

Increased Urinary Hydrogen Peroxide Levels Caused by Coffee Drinking

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Experiments with volunteers in Singapore have demonstrated that coffee drinking increases urinary hydrogen peroxide levels (Long, Halliwell, *Free Rad. Res.*, 32, 463—467 (2000)). We re-examined the effect of coffee drinking of healthy Japanese subjects on urinary hydrogen peroxide levels. A cup of brewed or canned coffee commercially available in Japan generated 120—420 μmol hydrogen peroxide in incubation in a neutral medium at 37 °C for 6 h. The increased levels were higher than those obtained from a cup of green tea extract or a glass of red wine. After the subject drank a cup of coffee, apparent hydrogen peroxide levels ($\mu\text{mol/g}$ creatinine) in urine collected 1—2 h after coffee drinking increased 3—10-fold compared to the levels before coffee drinking. The increased urinary hydrogen peroxide levels are likely derived mainly from 1,2,4-benzenetriol excreted in urine, because the major component that generates hydrogen peroxide is found to be 1,2,4-benzenetriol, and storing urine collected after coffee drinking increased hydrogen peroxide levels in a time-dependent fashion. Total hydrogen peroxide equivalent levels excreted in 3 h-urine after coffee drinking were estimated to be 0.5—10% that of coffee consumed. A residual amount of hydrogen peroxide may be retained or consumed in human bodies.

Key words 1,2,4-benzenetriol; hydrogen peroxide; coffee drinking; urine

The hydrogen peroxide content in brewed coffee is shown to be much higher than that in other foodstuffs.^{1—4} Although hydrogen peroxide is used as a food additive for food processing in Japan, residual hydrogen peroxide in processed foods is not permitted owing to its possible carcinogenicity in mice.⁵ 1,2,4-Benzenetriol (hydroxyhydroquinone) and related components are known to be present in roasted coffee beans,^{6,7} and our previous study has demonstrated that 1,2,4-benzenetriol is a major hydrogen peroxide generating component causing DNA single strand breaks *in vitro*.⁸ Although 1,2,4-benzenetriol is stable in a rather acidic brewed coffee, it generates 0.5—0.6 equimolar amounts of hydrogen peroxide in incubation under aerobic conditions at pH 7.4.⁹

In another course of studies on benzene toxicity, the toxicity of 1,2,4-benzenetriol was investigated as one of the intermediary metabolites of benzene. 1,2,4-Benzenetriol is mutagenic to *Salmonella typhimurium* TA97 and TA100 strains without metabolic activation¹⁰ through the generation of reactive oxygen species during autoxidation. 1,2,4-Benzenetriol cleaved DNA single strands through the metal ion- and reactive oxygen species-dependent pathway,^{11—13} which may participate in the carcinogenicity of benzene.^{14,15} Moreover, long-term supplementation of the component to mice remarkably induces lung lipid peroxidation, and enhances type IV and I allergy responses.¹⁶

It is important to elucidate the effects of drinking coffee containing 1,2,4-benzenetriol in humans. Recent experiments with healthy volunteers in Singapore have demonstrated that coffee drinking increases urinary hydrogen peroxide levels, suggesting that oxidative stress is induced in human bodies by coffee drinking.¹⁷ In the present study, we re-examined the effect of coffee drinking of healthy Japanese subjects on hydrogen peroxide excretion in urine. Coffee drinking by all the Japanese subjects increased apparent urinary hydrogen peroxide levels, and the total hydrogen peroxide equivalent amount excreted in urine was 0.5—10% that of coffee consumed.

MATERIALS AND METHODS

Materials 1,2,4-Benzenetriol (hydroxyhydroquinone) was obtained from Wako Pure Chemical Industries, Osaka, Japan. Brewed coffee was prepared by the extraction and filtration of 20 g ground roasted coffee beans (Mocha blend, regular medium fine) prepared by COOP Univ, Tokyo, Japan, with 140 ml of tap water heated at 75 °C in the usual manner. The pH value of the preparation was 5.1. This preparation was used and drunk without the addition of sugar or milk. Canned coffee (187 ml) with sugar, milk, spice, emulsifier and casein sodium salt (brand FIRE) prepared by Kirin Beverage Corporation, Tokyo, Japan, was also used and drunk. The pH value of this preparation was 6.4. Green tea extract was prepared by the extraction of 4.0 g green tea leaves (brand MACCHA-IRI-SENCHA) prepared by COOP, Tokyo, Japan, with 300 ml of tap water heated at 75 °C in the usual manner. The pH value of the preparation was 6.6. This preparation was directly used and drunk. Red wine (brand SYATO KATSUNUMA) prepared by Syato Katsunuma Company, Yamanashi, Japan, without antioxidants and preservative agents (pH 3.6) was used and drunk.

