Protective Effects of Baicalein against Cell Damage by Reactive Oxygen Species

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Baicalein (5,6,7-trihydroxy-2-phenyl-4*H*-1-benzopyran-4-one), a naturally occurring flavonoid, was found to prevent human dermal fibroblast cell damage induced by reactive oxygen species such as hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (BuOOH) and superoxide anions ($\cdot O_2^-$) in a concentration-dependent manner, and was more effective than the iron chelator, deferoxamine, hydroxyl radical ($\cdot$OH) scavengers such as dimethyl sulfoxide (DMSO) and ethanol (EtOH), the lipid peroxidation chain blocker, α-tocopherol (Vit. E) and the xanthine oxidase inhibitor, allopurinol. To probe the mechanism of cell defense, the reaction of baicalein with oxygen free radicals was investigated using electron spin resonance (ESR) spectrometry. Baicalein decreased the signal intensities due to the 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) spin adducts of $\cdot$OH, $\cdot O_2^-$ and *tert*-butyl peroxyl (BuOO$\cdot$) radicals in a concentration-dependent manner. The IC_{50} values, which are the 50% inhibition concentrations of baicalein for the free radicals, were 10, 45 and 310 μM, respectively. These results suggested that baicalein possesses free radical scavenging ability which prevents the fibroblast damage induced by these free radical species.

Key words baicalein; human dermal fibroblast; active oxygen scavenging; ESR; spin-trapping method

Baicalein (5,6,7-trihydroxy-2-phenyl-4*H*-1-benzopyran-4-one) is one of the naturally occurring flavonoids in *Scutellaria baicalensis* GEROGI. It has been reported that baicalein scavenges reactive oxygen species such as hydroxyl radicals ($\cdot$OH), superoxide anion radicals ($\cdot O_2^-$)[1] and singlet oxygens.[2] When a hypoxanthine (HX)–xanthine oxidase (XOD) system was used for generation of $\cdot O_2^-$, baicalein inhibited XOD.[3] This observation is consistent with the earlier study,[4] in which baicalein behaved as a non-competitive inhibitor of XOD. Baicalein has also been shown to strongly inhibit iron-dependent lipid peroxidation in microsomes[5,6] and mitochondria.[7] In addition, glomerular mesangial cell damage induced by hydrogen peroxide (H_2O_2) was inhibited by baicalein, whereas this compound did not react with H_2O_2.[8] Since an iron chelator was also found to attenuate the cell damage,[8] the involvement of $\cdot$OH, formed through the Fenton reaction, in cell damage is likely. Therefore, the reactivity of baicalein towards reactive oxygen species and inhibition of lipid peroxidation is expected to protect cells from oxidative stress by these species.

The problem of ozone layer depletion has become a matter of great concern.[9] Since this layer plays an important role as a UV filter, the increasing UV light acts directly on skin and causes severe complications such as premature aging, called "photoaging".[10] H_2O_2 has been reported to accumulate in skin fibroblasts exposed to UV irradiation and undergoes Fenton reaction to produce $\cdot$OH, which in turn damages the cells.[11—13] Therefore, in the present study we examined whether baicalein protects human dermal fibroblasts from damage induced with reactive oxygen species such as H_2O_2, *tert*-butyl hydroperoxide (BuOOH) and $\cdot O_2^-$, compared with the effects of an iron chelator and free radical scavengers. In addition, we studied the scavenging effects of baicalein on $\cdot$OH, $\cdot O_2^-$ and *tert*-butyl peroxyl (BuOO$\cdot$) radicals by using the ESR method to investigate the possible mechanism of action of baicalein. On the basis of the obtained results, the relationship between radical scavenging activity and the cell protective effect of baicalein is discussed.

Experimental

Materials Baicalein was purchased from Fluka Chemie AG (Switzerland) and used without further purification. Deferoxamine mesylate and methemoglobin (MetHb) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). HX, XOD, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA), ferrous sulfate heptahydrate ($FeSO_4\cdot 7H_2O$), hydrogen peroxide (H_2O_2) and allopurinol were from Wako Pure Chemical Ind. (Tokyo, Japan). *dl*-α-Tocopherol (Vit. E), L-glutamine and fetal bovine serum (FBS) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). *tert*-Butyl hydroperoxide (BuOOH) was obtained from Katayama Chemical Ind. (Osaka, Japan) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) from Labotec Company (Tokyo, Japan). All other chemicals used were of commercially available analytical reagent grade.

