황금 추출물이 사람의 glioma 세포에서 oxidant에 의한 세포손상에 미치는 효과

김성대, 정지천

동국대학교 한의과대학 내과학교실

Effect of *Scutellariae Radix* extraction (SRE) on oxidant-induced cell injury in huamn glimoa cells

Sung-Dae Kim, Ji-Cheon Jeong

Department of Internal Medicine, College of Oriental Medicine, Dongguk University

신경교세포에서 황금추출물이 반응성 산소기에 의한 세포 사망을 방지할 수 있는지를 확인하기 위하여 사람의 glioama 세포주인 A172 세포를 사용하여 H₂O₂의 독성작용에 대한 영향을 조사하였다. 세포 사망 정도는 tryptan blue exclusion과 MTT reduction assay로 평가하였다. H₂O₂는 세포 사망을 유도하였으며 또한 세포내 ATP 함량을 감소시켰으며, 이러한 효과는 황금 추출물에 의해 방지되었으며 그 효과는 농도 의존적으로 나타났다. H₂O₂에 의한 세포 사망은 잘 알려진 flavonoid인 quercetin과 철착염제인 phenanthroline에 의해 방지되었으나, 항산화제인 DPPD나 Trolox에 의해서는 영향을 받지 않았다. H₂O₂는 poly (ADP-ribose) polymerase를 활성화시켰으며, 이러한 효과는 황금, quercetin 및 phenanthroline에 의해 억제되었다. 황금 추출물은 유기 산화제인 t-buthyhydroperoxide 및 중금속인 수은에 의한 세포 사망을 방지하였다. 이러한 실험 결과는 황금 추출물이 H₂O₂에 의한 세포 사망을 방지하며 그 효과는 황금의 flavonoid 성분이 철과 결합하여 H₂O₂로부터 hydroxy radical의 생성을 억제함으로써 나타나는 것으로 추측된다.

Key Word : *Scutellariae Radix*, reactive oxygen species, cell death, PARP activation, human glioma cells.

I. INTRODUCTION

Reactive oxygen species (ROS) have been implicated in the pathogenesis of a number of neurodegener ative disorders.[1-4] The hydroxyl radical derived from H_2O_2 in the presence of iron is a powerful oxidizing agent that can chemically modify a number of cellular macro-molecules, including polyunsaturat ed fatty acids, DNA, and proteins.[5] Although lipid peroxidation has been considered as one of the best-known manifestations of oxidant-induced cell injury, whether lipid peroxidation is involved in the pathogenesis of oxidant-induced cell death remains controversial.[5,6] Some studies have demonstrated that oxidant-induced injury is not directly linked with lipid peroxidation in renal tubular cells.[7,8] Similar dissociation of lipid peroxi-dation from oxidant-induced cell injury has been shown in other studies using renal proximal tubule,[9] hepatocytes,[6] cultured pulmonary artery endothelial cells.[10,11] These data suggest that oxidant-induced cell death could result from a mechanism independent of lipid peroxidation.

DNA damage by ROS can lead to loss of cell viability by several mechanisms. One of these involves activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) by DNA damage. Several lines of evidence indicate that PARP is involved in the pathogenesis of cell injury caused by ROS in neuronal and nonneuronal cell systems.[12] Inhibition of PARP by benzamide and related compounds has been reported to protect primary neuronal cultures against the toxic effects of

glutamate and NMDA[13,14] and the H_2O_2-induced neurotoxicity.[15,16] However, the protective effect of PARP inhibitors against oxidant-induced cell injury is not universal. Yamamoto et al.[17] observed that the PARP inhibitors in hepatocytes did not affect the cell death induced by t-butylhydroperoxide (tBHP) and H_2O_2.

It has been reported that H_2O_2-induced cell death is associated with PARP activation but not lipid peroxidation in cultured renal cells,[7,8] cultured endothelial cells,[10,11] and murine fibroblasts[17] showing no protection of H_2O_2-induced cell death by antioxidants.

Scutellariae Radix extract (SRE) has been widely employed for the purpose of clearing away heat and toxic material in traditional Korean herbal medicine for centuries.[18] SRE contains numerous flavonoids.[19] It has been reported that SRE has antioxidant activity[20] and attenuates cell death induced by antimycin A and ischemia/reperfusion in cardiomyocytes.[21] In the present study, we examined the effect of SRE on the H_2O_2-induced cell death in A172 cells, a human glioma cell line. It has been suggested that glia cells play a role in protecting neurons from oxidative stress by establishing antioxidative defence systems.[22,23]

II. MATERIALS and METHODS

Scutellariae Radix extraction (SRE) preparation

300 g of crushed crude drug was extracted with methyl alcohol under reflux for 4 hr three times and the total extractive was evaporated under reduced pressure to give 46 g. The extract was dissolved in the incubation medium.

Culture of A172 cells.

A172 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained by serial passages in 75-cm² culture flasks (Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL) containing 10% heat inactivated fetal bovine serum (Gioco, BRL) at 37℃ in humidified 95% air/5% CO_2 incubator. When the cultures reached confluence, subcul ture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on 24-well tissue culture plates and used 1-2 days after plating when a confluent monolayer culture was achieved.

Cell viability assay.

