응용약물학회지, The Journal of Applied Pharmacology, 7, 112-120(1999)

Differential Antioxidant Effects of Ambroxol, Rutin, Glutathione and Harmaline

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(Received March 10, 1999; Accepted May 12, 1999)

Abstract-The protective actions of ambroxol, rutin, glutathione and harmaline on oxidative damages of various tissue components were compared. The mechanisms by which they prevent oxidative tissue damages were explored. Lipid peroxidation of liver microsomes induced by combinations of Fe^{2+} and ascorbate or Fe^{3+}, ADP and NADPH was inhibited by 50 μM of rutin, ambroxol, harmaline and glutathione. Ambroxol (100 μM) inhibited the degradation of hyaluronic acid by Fe^{2+}, H_2O_2 and ascorbate, and it was greater than that of harmaline, whereas hyaluronic acid degradation was not prevented by rutin and glutathione. The compounds used (100 μM) did not protect the degradation of cartilage collagen by xanthine and xanthine oxidase. Rutin, glutathione and harmaline decreased the degradation of IgG by xanthine and xanthine oxidase, while ambroxol did not attenuate degradation of IgG. Glutathione showed a scavenging action on H_2O_2. The compounds all showed scavenging actions on hydroxyl radical. Ambroxol and harmaline exhibited quenching effects on singlet oxygen. In conclusion, ambroxol, rutin, glutathione and harmaline may exert protective effects differently on tissue components against oxidative attack depend on kind of tissue component and free radical.

Keywords □ Differential antioxidant effect, Ambroxol, Rutin, Glutathione, Harmaline

Excessive production of reactive oxygen species is thought to cause damages of tissue components. Free radicals have been implicated as important mediators of tissue damages in postischemic tissue injury, atherosclerosis, diabetes and rheumatoid arthritis (Halliwell and Gutteridge, 1989a). They cause degradation of proteins (Winyard *et al.*, 1989), peroxidation of membrane phospholipids (Halliwell and Gutteridge, 1989a), fragmentation of carbohydrate polymers (Greenwald and Moy, 1980) and oxidation of deoxyribonucleic acid (Schraufstatter *et al.*, 1988). Reactive oxygen species attack cellular proteins and cause fragmentation and cross-linking. Oxidative damages of enzymes could change their susceptibility to proteolysis, which is involved in the regulation of enzyme degradation and in the accumulation of altered forms of enzymes during aging (Oliver *et al.*, 1987). The pathologic consequences of lipid peroxidation are associated with alteration of membrane function, loss of membrane integrity and inactivation of integral enzymes.

Endogenous and exogenous antioxidants are expected to protect tissue components against the oxidants. Ambroxol, trans-4[(2-amino-3,5-dibromobenzyl)amino]cyclohexanol HCl, is known to promote bronchial secretion and is used as an expectorant (Disse, 1987). Ambroxol inhibits lipid peroxidation of lung tissue caused by heat and H_2O_2 (Nowak *et al.*, 1994b) and protects collagen and hyaluronic acid against oxidative attack (Koh *et al.*, 1998). However, it does not prevent lung damage by paraquat and hyperoxia (Nemery *et al.*, 1992) and not inhibit peroxidation of liver lipids. In addition, ambroxol effectively scavenges hydroxyl radicals, while its removing action on H_2O_2 is uncertain (Nowak *et al.*, 1994a; Gillissen *et al.*, 1997).

Flavonoids, which are ubiquitous in photosynthesizing cells, have been reported to show multiple biological effects, including antioxidant ability (Havsteen, 1983). Flavonoids effectively inhibit metal ion-induced peroxidation of liposome (Arora *et al.*, 1998) and attenuate degradation of protein induced by Cu^{2+} and H_2O_2 (Park *et al.*, 1991). However, some phenolics exert pro-oxidant action and are oxidized to produce oxygen-derived species, such as H_2O_2 (Ochiai *et al.*, 1984). They cause a damage of DNA, which is enhanced by metal ions (Sahu and Gray, 1994).

Cellular sulfhydryl groups play a major role in maintenance of membrane structure and cell functions. Glutathione (GSH) can provide protection against endogenous and exogenous oxidants. A GSH significantly decreases cytotoxicity by tumor necrosis factor-α (Cavallo *et al.*, 1997) and completely inhibits lactoferrin-B-induced apoptosis in THP-1

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human monocytic leukemic cells (Yoo *et al.*, 1997). However, GSH also has pro-oxidant action and could evoke cellular damages by the formation of thiyl radicals (Halliwell and Gutteridge, 1989a). Exogenous GSH enhances myoglobin toxicity on mouse renal proximal tubular (HK-2) cells (Zager and Burkhart, 1998).