Determination of Hydrogen Peroxide in Beverages and Urine Hydrogen peroxide in beverage and urine samples was determined by the FOX-1 method using xylenol orange and ferrous ion with sorbitol chain amplification.¹⁸ The assay was performed by the standard addition method. The sample solutions were prepared as follows. To 5 tubes containing 1.0 ml of beverage or urine with or without 1.0 mg of catalase [EC 1.15.16] (bovine liver (20000 units/mg) from Sigma Chemical Company, St. Louis, MO, U.S.A.), a 1/100 volume of standard hydrogen peroxide solution was added to make final concentrations of hydrogen peroxide at 0, 5, 10, 15 and 20 μM . To 850 μl of water, 50 μl of each of the sample solutions, 50 μl of a solution of 5 mM ferrous ammonium sulfate in 0.5 M sulfuric acid and 50 μl of a solution of 2 mM xylenol orange in 2 M sorbitol were added, and the mixture was kept at 20 °C for 30 min. The absorbance at 560 nm of the solution was measured.

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Determination of Creatinine in Urine Creatinine in urine was determined by the usual method¹⁹⁾ using Creatinine-Test Wako (Wako). Urine was diluted 1/20 (v/v) with water. To 286 μl of the sample, 1.71 ml of deproteinizing solution was added, and the mixture was kept at 20 °C for 10 min. After the clear solution was made at 30 °C, 1.0 ml of picric acid solution and 1.0 ml of 0.75 M NaOH solution were added. The mixture was kept at 30 °C for 20 min, and absorbance of the solution at 520 nm was measured. A solution of 10 mg creatinine/100 ml was used as a standard solution.

Generation of Hydrogen Peroxide from Beverage An aliquot of the beverage was diluted 1/10 or 1/20 into 10 ml of 0.1 M Tris-HCl buffer (pH 7.4), with or without 100 μg of superoxide dismutase (SOD) [EC 1.15.1.1] (from bovine liver Cu,Zn-SOD (2600 units/mg) from Sigma), and the neutral mixture was incubated at 37 °C for the indicated periods up to 6 h. Hydrogen peroxide (μmol) was determined immediately after sampling.

Excretion of Apparent Hydrogen Peroxide in Urine after Coffee Drinking Ten healthy Japanese subjects, four males aged 20–30, two females aged 20–30, three male aged 30–40 and one male aged 60–70, of a laboratory of Tokyo University of Pharmacy and Life Science, were enlisted to drink coffee and collect urine by themselves. Before the experiments all the subjects agreed to participate in the experiments and to the coffee drinking-urine collection schedule. The subjects were obliged to take no coffee 24 h before the experiments of coffee drinking, and allowed to take their own usual foods and drinks. Before coffee drinking urine was collected, then a cup of coffee (150 ml for brewed coffee and 187 ml for canned coffee) was drunk within a period of 5 min. After coffee drinking urine was collected at 30 min intervals for 200 min. Hydrogen peroxide (μmol)/g creatinine was determined between 1 and 4 h after urine collection.

Time Course of Hydrogen Peroxide Levels in Urine or in 1,2,4-Benzenetriol-Added Urine Urine obtained before coffee drinking, urine obtained before coffee drinking mixed with 100 μM 1,2,4-benzenetriol, and 1 h-urine obtained after coffee drinking were kept at 20 °C for 5 h. Hydrogen peroxide (μmol) or hydrogen peroxide (μmol)/g creatinine was determined immediately after sampling.

Total Apparent Hydrogen Peroxide Levels in 3 h-Urine after Coffee Drinking The levels of hydrogen peroxide (μmol)/g creatinine in urine obtained before coffee drinking and in 3 h-urine obtained after coffee drinking were determined between 1 and 4 h after urine collection. Hydrogen peroxide equivalent (μmol) excreted in 3 h-urine after coffee drinking was calculated as follows: {hydrogen peroxide (μmol)/g creatinine (3 h-urine after coffee drinking)–hydrogen peroxide (μmol)/g creatinine (before coffee drinking)} \times g creatinine (3 h-urine after coffee drinking).