Culture Method for Fibroblasts Human dermal fibroblasts were maintained at 37 °C under 5% CO_2 in a culture medium containing Dulbecco's modified eagle medium supplemented with glutamine (0.1 mM) and FBS (5%, v/v) as reported.[14]

Evaluation of Baicalein for Protecting Human Fibroblasts Exposed to Reactive Oxygen Species Fibroblasts were distributed onto 96-well microplates at a density of 2×10^4 per well and cultured under 5% CO_2 at 37 °C for 24 h. The medium was then replaced with medium containing various concentrations of baicalein. After 1.5 h incubation, the cells were washed with Hanks balanced salt solution (HBS) containing $CaCl_2$ (1.26 mM) and $MgCl_2$ (0.81 mM), then exposed to HBS containing H_2O_2 (10 mM) or BuOOH (50 mM) for 1 h or a mixture of XOD (10 munits/ml) and HX (1.5 mM) for 1.5 h. Reactive oxygen species-induced fibroblast damage was expressed as survival rates, estimated by the neutral red method as described previously.[15] Baicalein was dissolved in 3 mol eq of aqueous NaOH. UV spectra of baicalein under such conditions were constant at least for 1.5 h. Vit. E was dissolved in ethanol (EtOH, 170 mM final concentration). Solutions of baicalein and other test compounds were prepared with culture medium or HBS just before use.

Evaluation of Reactivity of Baicalein with Free Radicals ESR spectra were recorded on a JES-FR30 free radical monitor (JEOL Ltd., Tokyo, Japan) at room temperature (22 °C) under the following conditions: magnetic field 341±5 mT, field modulation frequency 100 kHz, modulation amplitude width 0.1 mT, time constant 0.1 s, output power 4 mW and sweep time 2 min. Manganese(II) oxide doped in magnesium oxide was used as an external standard. Signal intensity due to the free radical-spin adducts was normalized as relative signal height against the standard signal of Mn(II).

$\cdot$OH was generated by the Fenton system. The reaction system for evaluating the $\cdot$OH scavenging effect of baicalein consisted of $FeSO_4\cdot 7H_2O$

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(2.25 μM), H_2O_2 (22.5 μM), various amounts of baicalein (in acetonitrile) and DMPO (94.5 mM) in a final volume of 200 μl. H_2O_2 was added to start the reaction at 30 s before monitoring ESR spectra.

$\cdot O_2^-$ was generated by the dimethyl sulfoxide (DMSO)–alkaline method as described previously.[16] Briefly, DMSO containing water (1%, v/v) and NaOH (5 mM) was allowed to stand at 25 °C for 30 min to give $\cdot O_2^-$ solution. The reaction mixture (240 μl final volume) for evaluating the $\cdot O_2^-$ scavenging effect of baicalein consisted of the $\cdot O_2^-$ solution, sodium potassium phosphate buffer (100 mM, pH 7.4), DTPA (0.1 mM) and DMPO (112.5 mM) with or without baicalein (in DMSO). ESR spectra were recorded at 30 s after addition of the $\cdot O_2^-$ solution (100 μl). In some experiments, HX (0.42 mM) and XOD (100 munits/ml) were used to produce $\cdot O_2^-$; other conditions were the same as described above.

tert-Butyl peroxyl radical (BuOO·) was prepared by the method of Akaike *et al.*[17] Briefly, reaction mixture (200 μl final volume) consisted of BuOOH (25 mM), MetHb (0.25 mg/ml), sodium potassium phosphate buffer (100 mM, pH 7.4), DTPA (0.025 mM), DMPO (11.25 mM) and various concentrations of baicalein (in DMSO). BuOOH was added to initiate reaction at 30 s before ESR measurement.

Microsomal Lipid Peroxidation Rat liver microsomal lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS), and was induced by Fe^{3+} and ascorbic acid as described previously.[6]

Statistics Statistical analysis was performed by Student's *t* test.

