Dietary Supplement of G-Rutin Reduces Oxidative Damage in the Rodent Model

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Dietary supplement of bioflavonoid rutin and calorie restriction were examined to investigate possible reduction in oxidative DNA damage, protein oxidation, and lipid peroxidation in animals. Rats were fed ad libitum 20% casein semipurified diet or a diet that was supplemented by 2.5% water soluble rutin derivative, 4^G-α-glucopyranosylrutin (G-rutin) for 18 days. Two other groups of rats were fed the respective diets at 60% of the mean food intake of the ad libitum fed animals for the same period. Urinary excretion of thymine glycol and thymidine glycol, indices of DNA base damage in the whole body, were significantly low in the G-rutin-supplemented groups. The protein carbonyl contents, a measure of protein oxidation, were significantly low in the liver of G-rutin-supplemented groups and calorie-restricted groups. The data indicate that G-rutin provides an antioxidant defense in rodents against free radical-caused oxidative damage of DNA and proteins.

Keywords: *Flavonoid; rutin; calorie restriction; thymine glycol; protein carbonyl content*

INTRODUCTION

Oxygen radicals are formed in vivo during normal aerobic metabolism and cause oxidative damage to DNA, protein, and unsaturated fats despite natural defense systems (superoxide dismutase, catalase, glutathione peroxidase) within the organism. The accumulation of unrepaired damage products may be related to the processes of aging and degenerative diseases. Oxidative damage to DNA, in particular, could be an important and critical factor in cytotoxicity and mutation leading to aging and cancer. Besides the endogenous enzymatic antioxidant defense system, there are exogenous nonenzymatic antioxidative mechanisms such as certain vitamins (α-tocopherol, β-carotene, and ascorbic acid) that protect against oxygen damage (Cathcart et al., 1984). Moreover, a diet rich in vegetables and fruit contains specific compounds that exhibit antioxidant activities (Ames, 1983; Saija et al., 1995). Simic and Bergtold (1991) showed that a diet rich in fruit and vegetables can reduce oxidative DNA-base damage in humans by measuring daily excretion of thymidine glycol (dTg) and 8-hydroxydeoxyguanosine in urine as biomarkers of oxidative DNA-base damage. A diet rich in vegetables contains several phenolic substances (flavonoid and nonflavonoid phenolic substances including catechins and others) in an appreciable amount, and the daily intake level of flavonoids is close to 1 g in the average human diet (Ames, 1983). Rutin (quercetin-3-rutinoside) is a bioflavonoid that was suggested as a potent radical scavenger (Negre-Salvayre, 1991; Grinberg et al., 1994; Saija et al., 1995; Haenen et al., 1997).

The present study was designed to determine whether dietary manipulation, especially supplementation of 4^G-α-D-glucopyranosylrutin (G-rutin), could reduce the levels of oxidative DNA-base damage by measuring urinary output of thymine glycol (Tg) and dTg in an animal model, an important experimental step to protect against oxidative DNA damage. G-rutin, which is formed by enzymatic transglycosylation (Suzuki and Suzuki, 1991), has been used as a food additive as an antioxidant and a colorant for processed foods because of its higher water solubility than that of rutin. Effects of G-rutin and calorie restriction on the accumulation of oxidized protein in liver and lipid peroxidation in plasma were also examined. Oxidative damage to protein in the liver of rats associated with aging was shown to be reduced by dietary restriction of protein or calories (Youngman et al., 1992).

We developed a new and simple method to measure Tgs (Tg plus dTg). It offers some advantages over the previous method (Cathcart et al., 1984; Bergtold et al., 1990) with respect to simplicity and rapidity. We report here that dietary rutin markedly inhibited the accumulation of oxidatively damaged DNA and proteins.

MATERIALS AND METHODS

Animals and Diets. Twenty-four male Sprague−Dawley rats (3 weeks of age) were obtained from Charles River Japan (Kanagawa, Japan). They were randomly divided into four groups of six animals and housed individually in metabolic cages at 22 ± 2 °C and 60 ± 5% relative humidity, with a 12 h light−dark cycle. Their body weight and food intake were measured daily. After 2 days of preliminary feeding of a stock pellet diet (CE-2, Nippon Clea, Tokyo, Japan), the first group was fed a normal diet (NA) and the second group was fed a G-rutin-supplemented diet (RA) ad libitum for 18 days, respectively. After 3 days of preliminary feeding of a stock diet, the third group (N60) received 60% of the amount consumed by the first ad libitum group (NA) and the fourth group (R60)

