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Protection by αG-Rutin, a Water-soluble Antioxidant Flavonoid, against Renal Damage in Mice Treated with Ferric Nitrilotriacetate

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The protective effect of αG-Rutin against ferric nitrilotriacetate (Fe-NTA)-induced renal damage was studied in male ICR mice. Fe-NTA induces renal lipid peroxidation, leading to a high incidence of renal cell carcinoma in rodents. Administration of αG-Rutin (50 μmol as rutin/kg) by gastric intubation 30 min after i.p. injection of Fe-NTA (7 mg Fe/kg) most effectively suppressed renal lipid peroxidation. Repeated i.p. injection of Fe-NTA (2 mg Fe/kg/day for the first 3 days and 3 mg Fe/kg/day for 12 days, 5 days a week) causes subacute nephrotoxicity as revealed by induction of karyomegalic cells in renal proximal tubules. A protective effect was observed in mice given αG-Rutin 30 min after each Fe-NTA treatment. To elucidate the mechanism of protection by αG-Rutin, the pharmacokinetics and hydroxyl radical-scavenging effect of αG-Rutin were investigated by HPLC analysis and by electron spin resonance (ESR) spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), respectively. When mice were given αG-Rutin (50 μmol as rutin/kg) by gastric intubation, rapid absorption into the circulation was observed. The plasma concentration of αG-Rutin reached the highest level 30 min after oral administration and then decreased to the control level within 60 min. αG-Rutin inhibited the formation of DMPO-OH in a concentration-dependent manner. Further, chelating activity of αG-Rutin to ferric ions was shown by spectrophotometric analysis. These results suggest that absorbed αG-Rutin works as an antioxidant *in vivo* either by scavenging reactive oxygen species or by chelating ferric ions and this serves to prevent oxidative renal damage in mice treated with Fe-NTA.

Key words: Flavonoid — Antioxidant — αG-Rutin — Ferric nitrilotriacetate — Renal damage

Nitrilotriacetic acid (NTA) is a synthetic aminopolycarboxylic acid that efficiently forms water-soluble chelate complexes with metal cations and has been used as a detergent builder in the U. S., Canada, and Europe. An experimental model of hemochromatosis was developed by using Fe-NTA.[1] It has been demonstrated that Fe-NTA induces acute and subacute renal proximal tubular necrosis followed by a high incidence (60–92%) of renal cell carcinoma in both rats and mice.[2–6] Fe-NTA in the presence of a reductant can induce DNA strand breaks *in vitro* as a result of generation of reactive oxygen species such as hydroxyl radicals.[7,8] It has been reported that intraperitoneal administration of Fe-NTA generates 8-hydroxydeoxyguanosine in rat kidney DNA[9,10] and produces 4-hydroxy-2-nonenal (HNE), an α,β-unsaturated aldehyde, in the rat renal proximal tubules through lipid peroxidation.[11] These results suggest that reactive oxygen species are involved in Fe-NTA-induced renal carcinogenesis. In humans, iron overload has been reported to be associated with an increased risk of cancer and increased overall death rate.[12] Recently, iron-induced carcinogenesis has been reviewed by Toyokuni[13] and Okada.[14]

Several antioxidants such as vitamin E,[15] glutathione,[16] probucol,[17] 2-mercaptoethane sulfonate and *N*-acetylcysteine[18] have been reported to show protective effects against Fe-NTA-induced nephrotoxicity. Flavonoids, which are widely distributed in the plant kingdom, are known to possess a wide range of biological properties such as anti-inflammatory and anticarcinogenic effects, and are considered as candidate chemopreventive or therapeutic agents against free radical-associated diseases.[19] Numerous *in vitro* studies of flavonoids have demonstrated that they are potential antioxidants and their antioxidant activity is due to their ability to scavenge free radicals and to chelate metal ions.[20–22]

αG-Rutin, which is formed by enzymatic transglycosylation,[23] has been used as an antioxidant and a colorant for processed foods in Japan, and contains mainly 4^G-α-D-glucopyranosylrutin and a small amount of isoquercitrin.[24] The equivalent ratio of quercetin glycoside as rutin in αG-Rutin is 80%. Therefore, the molar concentration of αG-Rutin is expressed as that of rutin in this study. The chemical structure of 4^G-α-D-glucopyranosylrutin, the main component of αG-Rutin, is shown in Fig. 1. Our previous study demonstrated that αG-Rutin inhibited lipid peroxidation induced by Fe-NTA/H_2O_2 and