Results

Protective Effects of Baicalein on Cell Damage Induced by Reactive Oxygen Species We examined whether baicalein has ability to prevent damage to human dermal fibroblast cells induced by reactive oxygen species such as H_2O_2, BuOOH and $\cdot O_2^-$, and compared the results with those of an iron chelator and radical scavengers. As shown in Fig. 1A, cell damage by H_2O_2 was inhibited by baicalein, depending upon the concentration, although baicalein alone did not show any effect on cell viability under the conditions examined (data not shown). Addition of deferoxamine (10 mM) as an iron chelator, or DMSO (700 mM) or EtOH (170 mM) as $\cdot$OH scavengers also significantly protected against cell damage (Fig. 1B), suggesting a possible role for $\cdot$OH generated through Fenton reaction in the cell damage process. From

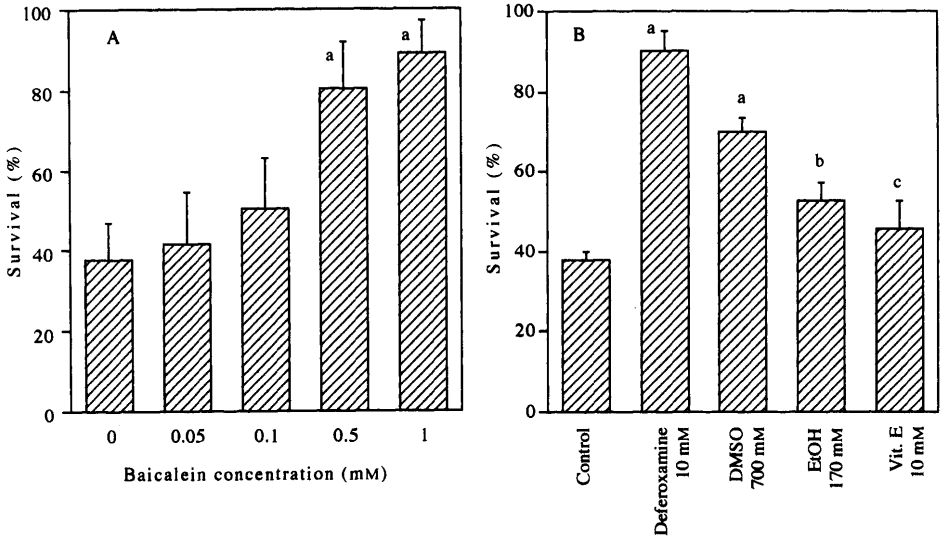

Fig. 1. Effect of Baicalein, Deferoxamine and Free Radical Scavengers on H_2O_2-Induced Fibroblast Damage

Fibroblasts were preincubated with baicalein (A) or other compounds (B) for 1.5 h before exposure to H_2O_2 (10 mM) for a further 1 h as described in the Experimental. Each column represents the mean±S.D. of four individual experiments. a, $p<0.01$ *vs.* control; b, $p<0.05$ *vs.* control; c, $p>0.05$ *vs.* EtOH group.