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was fed 60% of the amount consumed by the G-rutin-supplemented diet consumed by the second group (RA) for 18 days, respectively. The composition of the normal diet was 20% casein (Oriental Yeast Co., Tokyo, Japan), 0.3% D,L-methionine (Nihon Kayaku Co., Tokyo, Japan), 25% corn oil (Hayashi Chemical Co., Tokyo, Japan), 30% α-cornstarch (Nihon Nosan Kogyo Co., Kanagawa, Japan), 17% sucrose, 3.5% mineral mixture (AIN 76 composition, Nihon Nosan Kogyo Co.), 1% vitamin mixture (AIN 76 composition, Nihon Nosan Kogyo Co.), 0.2% choline bitartarate (Wako Pure Chemical Industries, Osaka, Japan), and 3% cellulose powder (Oriental Yeast Co.). In the G-rutin-supplemented diet, 2.5% of G-rutin (Toyo Sugar Refining Co., Ltd., Tokyo, Japan) was added to the normal diet at the expense of sucrose. G-rutin is a water soluble derivative of rutin synthesized from rutin and dextrin using glucanotransferase obtained from *Bacillus stearothermophilus* (Suzuki and Suzuki, 1991). The solubility of G-rutin is $\sim 3.0 \times 10^4$ times higher than that of rutin.

On the last 2 days of feeding, urine samples were collected for 48 h in a flask and stored at −20 °C until analysis. Thereafter, rats were anesthetized with sodium pentobarbital, and blood samples were drawn from the heart using heparinized needles and syringes. The plasma obtained by centrifugation was stored at −20 °C until use. After the animals were killed by bleeding, livers were removed immediately and stored at −20 °C until use.

Determination of Tg and dTg. The procedure involves isolation of *cis*-glycol by borate affinity chromatography (Cathcart et al., 1984), and high-performance liquid chromatographic (HPLC) separation and fluorometric detection of 3-hydoroxyquinaldine (3-HQ), which is the condensation product of *o*-aminobenzaldehyde (o-ABA) and acetol (an alkaline hydrolysis product of Tg and dTg). The condensation reaction that yields fluorescent 3-HQ from acetol and o-ABA has been used for measuring Tg in DNA from irradiated foodstuff (Pfeilsticker and Lucas, 1987) and animal tissues (Roberts and Friedkin, 1958). Application of the condensation reaction for measuring Tgs in urine results in a high background due to interfering substances, such as sugars in urine and excess o-ABA. To minimize the acetol formation from sugars and to maximize that from Tgs, the condensation reaction was carried out under controlled temperature and time. The supernatant of urine samples was adjusted to pH 8.8 by 0.2 M ammonium acetate and applied onto the top of a boronate affinity column (Affi-Gel 601; Japan Bio-Rad Laboratories, Tokyo, Japan). The boronate binding fraction, which contains the *cis*-diol fraction of Tg and dTg, was eluted with 10 mL of 0.5 mM acetic acid. The eluate (400 μL) was mixed with 50 μL of 4.5 M NaOH/0.01 M EDTA for conversion of Tg and dTg to acetol, followed by addition of 10 μL of 300 mM o-ABA dissolved in acetonitrile for condensation with acetol to form 3-HQ. The mixture was kept under 20 °C for 20 min and cooled in an ice−water bath for 20 min. The reaction was terminated by addition of 10 μL of 4.5 M HCl and 50 μL of 1.0 M KH_2PO_4 to adjust the pH. A 10 μL aliquot containing 3-HQ and o-ABA that remained was injected into the reverse-phase HPLC system using the conditions of a flow rate of 0.6 mL/min, column temperature at 30 °C, and 25% (v/v) acetonitrile/2.5 mM $(NH_4)_2HPO_4$ as mobile phase. The column effluent was monitored by fluorescence detection with excitation and emission wavelengths of 375 and 444 nm, respectively. The instrument for HPLC included a 600E system controller (Waters, Milford, MA), an F-1050 fluorescence spectrometer (Hitachi, Tokyo, Japan), and a Labochart-80 computing integrator (System Instrument, Tokyo, Japan). A series of two chromatographic columns (μ-Bondasphere C18, Waters, 150 mm × 3.9 mm i.d., 5 μm) were used.