RESULTS

The hydrogen peroxide generating potencies of a cup of coffee, a cup of green tea extract and a glass of red wine were compared. When a solution of slightly acidic brewed coffee in Tris-HCl buffer (pH 7.4) was incubated at 37 °C for up to 6 h, hydrogen peroxide levels gradually increased to 300–420 μmol (6–9-fold increase) compared to the initial

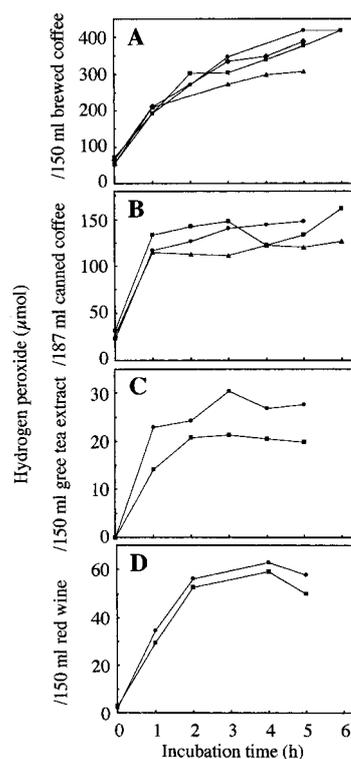


Fig. 1. Generation of Hydrogen Peroxide from Brewed Coffee (4 Extractions from the Same Lot of Roasted Coffee Beans) (A), Canned Coffee (3 Different Lots from the Same Brand) (B), Green Tea Extract (2 Extractions from the Same Lot of Green Tea Leaves) (C) and Red Wine (Two Determinations of the Same Lot) (D)

An aliquot of the beverage was diluted 1/10 or 1/20 into 10 ml of 0.1 M Tris-HCl buffer (pH 7.4) and the mixture was incubated at 37 °C for the indicated periods. Hydrogen peroxide levels were determined immediately after sampling. The amount of hydrogen peroxide in a cup or a glass of the beverage was calculated.

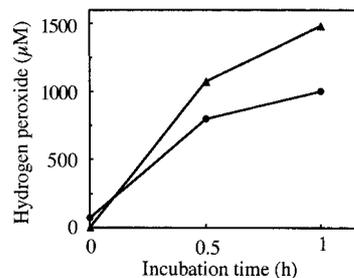


Fig. 2. Generation of Hydrogen Peroxide from Canned Coffee in the Absence and the Presence of SOD

An aliquot of canned coffee was diluted 1/20 into 10 ml of 0.1 M Tris-HCl buffer (pH 7.4) with (▲) and without (●) 100 μg of SOD, and the mixture was incubated at 37 °C for the indicated periods. Hydrogen peroxide levels were determined immediately after sampling.

level of 50 μmol (Fig. 1A). When a solution of canned coffee adjusted at pH 7.4 was similarly incubated, hydrogen peroxide levels increased to 120–140 μmol (4–5-fold increase) after 1 h as compared to the initial level of 30 μmol (Fig. 1B). When the neutral solution of canned coffee was incubated in the presence of SOD, the levels of hydrogen peroxide increased to a higher extent (Fig. 2), indicating the superoxide-dependent generation of hydrogen peroxide. It has been shown that 1,2,4-benzenetriol is the major hydrogen peroxide generating component in roasted coffee beans,⁸⁾ and pure 1,2,4-benzenetriol generates 0.5–0.6 equimolar amounts of

hydrogen peroxide under neutral conditions.⁹ Because the quantitative determination of 1,2,4-benzenetriol in coffee was unsuccessful, the amount of the component in coffee could not be estimated.

Similar incubation of green tea extract (Fig. 1C) and red wine (Fig. 1D) at neutral pH was done. Hydrogen peroxide levels of green tea extract increased to 20–30 μmol after 2 h compared to the initial level of 0 μmol , and those of red wine increased to 50–60 μmol after 2 h, compared to the initial levels lower than 5 μmol . The increases in hydrogen peroxide levels in green tea extract and red wine must be due to polyphenols contained in the beverages, because the polyphenols in these beverages generate hydrogen peroxide in neutral buffers.^{20,21} The hydrogen peroxide generating potency of coffee was the highest among these beverages.

