin, caused chromosomal damage in the CBMN assay system using WIL2-NS cells. In addition, on the basis

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Abbreviations: ATCC, American Type Culture Collection; B Ned, binucleated; BNC, binucleated cell; CBMN, cytokinesis-block micronucleus; Cyt-B, cytochalasin B; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HBSS, Hank’s balanced salt solution; H2O2, hydrogen peroxide; MN, micronucleus; M Ned BNC, micronucleated BNCed cell; NDI, nuclear division index; ROS, reactive oxygen species
of the report that phenolic compounds generated H$_2$O$_2$ in vitro, 18) we examined whether quercetin and the metabolites generated H$_2$O$_2$ under our CBMN assay conditions. Another aim of this study was to confirm whether preincubation of quercetin, quercetin-3-O-sulfate, and isorhamnetin protects against chromosomal damage caused by H$_2$O$_2$.

**Materials and Methods**

**Materials.** The chemicals used in this study were purchased from the following companies. RPMI-1640 medium, Hanks balanced salt solution (HBSS), fetal bovine serum (FBS) and antibiotics solution (5000 U/ml penicillin and 5 mg/ml streptomycin) were obtained from Gibco, BRL (Gaithersburg, MD, USA). Diff-Quik was purchased from International Reagent Corporation (Kobe, Japan) and H$_2$O$_2$ from Wako Pure Chemical Industries (Osaka, Japan). Horseradish peroxidase (Type II) was obtained from Sigma Chemical Co., Ltd. (Irvine, UK). The WIL2-NS (ATCC no. CRL8155) cell line was purchased from ATCC (Manassas, VA, USA). Quercetin, quercetin-3-O-sulfate, and isorhamnetin were obtained from Funakoshi (Tokyo, Japan). All other chemicals and solvents were of analytical grade or better.

**Cell culture, and treatments with flavonoids and H$_2$O$_2$.** WIL2-NS cells were cultured and maintained in RPMI-1640 medium containing 5% FBS, 1% antibiotic solution, and 1 mM glutamine at 37°C in a humidified atmosphere with 5% CO$_2$ (CO$_2$ incubator). One day before assay, the cells were seeded at a density of 0.3 x 10$^6$ cells/ml. On the assay day, the WIL2-NS cells were washed once with HBSS by centrifugation at 180 x g for 5 min, resuspended in HBSS at a density of 0.5 x 10$^6$ cells/ml, and used for the respective exposure studies. Cell suspensions (950 μl) were incubated for 30 min with various concentrations of flavonoids (in 50 μl) dissolved in HBSS. To evaluate the protective effects of quercetin and its metabolites on H$_2$O$_2$-chromosomal damage, the cells were washed twice with HBSS to eliminate these flavonoids outside of the cells, and then treated with H$_2$O$_2$.

**Cytokinesis-block micronucleus assay (CBMN assay).** The CBMN assay was done as described previously. 17) Briefly, immediately after the treatment with the flavonoid and/or H$_2$O$_2$, WIL2-NS cells were washed with HBSS and with RPMI-1640, then resuspended in RPMI-1640 medium containing 10% FBS, 1% antibiotic solution, 2 mM glutamine, and 4.44 μg/ml cyt-B at a cell density of 0.5 x 10$^6$ cells/ml. After 42 h of culture, the cells were harvested. Slides were prepared using a cytocentrifuge (Shandon Southern Products, Cheshire, UK). Before cytocentrifugation, DMSO was added at a final concentration of 5% to minimize clumping of the cells and thus optimize the recognition of cytoplasmic boundaries. The slides were air dried for 30 min and then fixed and stained using Diff-Quik.

**Micronuclei in binucleated (BNed) cells were scored using established criteria.** 19,20 A total of 500 viable cells (mononucleated, BNed, other multinucleated) were scored for the micronuclei. Chromosomal damage rates were expressed as the number of micronucleated binucleate cells (MNed BNC) per 1000 BNC. The nuclear division index was calculated by the formula suggested by Eastmond and Tucker21) using only viable cells to measure the ratios. In untreated WIL2-NS cells, 3–4% were not viable as assessed by trypan blue exclusion; these cells may have been spontaneously necrotic.