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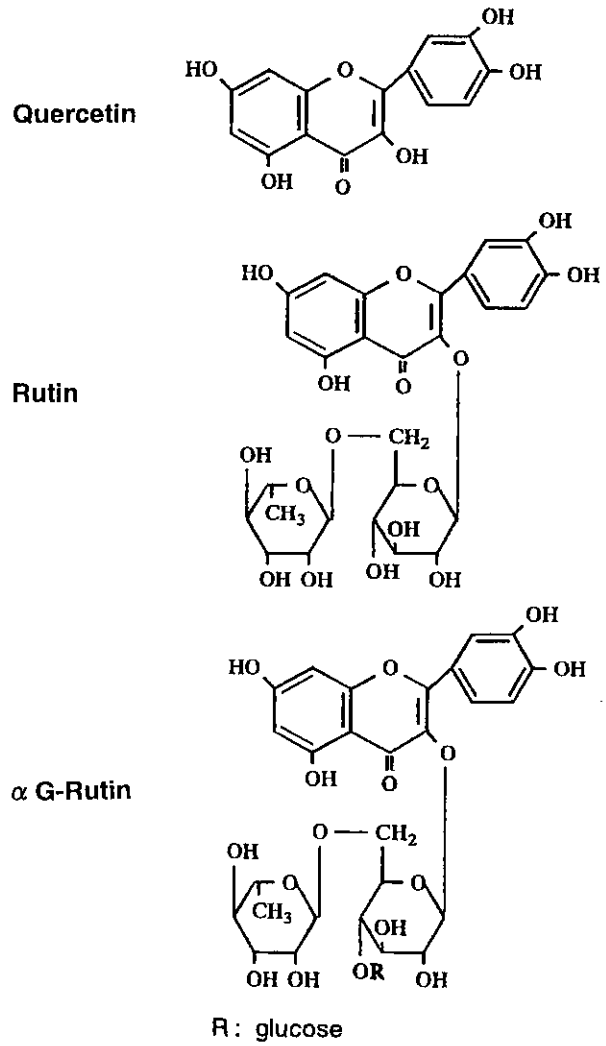


Fig. 1. Chemical structures of quercetin, rutin, and αG-Rutin (4^G-α-D-glucopyranosylrutin).

Fe^{2+}/H_2O_2 in a concentration-dependent manner and that preadministration of αG-Rutin by gastric intubation 30 min before γ irradiation reduced the frequency of micronucleated reticulocytes (MNRETs) in mouse peripheral blood.[25] It is known that γ-rays generate hydroxyl radicals in cells and induce DNA damage which leads to mutations and chromosomal aberrations.[26,27]

In the present study, the protective effect of αG-Rutin on renal oxidative damage in male ICR mice exposed to Fe-NTA was examined in order to evaluate the *in vivo* antioxidant and preventive activity of flavonoids. The association between the plasma level of αG-Rutin and antioxidant activity in mice treated with Fe-NTA, and the mechanism by which this water-soluble flavonoid works *in vivo* are discussed.

MATERIALS AND METHODS

Chemicals αG-Rutin was a kind gift from Toyo Sugar Refining Co., Ltd., Tokyo. Quercetin was from Funakoshi Co., Tokyo. α-Tocopherol was from Merck AG, Darmstadt, Germany. For Fe-NTA solution, ferric nitrate enneahydrate was purchased from Wako Pure Chem. Ind. Ltd., Osaka and nitrilotriacetic acid disodium salt from Nacalai Tesque, Kyoto. Other chemicals used were from Wako.

Animals and housing conditions Male ICR mice (9–10 weeks old, Japan SLC Inc., Hamamatsu) were housed in an air-conditioned room and fed a synthetic basic diet which consists of 38% corn starch, 25% casein, 10% α starch, 8% cellulose powder, 6% minerals, 5% sugar, 2% vitamins and 6% lard instead of corn oil (Oriental Yeast Co., Tokyo) *ad libitum* throughout the experiments. Five to six mice were assigned to each experimental group.