β-Carboline alkaloids are considered to have various pharmacological actions, such as monoamine oxidase inhibition (Fuller *et al.*, 1986) and anxiolytic effect (Barbaccia *et al.*, 1986). Harmane and related β-carbolines are reported to exert antioxidant effect and to inhibit oxidative damages of microsomal lipids, hyaluronic acid and cartilage collagen (Tse *et al.*, 1991; Cho *et al.*, 1995). They are suggested to have a scavenging action on hydroxyl radicals, while their protective mechanisms on lipid peroxidation are uncertain.

It has been shown that many antioxidants are known to exert dual actions on oxidative stress, and the responses of tissue components against oxidants may be different. From these respects, the present study investigated antioxidant abilities of ambroxol, rutin, glutathione an harmaline on several tissue components, and by which mechanisms they protect tissue components against oxidative attack were examined and compared. The present study reports the different protective effects of ambroxol, rutin, glutathione and harmaline on the oxidative damages of microsomal lipids, hyaluronic acid, cartilage collagen and immunoglobulin G.

MATERIALS AND METHODS

Chemicals

Ambroxol, rutin, glutathione (reduced form, GSH), harmaline, catalase (from bovine liver), dimethyl sulfoxide (DMSO), 1,4-diazabicyclo (2,2,2) octane (DABCO), hyaluronic acid (Grade III from human umbilical cord), cartilage collagen (from bovine trachea), human immunoglobulin G (IgG), xanthine, xanthine oxidase, ascorbic acid, NADPH, 2-thiobarbituric acid (TBA), ferricytochrome c, 2-α deoxyribose, 1,3-diphenylisobenzofuran (DPBF) and *o*-phenanthroline were purchased from Sigma-Aldrich Ltd. (St. Louis, M.O., U.S.A.). Other chemicals were of analytical grade.

Ambroxol is expressed as AMB, rutin as RUT, glutathione as GSH and harmaline as HAR.

Preparation of Rat Liver Microsomes

The microsomal fraction was prepared from the rat liver by the method of Appel *et al.* (1981). Male Sprague-Dawley rats weighing about 150 g were used and were killed by decapitation. Livers removed were placed in ice cold buffer I (0.25 M sucrose, 0.02 M Tris-HCl, 0.5 mM EDTA, pH 7.4) and were homogenized in 4 volume of buffer I using polytron homogenizer (Brinkman, Model PT-20). After removal of cell debris, nuclei and mitochondria by centrifugation for 10 min at 500, 1,000 and 10,000 g, the microsomal fraction was pelleted by centrifugation at 100,000 g for 60 min. The resultant pellets were suspended in homogenizing buffer II (0.12 M KCl, 0.05 M Tris-HCl, pH 7.4) and were centrifuged at 20,000 g for 20 min. The supernatants were recentrifuged at 100,000 g for 60 min, and the pellets were resuspended in buffer II. Protein concentration was determined using the Bradford method as described in the Bio-Rad protein assay kit.

Measurement of Lipid Peroxidation

Lipid peroxidation of microsomes was estimated measuring malondialdehyde concentration by thiobarbituric acid method. Liver microsomes (0.2 mg protein/ml) were suspended in the reaction mixture consisting of 150 mM KCl, iron, reducing agents and 50 mM NaH_2PO_4, pH 7.4. After 30 min incubation, the reaction was finished by adding 1.0 ml of 1% TBA in 50 mM NaOH and 1.0 ml of 2.8% trichloroacetic acid. The absorbance was measured at 532 nm (Gutteridge, 1981; Gutteridge *et al.*, 1982). The concentration of malondialdehyde was expressed as nmol/mg protein using the molar extinction coefficient of 1.56×10^5/M/cm (Buege and Aust, 1978).

Measurement of Hyaluronic Acid Degradation

Viscosity of hyaluronic acid was measured in a modified Cannon capillary viscometer, which is regulated by vacuum pressure. The reaction mixtures contained 1 mg/ml hyaluronic acid, 150 mM KCl and 50 mM KH_2PO_4 buffer, pH 7.5. The viscosity change of depolymerized hyaluronic acid was measured at 25°C and expressed as a flow time (sec).

Electrophoretic Analysis of Collagen Degradation

Cartilage collagen (200 μg/100 μl) was incubated with 0.2 mM xanthine and 10.5 mU/ml xanthine oxidase in the presence or absence of compounds 4 h at 37°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then performed by the method of Lammeli (1970) on 5% acrylamide slab gels using Mini-Protean II (Bio-Rad). SDS-PAGE was carried out at 40 mA for 75 min. The gels were stained with Coomassie Brilliant Blue R-250.