**Analysis of H$_2$O$_2$.** Production of H$_2$O$_2$ was evaluated using the phenol red method.22) Briefly, a reaction mixture (total 200 μl) consisting of flavonoids and/or H$_2$O$_2$ in HBSS was incubated for 30 min at 37°C. After the incubation, 50 μl of phenol red–horseradish peroxidase mixture (400 μg/ml of phenol red and 0.2 mg/ml of horseradish peroxidase) was added. Then 15 μl of 1 N NaOH was added to stop the reaction. The absorbance of the mixture was measured at 610 nm.

**Statistical analysis.** All assays were done in triplicate, i.e. for each dose tested, three cell suspensions were treated separately with flavonoids or H$_2$O$_2$ and each of these cell suspensions provided a separate culture for the CBMN assay. The results are presented as means±SEM for these triplicate experiments. Statistical analysis of data was done using ANOVA followed by a post hoc Fisher’s protected least significant difference test to find any significant differences. These analyses were done using a computer program (Stat View 4.5, SAS Institute Inc, Cary, NC, USA).

**Results**

**Chromosomal-damaging effects of quercetin and its metabolites**

To examine whether quercetin, quercetin-3-O-sulfate, and isorhamnetin themselves induced chromosomal damage in WIL2-NS cells, the cells were exposed to various concentrations of quercetin, quercetin-3-O-sulfate and isorhamnetin (5, 10 and 30 μM) for 30 min. The influence of the exposure on the parameters of the CBMN assay was determined (Table 1). The NDI value, a measure of cell division, was decreased by the treatment with flavonoid compared with the control ($p < 0.01$). The values for BNC (%), as index of cell ability and division, were not changed except with 5 μM quercetin-3-O-sulfate and 10 μM isorhamnetin (the exposure to isorhamnetin decreased the BNC value). When WIL2-NS cells were exposed to quercetin, chromosomal damage dose-dependently increased the value of MNed BNC, and significant increases were observed at 10 and 30 μM. In contrast, the exposure to quercetin-3-O-sulfate
followed by incubation at 37°C. sulfate, and isorhamnetin were added to RPMI-1640, O/C22 in the medium were measured. As shown in Table 2, BNC.

Generation of H₂O₂ on Addition of Quercetin, Quercetin-3-O-sulfate, and Isorhamnetin on HBSS

| Flavonoids              | NDI in viable cells | BNC (%) | MNI/1000 BNC
|------------------------|---------------------|---------|---------------
| Control                | 0                   | 71.4 ± 0.41 | 15.63 ± 3.89 |
| Quercetin              | 5                   | 72.7 ± 1.25 | 27.85 ± 5.24 |
|                        | 10                  | 72.2 ± 2.77 | 58.18 ± 9.16 |
|                        | 30                  | 73.6 ± 0.32 | 85.42 ± 5.87 |
| Quercetin-3-O-sulfate  | 5                   | 77.2 ± 0.58 | 11.68 ± 4.77 |
|                        | 10                  | 74.8 ± 2.58 | 22.39 ± 2.75 |
|                        | 30                  | 71.7 ± 0.58 | 29.36 ± 3.51 |
| Isorhamnetin           | 5                   | 70.5 ± 1.01 | 19.81 ± 2.72 |
|                        | 10                  | 66.4 ± 0.66 | 29.82 ± 6.45 |
|                        | 30                  | 69.7 ± 1.05 | 30.17 ± 5.22 |

Data represent the means ± SEM of three experimental determinations. Significantly different from control: **, p < 0.01, *, p < 0.05. Statistical analysis was by one-way analysis of variance followed by the least significant difference test, with significant difference at p < 0.01.

and isorhamnetin did not affect the number of MNed BNC.

Generation of H₂O₂ on addition of quercetin and its metabolites to RPMI-1640 medium

Various concentrations of quercetin, quercetin-3-O-sulfate, and isorhamnetin were added to RPMI-1640, followed by incubation at 37°C, and the levels of H₂O₂ in the medium were measured. As shown in Table 2, 10 μM H₂O₂ was generated in the medium incubated with 100 μM quercetin. However, quercetin-3-O-sulfate and isorhamnetin did not generate any. The same result applied to the examination using O₂ electrode (SUPER ORITECTER MODEL5, ORIENTAL YEAST, Tokyo, Japan) to measure H₂O₂.