Determination of Protein Oxidation. Weighed liver tissue sample (50−60 mg) was homogenized in 3 mL of 0.1 M potassium phosphate buffer (pH 7.4) containing digitonin (0.1%), EDTA (1 mM), leupeptin (0.5 μg/mL), pepstatin (0.7 μg/mL), and aprotinin (0.5 μg/mL). All reagents were from Sigma Chemical Co. (St. Louis, MO). Homogenized tissue was allowed to stand for 15 min at room temperature and then centrifuged (10000*g* for 20 min at 4 °C). The supernatant was used for determination of protein concentration and protein carbonyl content. Protein concentration was measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard. Protein carbonyl content was determined as described by Levine et al. (1990). The supernatant (30 μL, 0.1 mg of protein) was added to an equal volume of 20% (w/v) trichloroacetic acid, and the mixture was centrifuged (12000*g* for 10 min at 4 °C). The precipitate was dissolved in 0.5 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl and incubated for 1 h at 25 °C with stirring; 0.5 mL of 20% (w/v) trichloroacetic acid was added, and the mixture was centrifuged (12000*g* for 10 min at 4 °C). The precipitate was washed three times with 1 mL of ethanol/ethyl acetate (1:1), dissolved in 0.6 mL of 6 M guanidine hydrochloride/20 mM sodium phosphate buffer (pH 6.5), incubated for 15 min at 37 °C, and centrifuged (12000*g* for 10 min at 4 °C). The resulting supernatant was used for protein carbonyl determination. The carbonyl content was calculated from the maximum absorbance at 360 nm using a molar absorption coefficient of 22000 M^{-1} cm^{-1} (Jones et al., 1956). Results are expressed as nanomoles of DNPH incorporated per milligram of protein to give nanomoles of protein carbonyl per milligram of protein.

Table 1. Effects of Diet on Food Intake and Body Weight Gain

diet[a]	food intake[b] (g/18 days)	body weight gain[b]
NA	216.9 ± 4.0[a]	188.8 ± 3.0[a]
RA	191.9 ± 4.0[b]	168.9 ± 2.1[b]
N60	130.1 ± 0.2[c]	130.8 ± 1.3[c]
R60	115.0 ± 0.1[d]	121.0 ± 1.9[d]

[a] NA, ad libitum fed normal diet; RA, ad libitum fed rutin-added diet; N60, 40% fewer calories than normal diet; R60, 40% fewer calories than rutin-added diet. [b] The initial body weights of the NA and RA groups were 50.1 ± 0.8 and 50.2 ± 0.7 g, respectively, and those of the N60 and R60 groups were 55.5 ± 0.6 and 55.5 ± 0.9 g, respectively. The data represent means ± SEM of six animals. Means in a column followed by different superscript letters are significantly different ($p < 0.05$) by Duncan's multiple-range test.

Determination of Thiobarbituric Acid-Reactive Substances (TBARS). The level of TBARS in plasma was fluorometrically determined with a commercial kit (Wako Pure Chemical Industries, Osaka, Japan). The lipid peroxide level in the liver homogenate was measured according to the thiobarbituric acid method of Uchiyama and Mihara (1978). 1,1,3,3-Tetraethoxypropane was used as a standard for thiobarbituric acid reactions, and the results were calculated as nanomoles of malondialdehyde (MDA).

Other Assays. Ascorbic acid concentration in the plasma was determined according to the α,α′-dipyridyl method (Zannoni et al., 1974). Plasma α-tocopherol was determined fluorometrically (Abe and Katsui, 1975). Creatinine in urine was enzymatically determined with a commercial kit (Wako Pure Chemical Industries).

Statistical Analysis. The analysis of variance (ANOVA) procedure followed by Duncan's multiple-range test was used to determine statistical significance among groups (Duncan, 1955; Snedecor and Cochran, 1967). Values were considered significantly different at $p < 0.05$.

RESULTS

Body weight gains in groups fed G-rutin-supplemented diets (RA and R60) were significantly lower than those in groups fed non-G-rutin-supplemented diets (NA and N60, Table 1). The lower body weight gain in rats fed the G-rutin-supplemented diets might be due to lower energy intake. Furthermore, some flavonoids such as rutin inhibited intestinal motility and secretion (Di Carlo et al., 1993), which might affect body weight gain.