HPLC Analysis of IgG

IgG (2 mg/ml) was incubated with 0.2 mM xanthine and 8.5 mU/ml xanthine oxidase in a total volume of 200 μl for

20 h at 37°C. After incubation, the reaction mixtures were centrifuged for 5 min at 14,000 rpm and then were injected into high performance liquid chromatography (HPLC). The HPLC system was composed of Waters U6K injector, 501 pump, automated gradient controller, 484 turnable absorbance detector and 745B integrator. The column was Bio-Sil SEC250-5 (BIORAD 300 mm×7.8 mm). The mobile phase was consisted of 0.1 M sodium phosphate, 0.15 M NaCl and 0.01 M sodium azide, pH 6.8. The flow rate was 1.0 ml/min, and 10 μl of sample was injected into the column. Absorbance change was measured at 280 nm.

Assay of Scavenging Action on Reactive Oxygen Species

Superoxide anion produced was measured by reduction of ferricytochrome c. Measurement of superoxide production was done in 2 ml of reaction mixtures containing 75 μM ferricytochrome c, 0.2 mM xanthine, 10.5 mU/ml xanthine oxidase, antioxidants, 150 mM KCl and 50 mM NaH_2PO_4 buffer, pH 7.4. The amount of reduced cytochrome c was determined using an extinction coefficient of $2.1\times10^4 M^{-1}cm^{-1}$ at 550 nm (Cohen and Chovaniec, 1978).

The concentration of H_2O_2 was measured by the method of Koh *et al.* (1998). The reaction mixtures contained 120 mM KCl, 0.1 mM H_2O_2, 10 μM sodium azide and 50 mM Tris-HCl, pH 7.4. After incubation, the stopping solution (25 mg/ml of potassium biphthalate, 2.5 mg/ml NaOH, 82.5 mg/ml potassium iodide and 0.25 mg/ml ammonium molybdate) was added to the above mixture, and the absorbance change was measured at 350 nm.

Amount of hydroxyl radical generated was estimated from TBA reactivity of 2-α deoxyribose (Gutteridge, 1981; Halliwell and Gutteridge, 1981). The reaction mixtures consisted of 2 mM 2-α deoxyribose, 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2, 100 μM ascorbate, (or 0.2 mM xanthine and 42.1 mU/ml xanthine oxidase), 150 mM KCl and 50 mM NaH_2PO_4 buffer, pH 7.4. After 30 min incubation, the reaction was finished by addition of 1.0 ml of 1% TBA in 50 mM NaOH and 1.0 ml of 2.8% trichloroacetic acid. The absorbance change was measured at 532 nm, and the fluorescence was measured at the wavelengths of excitation, 532 nm and emission, 553 nm.

Quenching effects of designated compounds on singlet oxygen were measured bleaching of DPBF by U.V. irradiation (Sang Kyo GL-15 fluorescent bulb, 254 nm of wavelength, Japan). The reaction mixtures consisted of 67 μM DPBF and 50 mM NaH_2PO_4, pH 7.4.

Chelating actions of the compounds on ferrous ion were assayed measuring formation of ferrous ion-*o*-phenanthroline complex (Aust *et al.*, 1993). Oxidation of ferrous ion was measured in reaction mixtures containing 1 mM *o*-phenanthroline and was read at 512 nm.

Data Analysis

The results obtained in various experiments were analysed for level of significance using Student's t-test for paired data. The p value represents significant different data from no addition of antioxidants, oxidant scavengers or iron chelator. The data in Fig. 3, 5 and 6 were expressed as % inhibition. Data were expressed as means±S.D..

RESULTS

Inhibition of Microsomal Lipid Peroxidation by Ambroxol, Rutin, Glutathione and Harmaline

Iron is thought to catalyze oxidative tissue damages by formation of reactive oxidants. Liver microsomal lipids were oxidized by addition of $FeSO_4$ and ascorbate. Antioxidative abilities of several compounds in this experimental condition were compared. Fig. 1 shows that the 10 μM $FeSO_4$ and 100 μM ascorbate-induced lipid peroxidation was inhibited by 50 μM of ambroxol, rutin, glutathione and harmaline by 87.9%, 89.1%, 17.2% and 66.4%, respectively.