HPLC analysis of flavonoids in mouse plasma Mice were anesthetized with ethyl ether at different times after administration of αG-Rutin (50 μmol as rutin/kg) by gastric intubation and blood was drawn from the abdominal aorta. Mixtures of 0.5 ml of 0.05 *M* oxalic acid and 0.5 ml of plasma samples were applied to Sep-Pak C_{18} cartridges. These were washed with 0.05 *M* oxalic acid and methanol/water/0.5 *M* oxalic acid (25 : 73 : 2, v/v/v), then flavonoids were eluted with methanol, and the eluates were evaporated to dryness. The residue was dissolved in 100 μl of methanol, and analyzed on a JASCO HPLC system (Tokyo) using a Capcell Pak C_{18}-UG120 column (150×4.6 mm I.D., 5 mm particle size, Shiseido, Tokyo) with methanol/water/0.5 *M* oxalic acid (40 : 60 : 2, v/v/v) as the mobile phase and UV detection (372 nm). The column temperature was maintained at 50°C and the flow rate was 1.5 ml/min. This HPLC analysis was carried out by the method of Terada and Miyabe[28] with some modifications. Quantification of absorbed αG-Rutin was done by measuring peak areas based on calibration plots of the peak area of standard αG-Rutin at various concentrations.

Treatment with Fe-NTA The Fe-NTA solution was prepared just before use as previously described.[11] Briefly, 300 m*M* ferric nitrate enneahydrate and 600 m*M* NTA were mixed at the volume ratio of 1 : 2 (molar ratio, 1 : 4) and the pH was adjusted with sodium hydrogen carbonate to 7.4. In the first experiment on acute nephrotoxicity, the mice were given αG-Rutin by gastric intubation (50 μmol/kg) at different times before or after i.p. injection of Fe-NTA at a dose of 7 mg Fe/kg according to the administration protocol shown in Fig. 2. In the second experiment on subacute nephrotoxicity, the mice were divided into three groups that were given Fe-NTA (10 mice), Fe-NTA and αG-Rutin (10 mice), or no

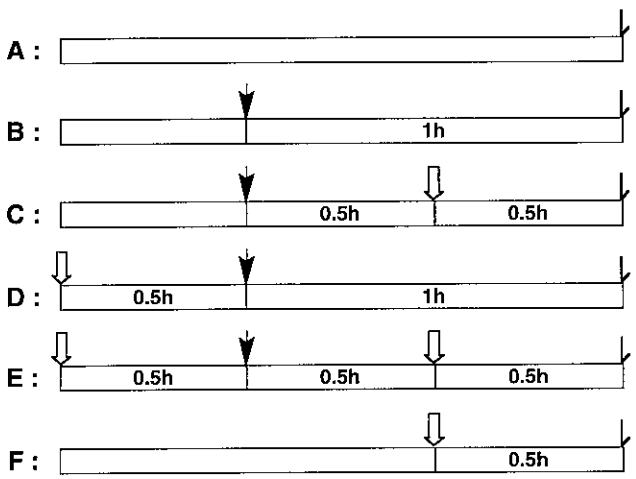


Fig. 2. Administration protocol of Fe-NTA and αG-Rutin. Fe-NTA (↓), 7 mg Fe/kg i.p. injection; αG-Rutin (⇩), 50 μmol as rutin/kg gastric intubation; measurement of TBARS (↓).

treatment (5 mice). The Fe-NTA dose was 2 mg Fe/kg/day for the first 3 days and 3 mg Fe/kg/day for 12 days (5 days a week). The mice were given repeated i.p. injection of Fe-NTA and gastric intubation of αG-Rutin (50 μmol/kg) 30 min after each Fe-NTA treatment. For histological observations, the mice were anesthetized with ethyl ether 1 h after the last treatment with Fe-NTA and the kidneys were immediately removed. Each kidney was rinsed with ice-cold physiological saline solution and fixed in 10% buffered formalin. Tissues were processed and stained with hematoxylin and eosin. The histology was classified into four categories (++, +, +−, −) according to the abundance of regenerative renal proximal tubular cells with karyomegalic cells, as follows: abundant (++), diffuse proximal tubular regeneration or more than 25 karyomegalic cells in two coronal sections of the kidney; scarce (+), 5 to 25 karyomegalic cells in two coronal sections of the kidney; equivocal (+−), no more than 5 karyomegalic cells in two coronal sections of the kidney; none (−), normal kidney.

Measurement of renal thiobarbituric acid-reactive substances (TBARS) Mice were anesthetized with ethyl ether 1 h after the treatment with Fe-NTA (7 mg Fe/kg). The left kidney of each mouse was removed and chilled in ice-cold physiological saline solution. Each kidney was rinsed with the saline solution, and homogenized with 40 m*M* phosphate buffer (pH 7.4). The tissue TBARS content was determined by the fluorometric method of Yagi[29] with some modifications. TBARS were expressed as malondialdehyde (MDA) equivalents. Protein determination was performed by the bicinchoninic acid method.[30]

Statistical analysis Statistical analyses were done by one-way ANOVA and Duncan's multiple range tests. The differences between data marked with different letters as superscripts are statistically significant.