Effect of the pretreatment of flavonoids on H₂O₂-induced chromosomal damage

WIL2-NS cells were pretreated with 1 μM quercetin, and quercetin-3-O-sulfate and isorhamnetin (1, 5, and 10 μM) for 1 h, then were exposed to 20 μM H₂O₂ for 30 min to examine the protective effects of the flavonoids against H₂O₂-induced chromosomal damage (Table 3). Pretreatment with either quercetin or its metabolites protected against these decreases in NDI and BNC (%) induced by H₂O₂. The NDI values after the pretreatment with quercetin-3-O-sulfate showed similar values to those of control cells.

Discussion

A considerable number of studies have been done on the metabolism of flavonoids. The typical flavonoid quercetin is present in foodstuff mainly as glycosides. The models of flavonoid absorption assumed that flavonoid glycosides were too polar to be absorbed from the small intestine and that the absorption was dependent on the cleavage of the β-glucoside linkage by colonic microflora. The question regarding the route of the flavonoid when are absorbed remains unsettled. Walle et al. (2000) found that both major glucosides of quercetin in the human, quercetin-monoglucoside and quercetin-diglucoside, are hydrolyzed in the human small intestine to the aglycon quercetin, 65–81% of which is then presumably absorbed. Many other researchers have confirmed that after eating foods containing quercetin glycoside or quercetin aglycon, quercetin presented as the glucuronide or sulfate form or further as methylated forms, these were conjugated to glucuronide or sulfate in plasma. And Senik et al. showed that quercetin glucuronides but not glucosides are present in plasma after administration of quercetin-3-glucoside or quercetin-4′-glucosides. While combination lipids and emulsifiers increased the absorption of orally administered quercetin in rats, free form quercetin or just methylated quercetins were not detected in the
plasma of these rats or human.\textsuperscript{20} It is important to clarify the safety and effectiveness of the flavonoids, but in most experiments on the genotoxicity or cell protection of flavonoids, the aglycon has been used.

Boulton \textit{et al.} found that quercetin was rapidly degraded along with the formation of numerous potentially biologically active products in the human hepatic cell line, Hep G2, and the human intestinal cell line, Caco-2.\textsuperscript{20} In these cell lines, UDP-glucuronosyltransferase (UGT) and sulfotransferase (PST) genes were clarified. The safety and effectiveness of the flavonoids, but in most experiments on the genotoxicity or cell protection of flavonoids, the aglycon has been used. In our present experiment similarly, 10 \textmu M of quercetin aglycon significantly induced chromosomal damage (\( p < 0.01 \)). On the other hand, pretreatment with quercetin (at 1 \textmu M) prevented H\textsubscript{2}O\textsubscript{2}-induced chromosomal damage to WIL2-NS cells. The reason why relatively lower concentration of quercetin induced DNA damage than former experiment may be that WIL2-NS cells are sensitive to reactive oxygen. The capacity of enzymes catabolizing reactive oxygen in this cell culture is unclear yet.

Long \textit{et al.} detected the generation of H\textsubscript{2}O\textsubscript{2} in various media with phenolic compounds added, and suggested those cellular effects of phenolic compounds \textit{in vitro} might be caused by H\textsubscript{2}O\textsubscript{2}.\textsuperscript{15} Other researchers demonstrated that the H\textsubscript{2}O\textsubscript{2} generation was closely related to the DNA-damaging efficiency of quercetin. In this study, using a photometric determination method with horseradish peroxidase, we examined whether quercetin, quercetin-3'-sulfate, and isorhamnetin generated H\textsubscript{2}O\textsubscript{2} in the same medium as in the CBMN assay. HBSS medium incubated with 100 \textmu M of quercetin for 30 min generated 10 \textmu M of H\textsubscript{2}O\textsubscript{2} (Table 2). While 10 \textmu M H\textsubscript{2}O\textsubscript{2} is enough to induce chromosomal damage in WIL2-NS cells, 30 \textmu M quercetin seemed to increase the value of MNed/1000 cells more than 10 \textmu M H\textsubscript{2}O\textsubscript{2} (data not shown). In addition, because 1 \textmu M of quercetin prevents chromosomal damage induced by 20 \textmu M of H\textsubscript{2}O\textsubscript{2} (Table 3), it seems quite probable that 30 \textmu M of quercetin generates far lower than 10 \textmu M of H\textsubscript{2}O\textsubscript{2}. But 30 \textmu M of quercetin induced larger number of chromosomal damages than this estimation (Table 2). H\textsubscript{2}O\textsubscript{2} is thought to react with metal ions, such as iron and copper, in the nucleus, and as a result of these reactions, free radicals (mainly the hydroxyl radical) are generated by the Fenton reaction close to DNA, resulting in chromosomal damage. The fact that quercetin itself generated H\textsubscript{2}O\textsubscript{2} deserves careful attention. The generation of H\textsubscript{2}O\textsubscript{2} seems not to be a main reason of chromosomal damage induced by quercetin. H\textsubscript{2}O\textsubscript{2} may be a byproduct rather than a cause of DNA damage by quercetin. Besides, the results indicated that the addition