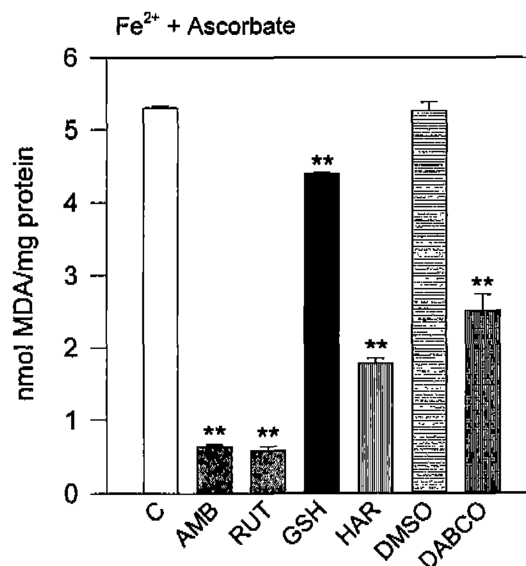

Fig. 1. Inhibition of Fe^{2+} and ascorbate-induced lipid peroxidation by ambroxol, rutin, glutathione and harmaline. Liver microsomes were incubated with 10 μM $FeSO_4$ and 100 μM ascorbate in the presence or absence (C) of 50 μM compounds and 10 mM oxidant scavengers. Values are means±S.D., n=4. $^{**}p<0.01$ by Student's t-test.

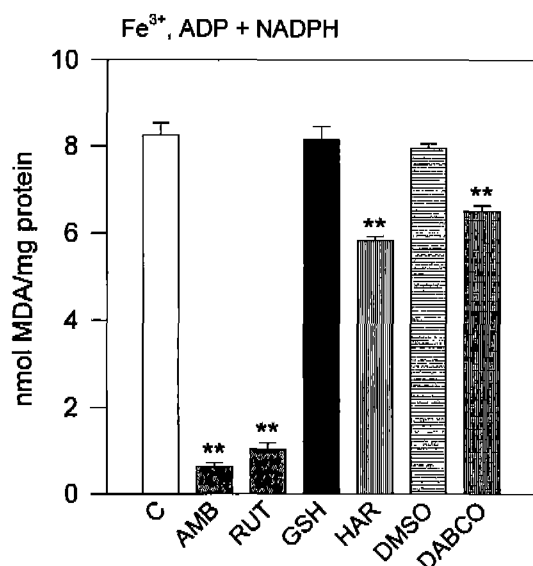

Fig. 2. Inhibitory effects of ambroxol and rutin on lipid peroxidation by Fe^{3+}, ADP and NADPH. Liver microsomes were incubated with 10 μM $FeCl_3$, 160 μM ADP and 100 μM NADPH in the presence or absence (C) of 50 μM compounds and 10 mM oxidant scavengers. Values are means±S.D., n=4-5. **$p<0.01$ by Student's *t*-test.

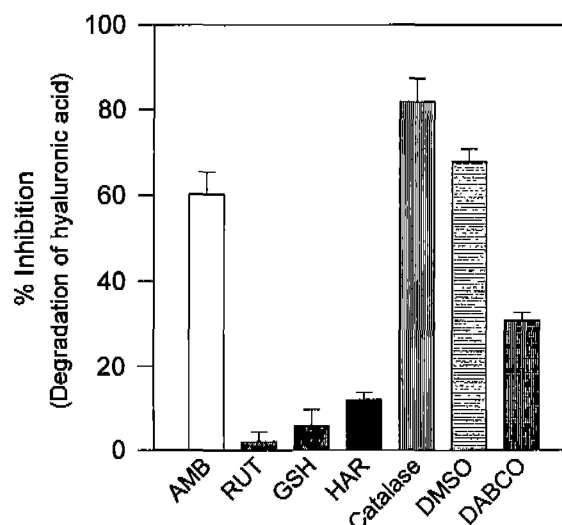

Fig. 3. Inhibition of Fe^{2+}, H_2O_2 and ascorbate-induced degradation of hyaluronic acid by ambroxol. Hyaluronic acid (1 mg/ml) was incubated with 10 μM $FeSO_4$, 500 μM H_2O_2 and 100 μM ascorbate in the presence of 100 μM test compounds, 30 μg/ml catalase, 10 mM DMSO and 10 mM DABCO for 1 h. Viscosity of intact hyaluronic acid was 29.5±0.4 sec (means±S.D., n=4). Equal concentrations of $FeSO_4$, H_2O_2 and ascorbate had a decreased viscosity of 11.0±0.6 sec. Values are expressed as % inhibition caused by the compounds and are means±S.D., n=4.

Depressant actions of compounds on the enzymatic lipid peroxidation of microsomes were studied. Ambroxol, rutin and harmaline (50 μM) showed protective effects on lipid peroxidation by 10 μM $FeCl_3$, 160 μM ADP and 100 μM NADPH, whereas the effect of glutathione was not observed (Fig. 2). The rank order of inhibitory potency was ambroxol>rutin>harmaline. At equal concentration, the protective effect of glutathione on peroxidation of microsomal lipids in both systems was less than that of the other antioxidants.