Electron-spin resonance (ESR) spectral measurement ESR spectra were measured at room temperature on an ESR spectrometer (JEOL X-Band Microwave JES-RE3X) according to the method of Inoue and Kawanishi.[7] 5,5′-Dimethyl-1-pyrroline-1-oxide (DMPO, Labotec) was used as the hydroxyl radical trapping agent. Fe-NTA (10 m*M* as Fe) 10 μl, 5.5 m*M* diethylenetriaminepentaacetic acid (DETAPAC, Sigma) 60 μl, DMPO 10 μl, and αG-Rutin (0–20 m*M*) 10 μl were well mixed in 60 m*M* sodium phosphate buffer (pH 7.0). After addition of 10 m*M* H_2O_2, aliquots of the reaction mixture were taken in a calibrated capillary and ESR spectra were measured immediately.

UV absorption analysis Five microliters of αG-Rutin (100 m*M* as rutin) or quercetin (100 m*M*) was added to 3 ml of phosphate buffer (100 m*M*, pH 7.4) and the mixture was kept at room temperature for 10 min. Then, UV absorption was measured with a U-3210 spectrophotometer (Hitachi). Absorption spectra were recorded again 10 min after adding 2.5 μl of Fe-NTA solution (100 m*M* as Fe) to the mixture, to allow the formation of the chelate complex.

RESULTS

The chemical structures of αG-Rutin (4^G-α-D-glucopyranosylrutin), rutin and quercetin are shown in Fig. 1. In order to evaluate the *in vivo* antioxidant activity of αG-Rutin, it is important to investigate the fate of αG-Rutin in mice after oral administration. Therefore, the level of αG-Rutin in mouse plasma after administration by gastric intubation (50 μmol as rutin/kg) was examined by HPLC analysis. The peak at a retention time of 2.5 min corresponded with that of standard αG-Rutin. As shown in Fig. 3A, the concentration of αG-Rutin in mouse plasma increased to the highest level 30 min after administration by gastric intubation and decreased to an undetectable level within 60 min. The plasma level of αG-Rutin did not increase any further when mice were given more than 50 μmol as rutin/kg of αG-Rutin (Fig. 3B). Calculating the concentration of this peak as rutin, the plasma level 30 min after administration of αG-Rutin (50 μmol as rutin/kg) by gastric intubation was about 4 μg/ml (6.7 μmol/liter). Quercetin was also detected at a retention time of 6.9 min in plasma obtained 30 min after dosing. αG-Rutin might be hydrolyzed, in part, by intestinal microbacteria before intestinal absorption. These results demonstrated that αG-Rutin was absorbed into the circulation system very rapidly.

As shown in Table I, renal TBARS was increased by Fe-NTA treatment (7 mg Fe/kg, group B), as previously