The effect of coffee drinking of ten Japanese subjects, female aged 20–30 (+ and \times), male aged 20–30 (\diamond , \blacktriangle , \blacksquare and \square), male aged 30–40 (\bullet , \circ and \blacklozenge) and male aged 60–70 (\triangle), on urinary hydrogen peroxide levels was examined (Fig. 3). Hydrogen peroxide was measured between 1 and 4 h after urine collection. Before coffee drinking, the urinary hydrogen peroxide levels in 8 of 10 Japanese subjects were estimated to be about 5 $\mu\text{mol/g}$ creatinine, whereas those in 2 subjects (\times and \circ) were higher at 25 and 30 $\mu\text{mol/g}$ creatinine. When a male subject (\bullet) took a cup of brewed coffee, the urinary hydrogen peroxide levels increased 6-fold in urine collected 2 h after coffee drinking, and decreased in urine collected thereafter (Fig. 3A). When each of 2 female and 8 male subjects took a cup of canned coffee, the urinary hydrogen peroxide levels of the subjects increased 3–8-fold in urine collected 2 h after coffee drinking and decreased in urine collected thereafter, whereas the levels greatly differed in the individual subjects (Fig. 3B). It is apparent that drinking a cup of coffee results in an immediate increase in urinary hydrogen peroxide levels.

In order to find whether the increased urinary hydrogen peroxide levels after coffee drinking reflected hydrogen peroxide excreted itself or 1,2,4-benzenetriol excreted, the time course of the increase in hydrogen peroxide levels in urine of the male subject (\bullet) collected before and 1 h after coffee drinking was followed by storing urine at 20 $^{\circ}\text{C}$ for 5 h in a stoppered tube or in a tube under aeration (Fig. 4). In this case, hydrogen peroxide was measured immediately after sampling. Urinary hydrogen peroxide levels before coffee drinking increased from 1 to 5 ($\mu\text{mol/g}$ creatinine) during storing the urine for 3–4 h, both in a stoppered tube and in a tube under aeration. Hydrogen peroxide levels in 1 h-urine after coffee drinking increased from 4 to 12 ($\mu\text{mol/g}$ creatinine) during storing urine for 2 h in a stoppered tube, and those increased from 6 to 16 ($\mu\text{mol/g}$ creatinine) in a tube under aeration for 2 h. Throughout the storing, the levels in urine after coffee drinking were always higher than those before coffee drinking. Urinary hydrogen peroxide levels collected after coffee drinking may be derived from 1,2,4-benzenetriol excreted during the storage of urine. This is supported by the fact that the incubation of 1,2,4-benzenetriol at 100 μM concentration in urine of the male subject (\bullet) collected before coffee drinking generated 40–60 μM hydrogen peroxide (0.4–0.6 equivalent of 1,2,4-benzenetriol) by storing for 5 h (Fig. 5). Hence, apparent urinary hydrogen peroxide levels after coffee drinking as shown in Fig. 3 may be de-

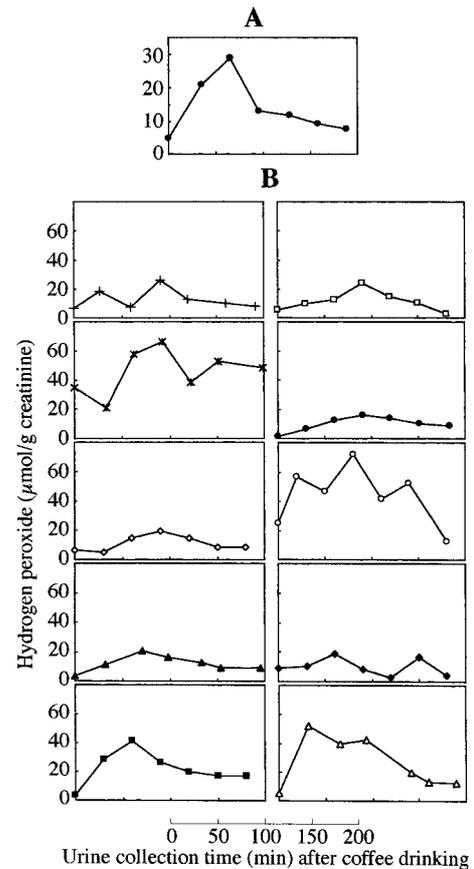


Fig. 3. Apparent Urinary Hydrogen Peroxide Levels after Drinking a Cup of Brewed Coffee (A) or Canned Coffee (B)

Subjects were female aged 20–30 (+ and \times), male aged 20–30 (\diamond , \blacktriangle , \blacksquare and \square), male aged 30–40 (\bullet , \circ and \blacklozenge) and male aged 60–70 (\triangle). Before coffee drinking urine was collected from each of the subjects, and then a cup of coffee (Fig. 1A) (A) or canned coffee (Fig. 1B) (B) was drunk within the period of 5 min. After coffee drinking urine was collected at every 30 min-interval during 200 min. Hydrogen peroxide ($\mu\text{mol/g}$ creatinine) in urine was determined between 1 and 4 h after urine collection.