Nonenzymatic and enzymatic peroxidations of lipids were decreased by DABCO, a quencher of singlet oxygen, but were not affected by DMSO, a scavenger of hydroxyl radical. Inhibitory effect of DABCO on enzymatic lipid peroxidation was smaller than that on nonenzymatic lipid peroxidation.

Inhibitory Effects of Ambroxol and Harmaline on Hyaluronic Acid Degradation

Reactive oxygen species react with hyaluronic acid to cause depolymerization and evoke a decrease of viscosity. The protective actions of several compounds on the Fe^{2+}, H_2O_2 and ascorbate-induced degradation of hyaluronic acid were investigated and compared. Fig. 3 shows that ambroxol and harmaline (100 μM) inhibited the degradation of hyaluronic acid by 10 μM Fe^{2+}, 500 μM H_2O_2 and 100 μM ascorbate. Equal concentrations of rutin and glutathione did not attenuate the degradation of hyaluronic acid. Inhibitory effect of ambroxol was greater than that of harmaline.

The degradative action of Fe^{2+}, H_2O_2 and ascorbate on hyaluronic acid was prevented by addition of 30 μg/ml catalase, 10 mM DMSO and 10 mM DABCO. The protective actions of oxidant scavengers on the oxidative damage of hyaluronic acid were dissimilar to their effects on lipid peroxidation of microsomes.

Effects of the Compounds on Degradation of Cartilage Collagen

The oxidative degradation of cartilage collagen was observed by an electrophoretic analysis. Cartilage collagen (200 μg/100 μl) was incubated with 0.2 mM xanthine and 10.5 mU/ml xanthine oxidase in the presence of 100 μM compounds or not for 4 h at 37°C. In this experimental condition, cleavage of single major band (approximate Mr. 120,000) was detected. As shown in Fig. 4, 100 μM of ambroxol, rutin, glutathione and harmaline did not show any protective effect on collagen degradation. Protective effects were also not observed at 1 h incubation in the same reaction mixture (data not shown). At given concentrations, ambroxol, glutathione and harmaline did not show inhibitory

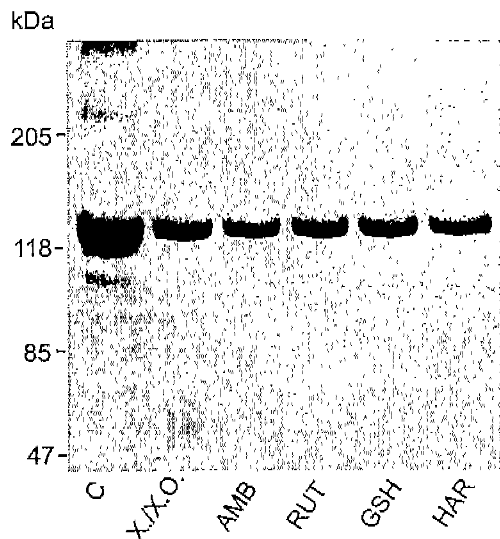

Fig. 4. Effects of the compounds on degradation of cartilage collagen by xanthine and xanthine oxidase. Lane 1, intact collagen, and lane 2, no addition of test compounds; lane 3, 100 μM of ambroxol; lane 4, rutin; lane 5, glutathione and lane 6, harmaline with 0.2 mM xanthine and 10.5 mU/ml xanthine oxidase. Reference proteins were myosin (Mr, 205,000), *β*-galactosidase (Mr, 118,000), bovine serum albumin (Mr, 85,000) and ovalbumin (Mr, 47,000).

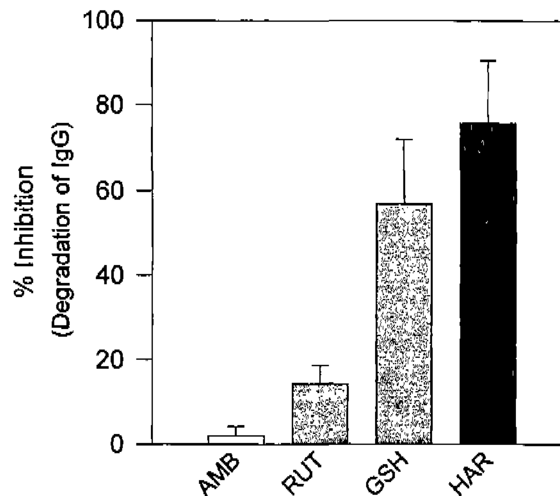

Fig. 5. Inhibition of xanthine and xanthine oxidase-induced degradation of IgG by glutathione and harmaline. IgG (2 mg/ml) was incubated with 0.2 mM xanthine and 8.5 mU/ml xanthine oxidase in the presence of test compounds. Values are expressed as % inhibition caused by the compounds and are means±S.D., n=3.

actions on xanthine oxidase activity. Despite inhibitory action of rutin on xanthine oxidase activity (33.9%), the collagen degradation by xanthine and xanthine oxidase was not inhibited by rutin.