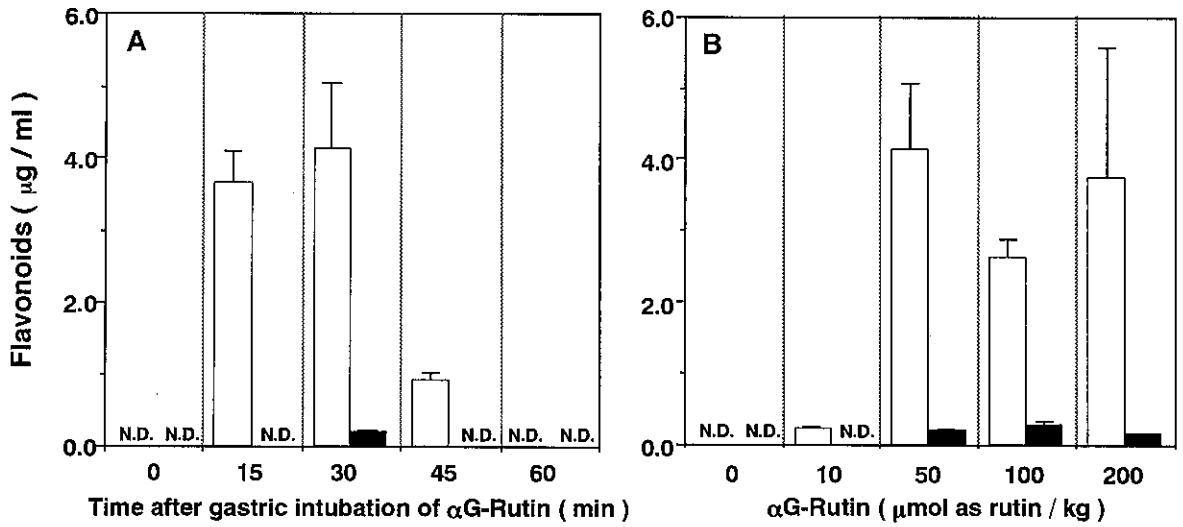


Fig. 3. A, Time course of plasma level of αG-Rutin. Mice were anesthetized with ethyl ether at different times after administration of αG-Rutin (50 μmol as rutin/kg) by gastric intubation and blood was then drawn from the abdominal aorta. The plasma samples were extracted by using Sep-Pak C_{18} cartridges. Column, Capcell Pak C_{18}-UG120; column temperature, 50°C; mobile phase, MeOH/H_2O/0.5 *M* oxalic acid (40 : 60 : 2, v/v/v); flow rate, 1.5 ml/min; injection volume, 10 μl; detection, UV 372 nm. B, Plasma level of αG-Rutin 30 min after administration of αG-Rutin at different doses (0–200 μmol as rutin/kg) by gastric intubation. Further details are as described in (A). □, αG-Rutin; ■, quercetin; N.D., not detected. Each value is the mean± SE ($n=5$).

Table I. Effect of αG-Rutin on Renal TBARS Induced by Fe-NTA at Different Administration Times

Group	TBARS (nmol MDA/mg protein)
A	$1.19\pm0.15^{a)}$
B	$2.80\pm0.47^{b)}$
C	$2.08\pm0.38^{c)}$
D	$2.96\pm0.47^{b)}$
E	$2.14\pm0.41^{c)}$
F	$1.12\pm0.11^{a)}$

a, b, c), Values (mean±SD) with different letters as superscripts are significantly different ($P<0.05$, $n=5$).
Administration schedule is shown in Fig. 2.
The mice were anesthetized with ethyl ether 1 h after the treatment with Fe-NTA (7 mg Fe/kg) and the left kidney was removed.

reported.[6,31] When mice were given αG-Rutin (50 μmol as rutin/kg) 30 min after i.p. injection of Fe-NTA (groups C and E), TBARS content was significantly decreased.

The effect of αG-Rutin on subacute nephrotoxicity by Fe-NTA was investigated. Fig. 4 shows typical histological observations of the renal proximal tubules of mice treated with or without Fe-NTA. Karyomegalic cells were observed in the renal proximal tubules of mice treated with Fe-NTA. Though karyomegalic cells were also observed in mice given αG-Rutin 30 min after each Fe-NTA treatment, their number was less than in mice given tap water (Table II). There was a difference in histology between mice given tap water and those given αG-Rutin (Fig. 4, Table II).

Fig. 5 shows ESR spectra of the hydroxyl radical spin adduct of DMPO generated from hydrogen peroxide and Fe-NTA in the presence of αG-Rutin at various concentrations. αG-Rutin inhibited the formation of DMPO-OH in a dose-dependent manner. In the UV absorption analysis, a shift of the peak at 367 nm (αG-Rutin) or 378 nm (quercetin) to the long-wave region was observed (Fig. 6). This shift indicated that αG-Rutin formed a chelate complex with ferric ions.

DISCUSSION

It has been reported that flavonoids act as strong antioxidants.[20–22] However, it has not been clear whether this is the case *in vivo*. In our previous study, we demonstrated that preadministration of flavonoids reduced the frequency of MNRETs in peripheral blood and suppressed lipid peroxidation in bone marrow and spleen when mice were exposed to γ-rays.[32] A good correlation ($r=0.717$) was observed between antioxidant activity (TBARS, OD 532 nm) against lipid peroxidation in-