Plasma concentrations of vitamin E in rats are shown in Table 2. A lower concentration was seen in the food-restricted groups (N60 and R60) compared to the ad

Table 2. Effects of Diet on Plasma Antioxidant Vitamins

diet[a]	vitamin E[b] (μg/mL)	vitamin C[b]
NA	5.5 ± 0.4[a]	7.7 ± 0.2[a]
RA	4.6 ± 0.1[a]	8.0 ± 0.3[a]
N60	2.8 ± 0.1[b]	7.2 ± 0.3[a]
R60	2.8 ± 0.1[b]	7.7 ± 0.3[a]

[a] NA, ad libitum fed normal diet; RA, ad libitum fed rutin-added diet; N60, 40% fewer calories than normal diet; R60, 40% fewer calories than rutin-added diet. [b] The data represent means ± SEM of six animals. Means in a column followed by different superscript letters are significantly different ($p < 0.05$) by Duncan's multiple-range test.

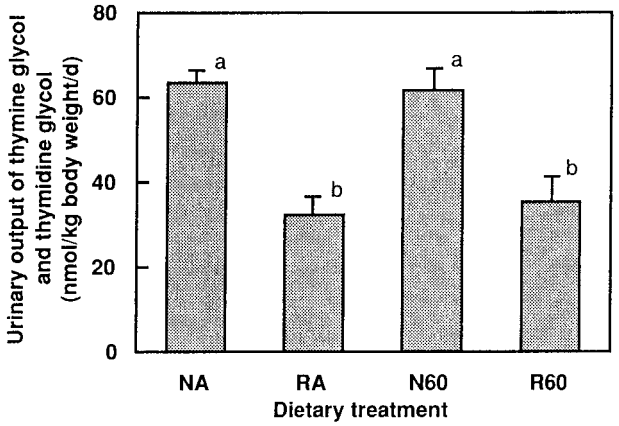

Figure 1. Effects of diets on urinary output of Tg and dTg. Each value represents the mean of six animals. Vertical bars indicate standard errors. Values with different letters are significantly different at $p < 0.05$. NA, ad libitum fed normal diet; RA, ad libitum fed rutin-added diet; N60, 40% fewer calories than normal diet; R60, 40% fewer calories than rutin-added diet.

libitum fed groups (NA and RA). The plasma level of vitamin E decreased as food intake decreased (Tables 1 and 2). The plasma vitamin C concentrations in the food-restricted groups (N60 and R60) were not significantly different from those in the ad libitum fed groups (NA and RA), nor was any significant difference observed in the concentrations of plasma vitamin C among the groups (Table 2).

G-rutin supplementation resulted in lower levels of urinary output of Tgs (nanomoles per kilogram of body weight per day) as compared to those of the non-G-rutin-supplemented groups (Figure 1). Food restriction produced no effect on the urinary output of Tgs irrespective of rutin supplementation. Urinary output of Tgs expressed per unit of creatinine (nanomoles per milligram of creatinine per day) was also calculated. The results expressed per creatinine were similar to those expressed per kilogram of body weight (results not shown).

The data in Figure 2 demonstrate that the level of oxidized proteins in the liver of rats decreased with the G-rutin-supplemented diet in the ad libitum fed groups (NA and RA). Figure 2 also shows that food restriction decreased protein carbonyl to the same extent as in the G-rutin-supplement groups: NA to N60 compared with NA to RA.

The plasma TBARS concentration in the ad libitum fed group (NA) was not significantly decreased by G-rutin supplementation (RA) (Figure 3). Food restriction also did not affect plasma TBARS concentration in normal diet (NA to N60); however, G-rutin supplementation decreased TBARS concentration in food-restricted group (N60 to R60). There were no significant differences among the groups in the liver TBARS concentrations (data not shown).

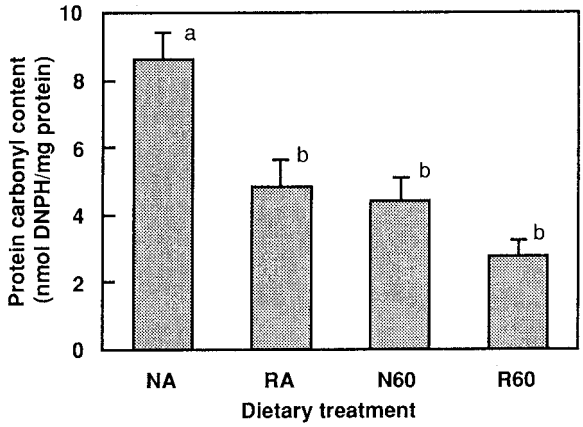

Figure 2. Effect of diets on liver protein carbonyl content. Each value represents the mean of six animals and is expressed as nanomoles of DNPH incorporated per milligram of protein. Vertical bars indicate standard errors. Values with different letters are significantly different at $p < 0.05$. NA, ad libitum fed normal diet; RA, ad libitum fed rutin-added diet; N60, 40% fewer calories than normal diet; R60, 40% fewer calories than rutin-added diet; DNPH, 2,4-dinitrophenylhydrazine.