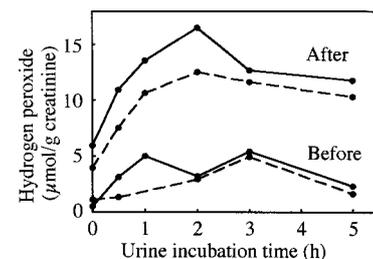


Fig. 4. Time Course of the Increase in Hydrogen Peroxide Levels in Urine during Storing

Urine obtained before coffee drinking, and 1 h-urine obtained after drinking a cup of canned coffee (Fig. 1B) by a male subject aged 30–40 (\bullet) were kept at 20 $^{\circ}\text{C}$ for 5 h in a stoppered tube (dotted line) or in a tube under aeration (solid line). Hydrogen peroxide ($\mu\text{mol/g}$ creatinine) was determined immediately after sampling.

rived from 1,2,4-benzenetriol excreted in urine.

Apparent hydrogen peroxide equivalent levels excreted in 3 h-urine after coffee drinking were determined. In this case, hydrogen peroxide was measured between 1 and 4 h after urine collection. When a cup of brewed coffee was taken by two male subjects (\bullet and \blacksquare), the amounts of hydrogen peroxide equivalents excreted differed not only by the individuals, but by coffee drinking in the same subjects. No more than 30 μmol equivalents of hydrogen peroxide correspond-

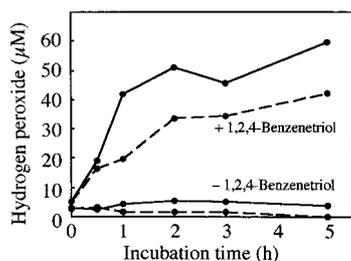


Fig. 5. Time Course of the Increase in Hydrogen Peroxide Levels in Urine Mixed with 1,2,4-Benzenetriol

Two urine samples (dotted and solid lines) collected from a male aged 30–40 (●) were mixed with 100 μM 1,2,4-benzenetriol, and the mixtures were kept at 20 $^{\circ}\text{C}$ for 5 h in a stoppered tube. Hydrogen peroxide (μmol) was determined immediately after sampling.

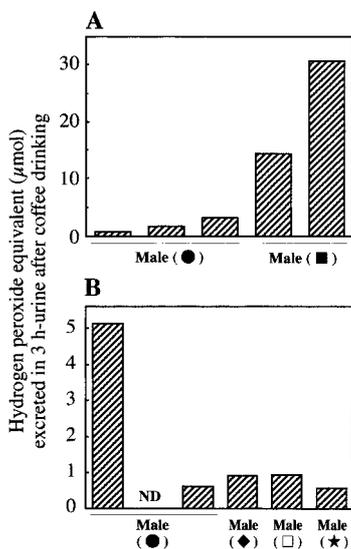


Fig. 6. Total Amounts of Hydrogen Peroxide Equivalent Excreted in 3 h Urine after Coffee Drinking

A cup of brewed coffee (Fig. 1A) was drunk by a male aged 30–40 (●, three times) and a male aged 20–30 (■, twice) (A), and a cup of canned coffee (Fig. 1B) was drunk by a male aged 30–40 (●, three times), a male aged 30–40 (◆), a male aged 20–30 (□) and a male aged 20–30 (★) (B). Hydrogen peroxide ($\mu\text{mol}/\text{g}$ creatinine of urine was determined between 1 and 4 h after urine collection. The amount of hydrogen peroxide (μmol) in 3 h-urine after coffee drinking was calculated as described in Materials and Methods section. ND indicates not detected.

ing to 10% that of brewed coffee drunk were excreted (Fig. 6A). When a cup of canned coffee was taken by four male subjects (●, ◆, □ and ★), the amounts of hydrogen peroxide equivalents excreted were different not only by the individuals but by the coffee drinking experiments of the same subjects. No more than 5 μmol equivalents of hydrogen peroxide corresponding to 5% that of canned coffee drunk were excreted (Fig. 6B). Hence, apparent urinary hydrogen peroxide equivalent levels excreted after coffee drinking was less than 10% that of the coffee consumed.