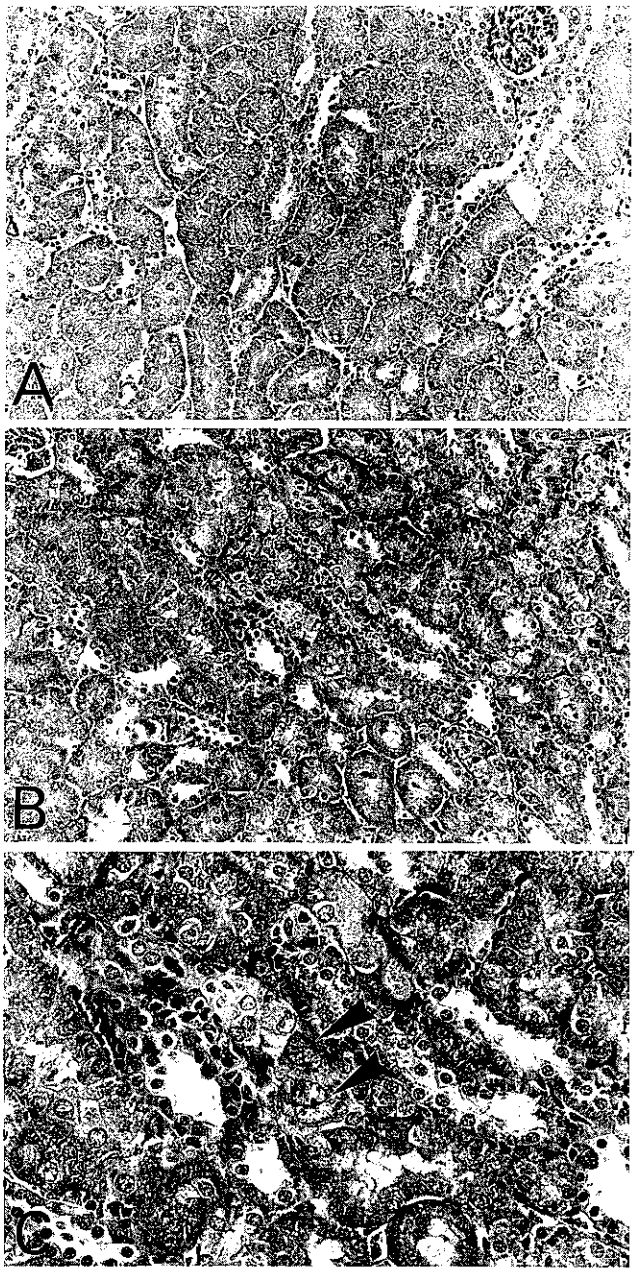


Fig. 4. Subacute nephrotoxicity in renal proximal tubules of mice treated with or without Fe-NTA. Mice were given repeated i.p. injection of Fe-NTA. The Fe-NTA dose was 2 mg Fe/kg/day for the first 3 days and 3 mg Fe/kg/day for 12 days (5 days a week). For histological observations, the mice were anesthetized with ethyl ether 1 h after the last treatment with Fe-NTA and the kidneys were immediately removed. Each kidney was rinsed with ice-cold physiological saline solution, then fixed in 10% buffered formalin and tissues were processed and stained with hematoxylin and eosin. A, Control kidney (×257); B, Diffuse proximal tubular regeneration. Note that most of the proximal tubular cells have nuclei with denser chromatin and more basophilic cytoplasm (×257), C, Karyomegalic cells. Note three bizarre giant cells with an extremely large nucleus (arrows, ×514).

duced by Fenton's reagent (Fe^{2+}/H_2O_2) and anticlastogenic activity (MNRETs, %) of 12 flavonoids.[33] In order to evaluate *in vivo* antioxidant action of flavonoids, it is important to investigate the fate of flavonoids *in vivo* after administration. Therefore, we first focused on the absorption of flavonoids.