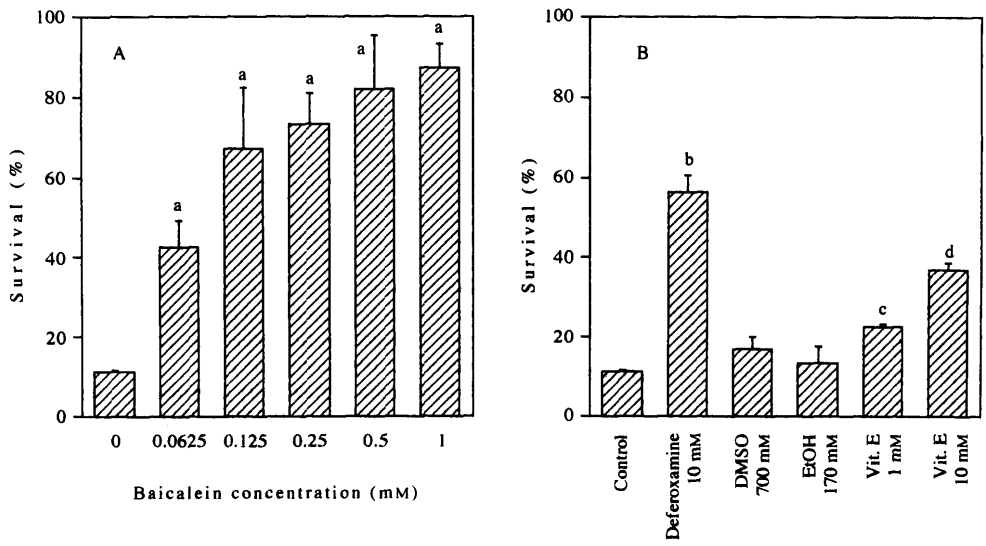

Fig. 2. Effect of Baicalein, Deferoxamine and Free Radical Scavengers on BuOOH-Induced Fibroblast Damage

Baicalein (A) and other compounds (B) were preincubated with fibroblasts for 1.5 h before exposure to BuOOH (50 mM) for a further 1 h as described in the Experimental. Each column represents the mean±S.D. of four individual experiments. a, $p<0.01$ *vs.* control; b, $p<0.05$ *vs.* control; c, $p<0.05$ *vs.* EtOH group; d, $p<0.01$ *vs.* EtOH group.

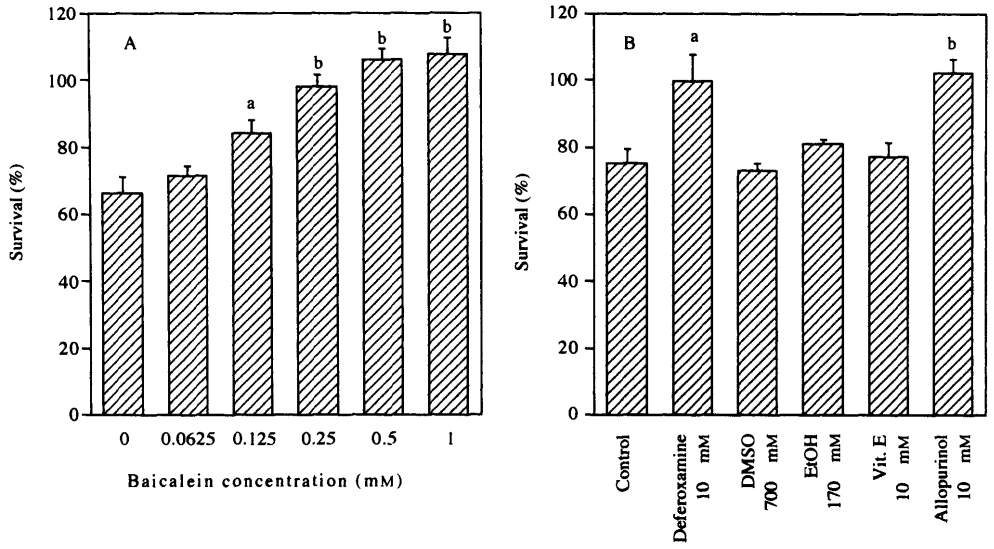

Fig. 3. Effect of Baicalein, Deferoxamine and Free Radical Scavengers on Fibroblast Damage Induced by HX–XOD

Baicalein (A) and other compounds (B) were preincubated with fibroblasts for 1.5 h before exposure to HX (1.5 mM) and XOD (10 munits/ml) for a further 1.5 h as described in the Experimental. Each column represents the mean±S.D. of four individual experiments. a, $p<0.05$ *vs.* control; b, $p<0.01$ *vs.* control.