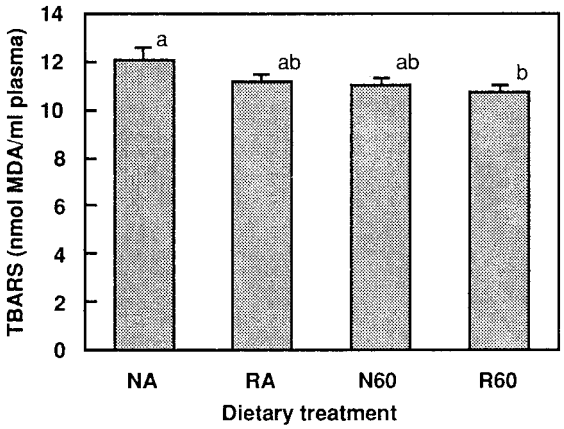

Figure 3. Effects of diets on plasma TBARS content. Each value represents the mean of six animals and is expressed in terms of nanomoles of MDA per milliliter of plasma. Vertical bars indicate standard errors. Values with different letters are significantly different at $p < 0.05$. NA, ad libitum fed normal diet; RA, ad libitum fed rutin-added diet; N60, 40% fewer calories than normal diet; R60, 40% fewer calories than rutin-added diet.

DISCUSSION

The idea that the accumulation of oxidative damage to DNA, protein, and lipid may play a major role in aging and degenerative diseases is now widely recognized. The present study was undertaken to determine whether dietary rutin, rich in vegetables in a human diet, could exert its antioxidant properties to prevent DNA damage and protein oxidation in the tissues of animal models.

The results indicated clearly that G-rutin added to the normal diet reduced urinary excretion of DNA damage products, Tg and dTg (Figure 1). There were no differences in the plasma concentrations of vitamin C among the groups tested (Table 2). The plasma vitamin E level was lower in the calorie-restricted groups (N60 and R60) than in ad libitum fed groups (NA

and RA), irrespective of G-rutin addition. Plasma vitamin E concentration was proportional to the amount of food intake. Lower vitamin E concentration in N60 than in NA or in R60 than in RA reflects the lower intake of dietary vitamin E. Therefore, it is difficult to explain the reduction of Tgs in relation to the plasma concentrations of vitamin C or vitamin E. The argument above does not rule out the possible mechanism in which rutin's antioxidative effect on DNA damage is due to its prevention of the loss of biological potency of vitamins (Crampton and Lloyd, 1950).

In contrast to G-rutin supplementation, the data in Figure 1 indicate that calorie restriction did not reduce the levels of DNA damage, because no difference in Tgs levels was observed between the pairs of the calorie-restricted groups: NA to N60 and RA to R60. The calorie restriction in the present study was done for 18 days; therefore, the period of calorie restriction might be too short to accomplish the repair of the DNA damage.

The average daily output for Tgs in the NA group was 63.7 nmol/kg (Figure 1). This value was 8.3 times higher than that obtained in the previous studies (Cathcart et al., 1984; Adelman et al., 1988) in which the value for Tg was 5.5 and that for dTg was 1.7, amounting to 7.2 nmol/kg per day for Tgs. The discrepancy between our data and those of Cathcart et al. (1984) and Adelman et al. (1988) must be due to the different methods employed in the assay of Tgs. Possible modified base products resulting from the attack of hydroxyl radicals on the base in DNA were considered to be 5-hydroxymethyluracil from thymine, 8,8-hydroxyguanine from guanine, and others (Aruoma, 1991). Considering the chemical structures, it is difficult to assume that some of the modified base products will yield acetol, which couples with o-ABA to give 3-HQ, because the formation of acetol requires the presence of a methyl group attached to carbon 5 of uracil or cytosine if thymine or cytosine is regarded as the parent base. Another possible parent base that forms acetol by alkaline hydrolysis is 5-methylcytosine (5-MeC). When 5-MeC in DNA and RNA is oxidized and 5-MeC glycol is formed and removed from DNA or RNA, our method would measure it as Tgs. The Tgs in urine of rats in our experiment was not derived from the diet because they were fed a nucleic acid-free semipurified diet. Contradictions in the urinary levels of Tgs between our data and those of Ames' group cannot be explained and should be resolved in the future.