Inhibitory Effects of Rutin, Glutathione and Harmaline on Degradation of IgG

Immunoglobulin (IgG) was incubated with 0.2 mM xanthine and 8.5 mU/ml xanthine oxidase for 20 h at 37°C. Injection of 10 μl of IgG (2 mg/ml) into HPLC column showed a 1,079,251±81,848 (mean±S.D., n=3) area of single peak. Treatment of IgG with xanthine and xanthine oxidase caused a decreased area of single peak and showed a 833,802±55,362 of area. Incubation with 50 μM of rutin, glutathione and harmaline decreased the degradation of IgG induced by xanthine and xanthine oxidase (Fig. 5). In this reaction, inhibitory effect of rutin may be ascribed to its suppressive action on xanthine oxidase activity. Harmaline exhibited a most potent protective effect on the oxidative damage of IgG, while ambroxol did not affect degradation of IgG.

Table I. Scavenging effects of rutin and glutathione on superoxide anion and hydrogen peroxide

Compounds	Superoxide anion (nmol/min)	Hydrogen peroxide (% removing effect)
No addition	0.245±0.009	-
Ambroxol 100 μM	0.239±0.001	1.6
Rutin 100 μM	0.149±0.016**	9.4
Glutathione 100 μM	0.244±0.005	100.0
Harmaline 100 μM	0.247±0.001	1.3

Superoxide anion was assayed (means±S.D.) in the reaction mixture consisted of 0.2 mM xanthine and 10.5 mU/ml xanthine oxidase. H_2O_2 was incubated with compounds for 30 min, and values are means of 3 experiments. **p<0.01 by Student's t-test.

Scavenging Effects of the Compounds on Reactive Oxygen Species

The scavenging actions of compounds on superoxide anion were measured with reduction of ferricytochrome c by xanthine and xanthine oxidase. Table I shows that ambroxol, glutathione and harmaline (all 100 μM) did not exhibited scavenging actions on superoxide anion produced by 0.2 mM xanthine and 10.5 mU/ml xanthine oxidase. The removing action of rutin on superoxide anion may be attributable to its suppressive action on xanthine oxidase activity.

Hydrogen peroxide is considered as a precursor for more reactive oxygen species. H_2O_2, which was effectively removed by 10 μg/ml catalase, was decomposed by adding 100 μM of glutathione. A glutathione markedly decomposed H_2O_2, and within 1 min post-addition, H_2O_2 (100 μM) was com-

Table II. Scavenging effects of ambroxol and harmaline on hydroxyl radical

Compounds	Deoxyribose degradation	
	Fe^{3+}, EDTA, H_2O_2, ascorbate	X/X.O.
No addition	1.026±0.011	76.1±2.1
Ambroxol 100 μM	0.701±0.014**	57.0±0.8**
Rutin 100 μM	0.823±0.021**	46.4±3.2**
Glutathione 100 μM	0.962±0.021**	63.9±4.4*
Harmaline 100 μM	0.716±0.038**	59.2±3.7**
DMSO 10 mM	0.099±0.004**	56.3±5.3**
Sodium formate 10 mM	0.191±0.006**	-

2-α-Deoxyribose (2 mM) was treated either with 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2 and 100 μM ascorbate (expressed as △absorbance) or with 0.2 mM xanthine and 42.1 mU/ml xanthine oxidase (expressed as fluorescence unit). Values are means±S.D., n=4-5. *$p<0.05$, **$p<0.01$ by Student's t-test.

pletely decomposed. However, the same concentrations of ambroxol, rutin and harmaline did not show removing actions on H_2O_2.

The scavenging actions of the compounds on hydroxyl radical were assayed by the degradation of 2-α deoxyribose either by 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2 and 100 μM ascorbate or by 0.2 mM xanthine and 42.1 mU/ml xanthine oxidase. The production of hydroxyl radical in both systems was inhibited by addition of ambroxol, rutin, glutathione and harmaline at 100 μM (Table II). In contrast to Fe^{3+}, EDTA, H_2O_2 and ascorbate system, the deoxyribose degradation by xanthine and xanthine oxidase system was much less sensitive to hydroxyl radical scavengers, DMSO and sodium formate.

U.V. irradiation evoked conversion of DPBF to dibenzoyl benzene. Fig. 6 showed inhibitions of U.V. irradiation-induced DPBF oxidation by ambroxol and harmaline (100 μM), while equal concentrations of rutin and GSH did not revealed detectable effects.