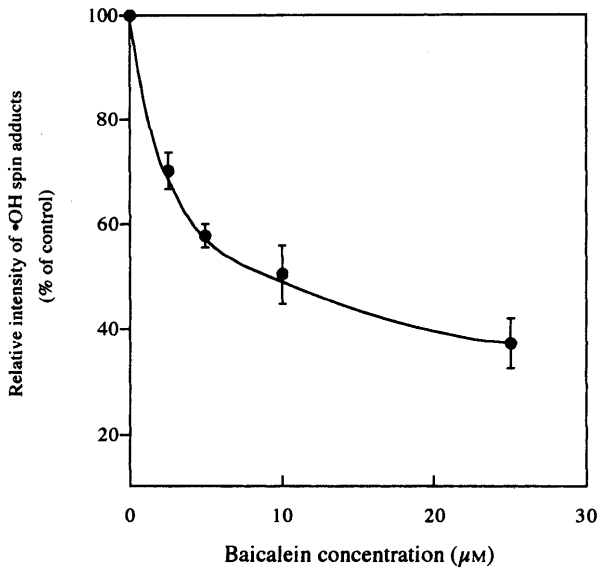

Fig. 4. Effect of Baicalein on •OH

Reaction mixtures contained $FeSO_4 \cdot 7H_2O$ (2.25 μM), H_2O_2 (22.5 μM), DMPO (94.5 mM) and varying concentrations of baicalein. ESR spectra were recorded at 30 s after the addition of H_2O_2. Other conditions were as described in the Experimental section. Each point represents the mean±S.D. of 3 experiments.

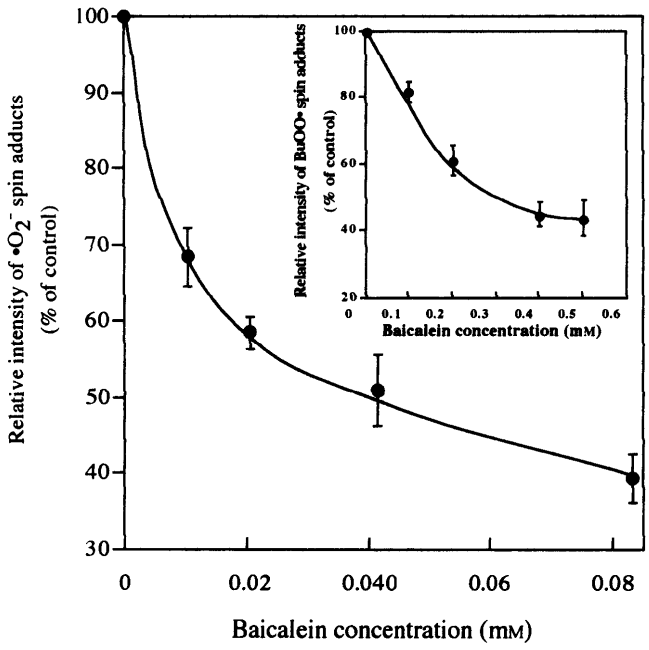

Fig. 5. Effect of Baicalein on $\cdot O_2^-$ and BuOO•

Conditions for $\cdot O_2^-$ and BuOO• (inset) experiments were the same as described in the Experimental section. Each point represents the mean±S.D. of three individual experiments.

these results, it was discovered that baicalein protects the cells against •OH at lower concentrations than deferoxamine and •OH scavengers.

On the other hand, baicalein prevented BuOOH-induced cell damage at low concentrations and cell survival was approximately 70% at 0.125 mM of baicalein (Fig. 2A). Deferoxamine at 10 mM and Vit. E at 1 and 10 mM also exhibited significant protective activities (Fig. 2B). These results suggested the involvement of lipid peroxidation in cell damage under the conditions used.

Baicalein enhanced significantly the survival of fibroblast cells exposed to the $\cdot O_2^-$ generating system of HX–XOD (Fig. 3A). Addition of 10 mM of deferoxamine and allopurinol also protected against cell damage. On the other hand, neither •OH scavengers such as DMSO and EtOH, or Vit. E exhibited any activities against $\cdot O_2^-$ induced cell damage as shown in Fig. 3B. When the cells were simultaneously treated with HX–XOD in the presence of catalase, superoxide dismutase, deferoxamine or •OH scavengers, cell survival increased (data not shown). These results suggested that cell damage mainly resulted from •OH radicals derived by iron-catalyzed Harber–Weiss reaction of $\cdot O_2^-$.[18]

Free Radical Scavenging Activity of Baicalein Since the involvement of •OH, $\cdot O_2^-$ and peroxides in fibroblast cell damage was postulated, the scavenging activity of baicalein against these free radicals was examined using the ESR spin-trapping method. Figure 4 shows the effect of baicalein on •OH formed with the Fenton system. Baicalein decreased the