DISCUSSION

1,2,4-Benzenetriol has been investigated as a toxic intermediary metabolite of benzene. The compound generates reactive oxygen species,^{8–13} and the hydrogen peroxide generating potency of the compound is much higher than those of other polyphenolics.^{8,9} The compound is mutagenic to *Salmonella* bacteria¹⁰ and cleaves DNA single strands^{8,9,11–13}

in *in vitro* studies, which is suggested to contribute to the carcinogenicity of benzene.^{14,15} Animal studies of the compound have shown that long-term supplementation of the component to mice remarkably induces lung lipid peroxidation, and enhances type IV and I allergy responses.¹⁶

Our previous study has demonstrated that roasted coffee beans contain 1,2,4-benzenetriol as a major hydrogen peroxide-generating component, causing DNA single strand breaks.^{8,9} The presence of this component in coffee has been clarified by its isolation from the complex coffee mixture.⁸ Pure 1,2,4-benzenetriol autoxidizes generating reactive oxygen species, superoxide, hydrogen peroxide and hydroxyl radical in a neutral medium.^{9,14} Quantitative determination of this component in brewed and canned coffee and also in urine was attempted but unsuccessful. The amount of hydrogen peroxide generated from a cup of brewed or canned coffee was estimated to be 120–420 μmol (4–14 mg), and the hydrogen peroxide generating potency of a cup of coffee was much higher than a cup of green tea extract or a glass of red wine, both containing polyphenols that generate hydrogen peroxide.^{20,21}

It is important to examine the toxicological effects of drinking coffee containing 1,2,4-benzenetriol in human subjects. Recent experiments with healthy volunteers in Singapore have demonstrated that coffee drinking increases urinary hydrogen peroxide levels and suggest that oxidative stress is induced by coffee drinking in human bodies.¹⁷ In the present study, we re-examined the effect of coffee drinking of healthy Japanese subjects on hydrogen peroxide excretion in urine. The results obtained here showed that coffee drinking of all the Japanese subjects increased urinary hydrogen peroxide levels to the greater extent than that obtained in the Singapore experiment. However, the total urinary hydrogen peroxide equivalents excreted after drinking of a cup of coffee did not exceed 30 μmol , corresponding to 10% that of coffee consumed. More than 90% of the hydrogen peroxide generated by coffee drinking appears to be retained or consumed in the bodies. Generated hydrogen peroxide may be degraded by catalase and glutathione peroxidase in the bodies, or may otherwise induce oxidative stress to the bodies.

It is known that hydrogen peroxide is detected in healthy human urine.^{22–25} Urinary hydrogen peroxide levels of Japanese subjects without coffee drinking may be less than 5 $\mu\text{mol}/\text{g}$ creatinine, because 8 of 10 subjects showed this value between 1 and 4 h after urine collection (Fig. 3). However, the hydrogen peroxide levels of urine immediately after collection was much lower, because the hydrogen peroxide levels increased 5-fold during storage for 2 h after urine collection. This result is consistent with earlier observations showing that urinary hydrogen peroxide levels slowly increase on allowing urine to stand in air.²⁵ The increase in hydrogen peroxide levels during urine storage may be due to polyphenols that are taken from general foods. Observed higher hydrogen peroxide levels in urine of 2 of 10 subjects (Fig. 3), 25 and 35 μmol hydrogen peroxide levels/g creatinine, may be due to the intake of greater amounts of polyphenols in foods.

1,2,4-Benzenetriol is a strong reductone and can serve as an antioxidant in the bodies. However, it has the adverse effect generating hydrogen peroxide during autoxidation in the presence of oxygen.⁹ Which effect of the compound prevails,

antioxidant or prooxidant, may depend on the locus of tissues and cells. Long-term supplementation of the component to mice increased lung lipid peroxidation, whereas it decreased liver and kidney lipid peroxidation.¹⁶⁾ Supplementation of the component to rats induced lung and heart lipid peroxidation (unpublished results). Supplementation to mice increased allergen-induced type IV contact allergy responses and type I respiratory allergy responses.¹⁶⁾ The intake of 1,2,4-benzene-triol can induce oxidative damage, depending on the tissues or the cells. Further toxicological studies for this component are necessary.

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