Iron chelating actions of compounds used were examined using o-phenanthroline. As can be seen in Fig. 7, rutin (100 μM) attenuated autoxidation of Fe^{2+}, which was markedly inhibited by 1 mM DTPA, a iron chelator, and exhibited a chelating action on iron. On the other hand, same concentrations of ambroxol and harmaline did not show iron chelating actions.

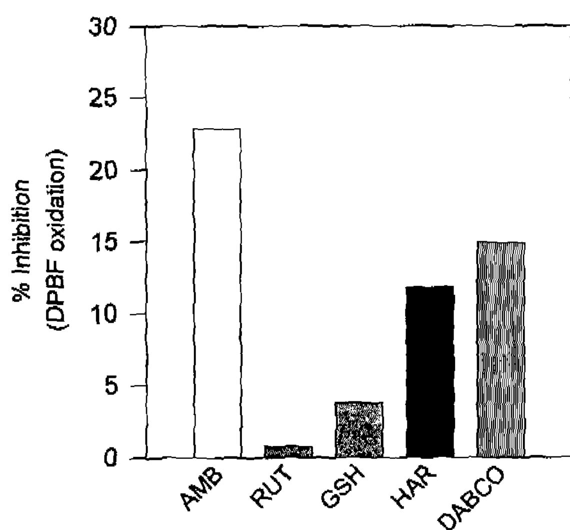

Fig. 6. Effects of ambroxol and harmaline on DPBF oxidation. The oxidation of DPBF in the presence of 100 μM test compounds or 20 mM DABCO was induced by U.V. irradiation. The data represent mean values of three experiments.

DISCUSSION

An increased production of free radicals in pathologic and drug toxicity states contributes to the damages of tissue components. The reactive oxygen species-induced cytotoxicity is catalyzed by metal ions, particularly iron (Halliwell and Gutteridge, 1989b). Oxidoreduction of metal ions plays a major role in the damages of cellular macromolecules, including lipid peroxidation. Iron released from iron- containing pro-

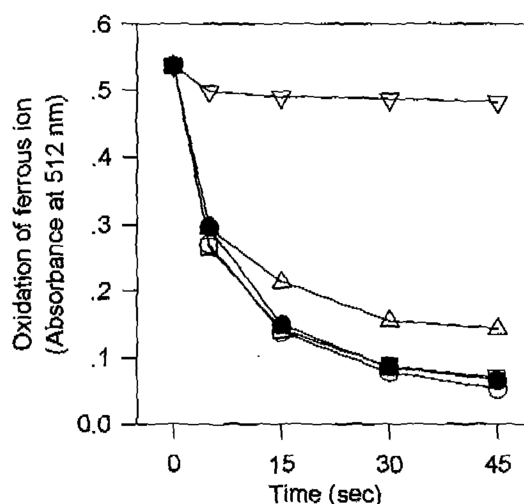

Fig. 7. Effects of the compounds on oxidation of ferrous ion. Autoxidation of ferrous ion in the presence of 100 μM test compounds or 1 mM DTPA was measured as described in Materials and Methods. ○, no addition; ●, ambroxol; △, rutin; □, harmaline; ▽, DTPA. The data represent mean absorbances of three experiments.

teins effectively catalyzes formation of highly reactive oxidants, such as hydroxyl radical and singlet oxygen (Fong *et al.*, 1976; Halliwell and Gutteridge, 1989b). The autoxidation of iron appears to produce reactive oxygen metabolites, such as hydroxyl radical, perferryl ion and ferryl ion.

Many antioxidants, including flavonoids and GSH, have been reported to have both antioxidant abilities and pro-oxidant actions. β-Carboline alkaloids, including harmaline, are reported to protect tissue components against the oxidants, but its antioxidant mechanism has not been elucidated. In this respect, antioxidant abilities of ambroxol, rutin, glutathione and harmaline on the oxidative damages of various tissue components were investigated and compared.

Lipid peroxidation of liver microsomes by Fe^{2+} and ascorbate was inhibited by catalase and DABCO, while the peroxidative action of Fe^{3+}, ADP and NADPH was inhibited by DABCO only. Thus, the peroxidative action of Fe^{2+} plus ascorbate on microsomal lipids may different from the action of Fe^{3+}, ADP and NADPH. No inhibitory effect of DMSO on both Fe^{2+} and ascorbate-and Fe^{3+}, ADP and NADPH-induced lipid peroxidation supports that iron-oxygen complexes are involved in the iron and reducing agents-induced lipid peroxidation rather than highly reactive oxygen species, such as hydroxyl radical (Halliwell and Gutteridge, 1989b). The peroxidative actions of both systems were inhibited by the antioxidants used in the present study, except no effect of glutathione on the peroxidative action of Fe^{3+}, ADP and NADPH. Inhibitory effects of ambroxol and rutin were similar each other and were greater than those of glutathione and harmaline.