<table>
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<th>H\textsubscript{2}O\textsubscript{2} (\textmu M)</th>
<th>Flavonoids pretreated (\textmu M)</th>
<th>NDI in viable cells</th>
<th>BNC (%)</th>
<th>MN/1000 BNcells</th>
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<tr>
<td>20</td>
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<td>Quercetin-3-O-sulfate</td>
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<td>114.08 ± 17.55</td>
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<td>20</td>
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<tr>
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<td>2.07 ± 0.05</td>
<td>70.3 ± 1.16</td>
<td>9.31 ± 1.16</td>
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</table>

Data represent the means±SEM of three experimental determinations. Significantly different from cells exposed 20 \mu M H\textsubscript{2}O\textsubscript{2}; *\( p < 0.01 \). Statistical analysis was by one-way analysis of variance followed by the least significant difference test, with significant difference at \( p < 0.05 \).
of quercetin-3-0-sulfate and isorhamnetin and incubation in HBSS did not generate H2O2. As one of mechanisms of oxidative DNA damage induced by quercetin in the presence of Cu(I), Yamashita et al. presented the reaction of DNA-Cu(I) and H2O2 generating from O2− causes oxidative DNA-damage via DNA-Cu(II)/OOH.9,33) On this idea, it is essential for an effective redox reaction that quercetin aglycon have both OH in positions of 3 and 3′ of its catechol structure. The result of this study supported the requirement of OH in its position.

Now, as for a protective effect of quercetin against chromosomal damage and cytotoxicity by H2O2, to compare quercetin, isorhamnetin, and quercetin-3-0-sulfate in CBMN assay, WIL2-NS cells were preincubated with these reagents, before exposure to H2O2. Interestingly, in our present study, preincubation of quercetin-3-0-sulfate and isorhamnetin protected from chromosomal damage of WIL2-NS caused by H2O2, while quercetin-3-0-sulfate and isorhamnetin did not induce chromosomal damage. These effects were lower than quercetin. While Ratty and Das demonstrated that the presence of polyhydroxylated substitutions on ring A and B of flavonoid with a 2,3-double bond, a free 3-hydroxyl substitution, and a 4-keto group confer potent antioxidant effects of flavonoid on nonenzymatic lipid peroxidation.34) According to several researchers, it is responsible for free radical scavenging ability that flavonoids have a catechol group in B ring (just with O-dihydroxyl structure at 3′- and 4′-position). Quercetin 3-O-sulfate has catecol group for radical-scavenging effect, but at the 3-position in C-ring, it lacks a free hydroxyl group. Also, isorhamnetin possess only one free hydroxyl group at 3-position. To make clear the mechanism of chromosomal damaging or protective effects of quercetin, in future, metabolism of quercetin in this cell must be examined. Moon et al. showed that the plasma quercetin level can be maintained in the range of 10−7−10−6 M by periodic ingestion of 100–200 g of onion/day, although the level of quercetin in the plasma may be transiently elevated for a few hours after the intake.15) Hollman et al. reported that the concentration of quercetin was 0.6 μM in human plasma after ingestion of 150 g of fried onions containing the equivalent of 64 mg of quercetin glycoside.36) Regular intake of quercetin by supplement is expected not to be excess concentration in plasma. In addition, these observations on present study support the view that quercetin has antioxidative effects, and furthermore, protective effects against chromosomal damage in vivo.

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