Table 1. Effect of Baicalein on Fibroblast Damage and Free Radical Species

Compound[a]	Fibroblast survival (%)			IC_{50} (μM) by ESR method			Microsomal lipid peroxidation
	H_2O_2	HX–XOD	BuOOH	·OH	$\cdot O_2^-$	BuOO·	TBARS (Inhibition %)
No addition	37	70	11				0
Baicalein (0.5 mM)	80	100	81	10	45,[b] 1[c]	310	100 at 10 μM
Vit. E (10 mM)	41	76	37	170	>500[c]	141	11 at 50 μM
Gallic acid				78	1[c]	153	
Ascorbic acid				2.3	17.4[c]	106	
Deferoxamine (10 mM)	95	99	56				100 at 10 μM
DMSO (700 mM)	70	69	15				0 at 140 mM
EtOH (170 mM)	55	80	13				0 at 170 mM

a) Concentrations are for fibroblast experiments. *b*) DMSO–alkaline method. *c*) HX–XOD system.

intensity of the DMPO–OH adduct signal in a concentration-dependent manner. The 50% inhibition concentration (IC_{50}) of baicalein against ·OH was found to be 10 μM under the conditions used.

Baicalein also exhibited scavenging activity against $\cdot O_2^-$ generated from DMSO–alkaline solution and BuOO· generated from MetHb and BuOOH (Fig. 5), the IC_{50}'s for $\cdot O_2^-$ and BuOO· were 45 and 310 μM, respectively.

Table 1 summarizes the effects of baicalein on cell damage caused by the three reactive oxygen species, IC_{50} values estimated by ESR spin trapping and TBARS values. Baicalein showed stronger scavenging or antioxidative activities against ·OH and $\cdot O_2^-$ than the other well-known scavengers in terms of IC_{50} values.

Discussion

Since human fibroblast cells have been shown to be sensitive to reactive oxygen species,[14] we used these cells in our present study. Low concentrations of baicalein were found to protect human dermal fibroblast cells from damage by reactive oxygen species such as H_2O_2, BuOOH and $\cdot O_2^-$ (Figs. 1A, 2A, 3A). H_2O_2-induced cell damage was partially prevented by relatively high concentrations of deferoxamine (10 mM) and by ·OH scavengers such as DMSO and EtOH (700 and 170 mM, respectively) (Fig. 1B). H_2O_2 has been reported to penetrate through cell membranes[19] and accumulation of intracelluar H_2O_2 thereby accelerates ·OH formation, which in turn damages the fibroblast cells.[12] Therefore, intracellular formation of ·OH under the present conditions should be considered. Since baicalein scavenged ·OH (Fig. 4), it is possible that baicalein prevents the cell damage by H_2O_2, which generates ·OH in the presence of biometals such as iron by Fenton-like reaction ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+\cdot OH+OH^-$).[20]

When the cells were exposed to lipid hydroperoxides including BuOOH, levels of both intracellular malondialdehyde[21] and reactive oxygen species[22] increased, but these were inhibited by treatment with deferoxamine and Vit. E. These results indicated the participation of lipid peroxidation in cell damage. In support of these observations, BuOOH-induced human dermal fibroblast cell damage was protected by deferoxamine and Vit. E, as inhibitors of the lipid peroxidation chain reaction (Fig. 2B). Baicalein was found to be more effective for protection of the cells than deferoxamine and Vit. E (Fig. 2A). On the other hand, formation of an alkyl radical (Bu·) and an alkoxyl radical (BuO·) was also identified under these conditions,[17] in which partial inhibition by DMSO suggested participation of ·OH in cell damage. H_2O_2 formed during lipid peroxidation[23] has been reported to undergo the Fenton reaction to injure cells. Although the detailed mechanism of BuOOH-induced cell damage has not yet been fully elucidated, BuOOH is thought to be decomposed by iron in the cells, followed by initiation of lipid peroxidation, which results in sequential cell damage. The scavenging ability of baicalein for organic peroxyl radicals (Fig. 6) suggests participation in the protection against BuOOH-induced fibroblast damage.