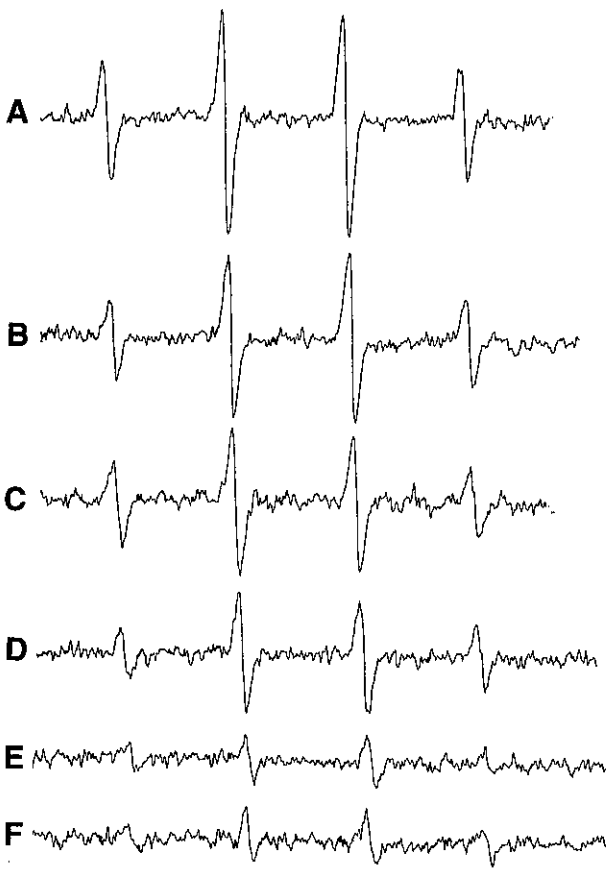
αG-Rutin was selected because of its water solubility, although it is not a naturally occurring flavonoid. We have already reported that αG-Rutin inhibited lipid peroxidation induced by Fe-NTA/H_2O_2 or Fe^{2+}/H_2O_2 in a concentration-dependent manner and that preadministration of αG-Rutin by gastric intubation 30 min before γ irradiation reduced the frequency of MNRETs in mouse peripheral blood cells.[25] HPLC analysis showed that αG-Rutin was efficiently absorbed and disappeared rapidly from plasma. The highest peak (retention time, 2.5 min) was observed 30 min after dosing (Fig. 3A).

Intraperitoneally injected Fe-NTA is absorbed into the portal vein through the mesothelium and passes into the circulation via the liver. Some fraction of Fe-NTA is filtered through the glomeruli into the lumen of the renal proximal tubules, where the glutathione degradation product cysteine is produced by γ-glutamyltranspeptidase and a nonspecific radical scavenger, albumin, is absent. Therefore, it is considered that Fe^{3+}-NTA is reduced by cysteine to Fe^{2+}-NTA, a Fenton-like reaction easily takes place, and reactive oxygen species are generated there.[13] This action mechanism is supported by the experimental findings of an increase in renal TBARS,[6,31] the presence

Table II. Effect of αG-Rutin on Subacute Nephrotoxicity as Revealed by Induction of Karyomegalic Cells in Renal Proximal Tubules of Mice Treated with Fe-NTA

Group	Treatment[a]	No. of mice used	No. of deaths in 3 weeks	Karyomegalic cells[b,c]			
				++	+	+−	−
A	Fe-NTA, Tap water	10	2	3	4	1	0
B	Fe-NTA, αG-Rutin	10	1	2	1	3	3
C	Control	5	0	0	0	0	5

a) Fe-NTA, Repeated i.p. injection (2 mg Fe/kg/day for the first 3 days and 3 mg Fe/kg/day for 12 days, 5 days a week). αG-Rutin, Gastric intubation (50 μmol as rutin/kg) 30 min after each Fe-NTA treatment.
b) Mice were anesthetized with ethyl ether 1 h after the last Fe-NTA treatment. The kidneys were immediately removed.
c) ++, diffuse proximal tubular regeneration, or more than 25 karyomegalic cells in two coronal sections of the kidney.
+, 5 to 25 karyomegalic cells in two coronal sections of the kidney.
+−, No more than 5 karyomegalic cells in two coronal sections of the kidney.
−, Normal kidney.



of HNE in the proximal tubules,[11] and formation of 8-hydroxydeoxyguanosine in kidney DNA.[9,10] Renal TBARS content has been reported to increase and to reach the highest level 1 h after the i.p. injection of Fe-NTA.[6,31] When mice received αG-Rutin by single gastric intubation 30 min after i.p. injection of Fe-NTA, protective effects against lipid peroxidation were observed in mice (Table I). These results suggest that the antioxidant activity of αG-Rutin in mice is associated with its plasma level. It is of note that αG-Rutin works as an antioxidant even after Fe-NTA administration. Therefore, there is a possibility that αG-Rutin could be used as an antidote for acute intoxication with certain transition metals, including iron.

To find a clue to the antioxidant mechanism of αG-Rutin, its hydroxyl radical-scavenging effect was investigated by ESR spin trapping with DMPO. αG-Rutin

Fig. 5. ESR spectra of DMPO-OH produced in the reaction system of hydrogen peroxide and Fe^{3+}-NTA with various concentrations of αG-Rutin. The reaction mixture contained 10 μl of Fe-NTA (0.01 *M* as Fe), 60 μl of diethylenetriaminepentaacetic acid (DETAPAC) (5.5 m*M*), 10 μl of DMPO, 10 μl of αG-Rutin (0–80 m*M*), and 10 μl of H_2O_2 (10 m*M*) in 60m*M* sodium phosphate buffer (pH 7.0). All spectra were recorded at room temperature. αG-Rutin (m*M*): A (0), B (0.025), C (0.125), D (0.5), E (2), F (8).