Dietary G-rutin is reported to be a potent antioxidant in animals treated with ferric nitrilotriacetate (Shimoi et al., 1997), which generates free radicals damaging DNA, lipid, and protein in tissues (Toyokuni et al., 1994; Nagasawa et al., 1997). The present results support the conclusions that part of the G-rutin was absorbed in rats and that dietary G-rutin is a powerful radical scavenger even in normal animals, although we did not measure the digestibility or the concentration of rutin and/or its metabolite in plasma and tissues. Shimoi et al. (1997) have demonstrated that a rapid absorption into the circulation was observed when mice were given G-rutin by gastric intubation, suggesting that G-rutin is absorbed without hydrolysis. On the other hand, G-rutin may be hydrolyzed to rutin by α-amylase, because it has a glucose bound to rutin in a molecule. However, absorption of dietary rutin across the intestine is not fully understood. Manach et al. (1997) have reported that plasma quercetin concentration was maintained at a high level by dietary supplementation of rutin as well as qurecetin. They showed rutin was absorbed more slowly than quercetin, because it must be hydrolyzed by the cecal microflora. Hollman et al. (1995) have shown that appreciable amounts of quercetin and its glycosides could be absorbed in human subjects. Recently, Gee et al. (1998) have suggested that quercetin glucosides interact with the intestinal glucose transport pathway. Thus, rutin and G-rutin are considered to be absorbed and act as antioxidants.

It is generally accepted that caloric restriction reduces the production of reactive oxygen species (Youngman et al., 1992; Sohal and Weindruch, 1996). The data in Figure 2 suggest that caloric restriction inhibits oxidative damage against proteins as significantly as rutin does, but there were no combined effects of rutin and calorie restriction on the accumulation of oxidatively damaged protein. These results seem to indicate that reduced production of oxygen radicals due to calorie restriction commensurated with the reduction of oxygen radicals resulting from the amount of rutin fed. Plasma vitamin E levels were low in the N60 group compared to the RA group, despite protein carbonyl levels being the same. This seems to indicate that vitamin E concentrations are not the sole and direct determinant of the oxidative modification of proteins. Rutin and/or its metabolite may contribute to the reduction of protein oxidation in collaboration with vitamin C or without it. Vitamin C acts as the first defense in the plasma as a preventive antioxidant (Niki, 1993). Rats synthesize the amount of ascorbic acid they need, resulting in the absence of proportional relationships between the plasma concentration of vitamin C and the amount of food intake (Tables 1 and 2). However, it is difficult to determine what is effective as an antioxidant, but the effect of rutin as a radical scavenger in cooperation with or without vitamin C cannot be excluded. Synergistic effects of rutin with calorie restriction on lipid peroxide in plasma was observed, but neither rutin alone nor calorie restriction alone exhibited any antioxidant activities on lipid peroxidation in plasma (Figure 3). Thus, dietary antioxidants and calorie restriction may have different mechanisms and target molecules against free radical-mediated oxidation.

In conclusion, dietary G-rutin markedly inhibited the accumulation of oxidatively damaged DNA and proteins. Rutin is almost insoluble in water, whereas G-rutin is water soluble. Therfore, G-rutin may be able to be used as an additive in beverages. The present observation prompts us to entertain the idea that dietary addition of antioxidants such as G-rutin could extend functional lifespans, based on the view that lowering the level of oxidative stress leads to an extended lifespan, which is the free radical hypothesis of aging (Orr and Sohal, 1994).

ABBREVIATIONS USED

o-ABA, o-aminobenzaldehyde; G-rutin, α-glucosyl rutin; 3-HQ, 3-hydroxyquinaldine; MDA, malondialdehyde; 5-MeC, 5-methylcytosine; NA, normal diet; N60, 60% of the normal diet; RA, G-rutin-supplemented diet; R60, 60% of the G-rutin-supplemented diet; TBARS, thiobarbituric acid-reactive substance; Tg, thymine glycol; dTg, thymidine glycol; Tgs, Tg plus dTg.

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