Protective effects of ambroxol, rutin, glutathione and harmaline on the oxidative damages of hyaluronic acid and collagen were studied. Hyaluronic acid exposed to Fe^{2+}, H_2O_2 and ascorbate causes depolymerization and shows a decrease of viscosity (Lee *et al.*, 1985). In contrast, to microsomal lipids, the oxidative degradation of hyaluronic acid induced by Fe^{2+}, H_2O_2 and ascorbate appears to be ascribed to H_2O_2 and hydroxyl radical. Ambroxol and harmaline inhibited degradation of hyaluronic acid induced by Fe^{2+}. H_2O_2 and ascorbate, while the effects of rutin and glutathione were not detected. The marked protective action of ambroxol on the microsomal lipids and hyaluronic acid indicates that it has potent and effective antioxidant ability. The compounds all (100 μM) did not affect the degradation of cartilage collagen caused by xanthine and xanthine oxidase. However, the same concentrations of ambroxol and harmaline are reported to inhibit the degradation of cartilage collagen induced by Fe^{2+}, H_2O_2 and ascorbate (Cho *et al.*, 1995; Koh *et al.*, 1998). On the other hand, the xanthine/xanthine oxidase-induced degradation of IgG was inhibited by rutin, glutathione and harmaline, whereas ambroxol did not show an inhibitory effect. In addition, responses of oxidatively damaged tissue components to oxidant scavengers were also different. These findings indicate that biological effects of antioxidants on the oxidative stress-induced cytotoxicity may be different depend on the kind of tissue component. Cartilage collagen was relatively resistant to oxidative attack, and its destruction required long exposure time to oxidants. Thus, it is suggested that the degradation of cartilage collagen does not well respond to antioxidants. In these experimental conditions, ambroxol exhibited most effective and protective action on the oxidative damages of lipids and hyaluronic acid. Rutin showed effective protective action on oxidative damage of lipids, glutathione on that of IgG and harmaline on those of lipids and IgG.

Antioxidants are thought to exert their protective actions on oxidative tissue damages by scavenging action on reactive oxygen species and chelating action on metal ions (Havsteen, 1983; Gillissen *et al.*, 1997). The scavenging actions of ambroxol, rutin, glutathione and harmaline on reactive oxygen species were examined. Superoxide anion and H_2O_2 are generally accepted as less reactive oxygen species to initiate tissue damages, including lipid peroxidation. The present data demonstrate that ambroxol, rutin and harmaline do not have scavenging effects on superoxide anion and H_2O_2, except remarkable decomposing action of glutathione on H_2O_2.

Highly reactive hydroxyl radical is known to damage most types of cellular macromolecules, except lipids. This radical can be detected sensitively by measuring TBA reactivity of 2-α deoxyribose (Aruoma. 1994). The present data indicate that ambroxol, rutin, glutathione and harmaline have scavenging effects on hydroxyl radical produced either by Fe^{3+}, EDTA, H_2O_2 and ascorbate or by xanthine and xanthine oxidase. Inhibitory effect of rutin on the production of hydroxyl radical by xanthine and xanthine oxidase may be partially attributed to its inhibitory action on xanthine oxidase activity. Deoxyribose degradation induced by Fe^{3+}, EDTA, H_2O_2 and ascorbate reaction system and by xanthine and xanthine oxidase system is thought to differently respond to hydroxyl radical scavengers. Inhibition of U.V. irradiation-induced oxidation of DPBF by ambroxol and harmaline postulates their

scavenging actions on singlet oxygen. From the present data, the compounds, ambroxol, rutin, GSH and harmaline may have different scavenging actions on reactive oxygen species.

In agreement with previous data (Havsteen, 1983; Sestili *et al.*, 1998), rutin, a flavonoid, exhibited a chelating action on iron and may provide partly a protective effect on lipid components against oxidative attack through chelating action. However, in Fe^{2+}, H_2O_2 and ascorbate-induced degradation of hyaluronic acid, rutin did not show an inhibitory effect, and a minor role of iron in this degradation was suggested. Meanwhile, ambroxol, GSH and harmaline appear to exert protective actions on oxidative tissue damages by removing highly reactive oxidants, which are independent upon metal chelation.

In conclusion, the protective effects of antioxidants on oxidative stress-induced tissue injuries may be exerted differently depend on kind of tissue component and free radical.

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