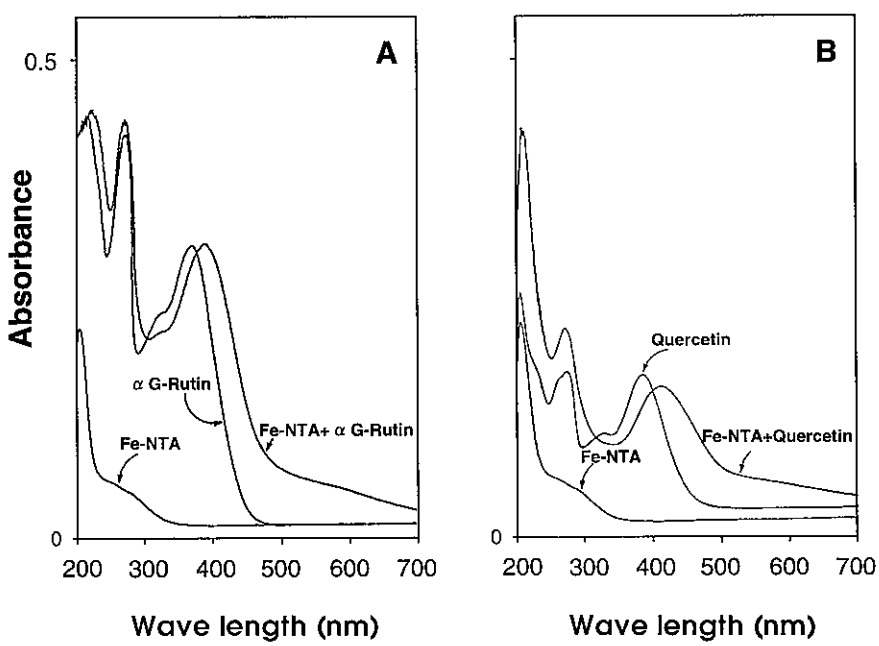


Fig. 6. Absorption spectra of flavonoids, Fe-NTA and their mixtures. Concentrations of flavonoids and Fe-NTA were 0.166 m*M* and 0.083 m*M*, respectively. A, αG-Rutin; B, quercetin.

inhibited the formation of DMPO-OH in a concentration-dependent manner (Fig. 5). On the other hand, chelating ability of rutin to form complexes with iron ions has been reported.[34] Fig. 6 shows the chelating ability of αG-Rutin with ferric ion. These results indicate that the absorbed αG-Rutin may either scavenge reactive oxygen species induced by Fe-NTA or form ferric ion-αG-Rutin chelate complexes which do not have pro-oxidant activity, in renal proximal tubules.

In the present study, we obtained evidence of very rapid absorption of αG-Rutin and its transient existence in mouse plasma. In our previous study, we examined the effect of αG-Rutin on subacute toxicity by Fe-NTA. When mice were treated with repeated i.p. injections of Fe-NTA and tap water, only one mouse remained alive on the 19th day. However, survival was much better when mice were given αG-Rutin solution (3.3 m*M*) as drinking water *ad libitum*.[25] These results suggest that the maintenance of plasma level of αG-Rutin might improve the antioxidant status *in vivo*. We also found that the number of karyomegalic cells, reflecting induction of morphological changes, observed in the proximal renal tubules of mice given i.p. injection of Fe-NTA for 19 days was less in mice given αG-Rutin than in mice given tap water (Table II). Taking into consideration that reactive oxygen species are involved in carcinogenesis, the absorbed αG-Rutin may play a role as a cancer chemopreventive agent.

Some flavonoids (for example, quercetin) have been reported to be mutagenic and clastogenic in bacteria and mammalian cells,[35,36] but not carcinogenic in rodents.[37] It has been suggested that lack of carcinogenicity of quercetin is due to its poor absorption from intestinal tract[38] and functional properties such as antiproliferative activity towards tumor cells.[39] We showed that αG-Rutin at the plasma level of 4 μg/ml exerts antioxidant activity in Fe-NTA treated mice. Therefore, further investigations to elucidate the daily intake, absorption and metabolism of plant flavonoids are required in order to establish whether they are useful antioxidants and chemopreventive food factors against free radical-associated diseases such as cancer in humans.

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