The produced $\cdot O_2^-$ has been proposed to contribute to biological damage.[24–26] As a possible concept, $\cdot O_2^-$ undergoes dismutation and generates ·OH through iron-catalyzed Harber–Weiss reaction ($3\cdot O_2^- + 2H^+ \rightarrow \cdot OH + OH^- + 2O_2$).[18] Among the test compounds, deferoxamine and allopurinol showed protection against $\cdot O_2^-$-induced fibroblast damage (Fig. 3B). In contrast, ·OH radical scavengers, as well as catalase and superoxide dismutase, were effective only when added to the cells simultaneously with the $\cdot O_2^-$ generating system (data not shown). These results suggest the presence of complicated mechanisms in cell damage. The ·OH formation catalyzed by Harber–Weiss reaction may occur in fibroblasts. An alternative injury pathway may also be possible, *e.g.* extracellular attack by ·OH derived from Harber–Weiss reaction, since the charged $\cdot O_2^-$ species penetrates biological membranes only very slowly, unlikely H_2O_2.[27] In both cases, direct scavenging of $\cdot O_2^-$ by baicalein may inhibit the cytotoxic processes in the cells.

Since the involvement of ·OH, BuOO· and $\cdot O_2^-$ in cell damage was suggested as described above, the scavenging ability of baicalein for these free radical species was examined by using the ESR spin-trapping method. In the present study, ·OH, BuOO· and $\cdot O_2^-$ were generated by the Fenton system, MetHb–BuOOH system and DMSO–alkaline solution, respectively. A non-enzymatic method using DMSO–alkaline solution[16] was employed for evaluating the scavenging ability of baicalein against $\cdot O_2^-$, since it excluded the possibility of inhibition of XOD by baicalein. Results indicated that the intensity of each free radical-spin adduct was decreased with increasing concentration of baicalein. The IC_{50} values of baicalein for ·OH, $\cdot O_2^-$ and BuOO· were 10, 45 and 310 μM, respectively (Table 1), suggesting that baicalein has comparatively good scavenging ability for these free radical species.

It has been reported that a catechol or pyrogallol moiety

on the hydroxybenzoic acid plays a crucial role in radical scavenging activity.[28,29] Flavonoids (Fl-OH) have been proposed to scavenge free radicals by the following reactions[30]:

$$ROO\cdot + Fl\text{-}OH \rightarrow ROOH + FlO\cdot$$

$$\cdot OH + Fl\text{-}OH \rightarrow H_2O + FlO\cdot$$

In baicalein the occurrence of similar reactions is also assumed. Under the present conditions, however, we could not detect any free radical species derived from baicalein by ESR. Studies on the detection of free radical species derived from baicalein are underway in our laboratory.

Transition metal ions such as iron play a key role in generation of reactive oxygen species, and is closely related to the redox state of iron. Thus, it is possible that baicalein inhibits fibroblast damage by complex formation with iron. It has been reported that the free radical scavenging activities of thujaplicins are due, in part, to the chelating ability of the compounds with iron.[31] Such an effect is also known in flavonoids.[30,32] We have previously demonstrated that baicalein, with high affinity for membranes, inhibits lipid peroxidation by forming an inert complex with iron in the microsomal membrane.[6] Recent research has shown that baicalein enhances the auto-oxidation of ferrous ions.[33] However, the possibility of involvement of the auto-oxidative characteristic of baicalein in cell protective activity is not excluded, because an excess amount of iron was needed to reverse the inhibition.[6]

In spite of a lower ability to scavenge BuOO· than Vit. E (Table 1), baicalein was found to be more potent against BuOOH-induced fibroblast damage (Fig. 2 and Table 1), as well as against ascorbic acid-Fe^{3+} induced microsomal lipid peroxidation than Vit. E (Table 1). Therefore, broad effectiveness of baicalein against cell damage by reactive oxygen species is proposed.

The formation of ·OH, H_2O_2[12] and TBARS[34,35] in fibroblast cells have been observed under UV irradiation. Therefore, attention has been focused on the striking chemical and biological reactivities of oxygen radicals in the pathogenesis of various biological disorders, *e.g.*, inflammation, ischemia, lipid peroxidation, carcinogenesis, viral infection and aging.[36] The present study provides new insight into the antioxidative characteristics of baicalein, and suggests its usefulness for the treatment of active oxygen related pathological conditions.

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