The cell viability was estimated by a trypan blue exclusion assay. Cells were grown to confluence in 24-well dishes, incubated in the HBSS containing H_2O_2 at 37℃ in 95% air/5% CO_2, and then harvested using 0.025% trypsin. Cells were incubated with 4% trypan blue solution. Cells failing to exclude the dye were considered nonviable, and the data are expressed as percentage of nonviable cells.

In some experiments, the cell viability was evaluated using a MTT assay.[24] Tetrazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye and are therefore useful for the measurement of cell viability. The cells were gently washed with Hanks' balanced salt solution (HBSS, Sigma Co, USA), and exposed to H_2O_2. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 2 hr at 37℃, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 110 μl of dimethyl sulfoxide. A 100 μl aliquot of each sample was then translated to 96-well plates and the absorbance of each well was measured at 550 nm with ELISA Reader (Bio-Tek instrument, EL, 311). Data were expressed as a percentage of control measured in the absence of H_2O_2. Unless stated otherwise, the cells were treated with 0.5 mM H_2O_2 for 120 min. Test reagents were added to the medium 30 min before H_2O_2 exposure.

Measurement of lipid peroxidation.

Lipid peroxidation was estimated by measuring total malondialdehyde (MDA) production, utilizing the lipid peroxidation assay kit (Calbiochem). Protein was measured by the method of Bradford.[25]

Measurement of ATP content.

ATP levels were measured on cells with a luciferin-luciferase assay. After an exposure to H_2O_2, the cells were solubilized with 500 μl of 0.5% Triton X-100 and acidified with 100 μl of 0.6 M perchloric acid and placed on ice. Then cell suspension was diluted with 10 mM potassium phosphate buffer containing 4 mM $MgSO_4$ (pH 7.4), and 100 μl of 20 mg/ml luciferin-luciferase was added to 10 μl of diluted sample. Light emission was recorded at 20 sec with a luminometer (MicroLumat LB96P, Berthold, Germany). Protein content was determined on a portion of the cell sample.

Measurement of PARP activity.

Cells were treated with H_2O_2 and preincubated for 10 min in a buffer containing 28 mM NaCl, 28 mM KCl, 2 mM $MgCl_2$, 56 mM Hepes (pH 7.5), 0.01% digitonin, and 125 mM NAD (containing 0.25 Ci [³H]NAD). Permeabilized cells were incubated for 5 min at 37℃, and the protein ribosylated with [3H]NAD

was precipitated with 200 μl of 50% (w/v) trichloroacetic acid. After washing twice with trichloroacetic acid, the protein pellet was solubilized with 200 1 of 2% (w/v) sodium dodecyl sulfate in 0.1 M NaOH and incubated at 37℃ overnight, and the radioactivity was determined by scintillation counting.

Reagents.

Catalase, deferoxamine, 1,10-phenanthroline, dithiothreitol (DTT), glutathione (GSH), hydrogen peroxide (H_2O_2), t-butylhydroperoxide (tBHP), Trolox, 3-aminobenzamide (3-AB), and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). N,N' -diphenyl-p-phenylenediamine (DPPD) and dimethylthiourea (DM TU) were purchased from Aldrich Chemical (Milwaukee WI). All other chemicals were of the highest commercial grade available.

Statistical analysis.

The data are expressed as mean $\pm$ SE and the difference between two groups was evaluated using Student' s t-test. The data in Table 2 were evaluated by a one-way analysis of variance followed by the Tukey's test. A probability level of 0.05 was used to establish significance.

III. RESULTS

Effect of SRE on H_2O_2 induced loss of cell viability

In order to determine if SRE exerts protective effect against H_2O_2-induced cell death, cells were exposed to 0.5 mM H_2O_2 for 120 min in the presence of various concentrations of SRE. The results are summarized in Fig. 1, SRE prevented H_2O_2-induced cell death as estimated by a trypan blue exclusion assay in dose-dependent fashion and a significant protection was present at 0.05 and 0.1% (Fig 1A). Similar results are observed by a MTT reduction assay (Fig. 1B). However, SRE did not exert any effect in the control cells untreated with H_2O_2 (data not shown).

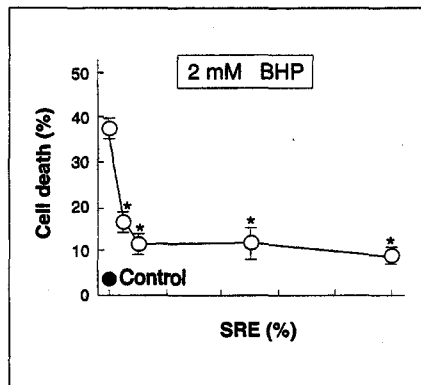## Effect of SRE on tBHP-induced cell death

Previous studies suggest that the underlying mechanism of H_2O_2-induced cell death is different from that of tBHP-induced death in human glioma cells.[26] Therefore, the effect of SRE on tBHP-induced cell death was examined. As shown in Fig. 2, SRE prevented tBHP-induced cell death and its protective effect was more prominent than that in H_2O_2-induced cell death. SRE prevented tBHP-induced cell death even as low as 0.005%, whereas it exerted the protective effect against H_2O_2-induced cell death at concentrations higher than 0.05%.
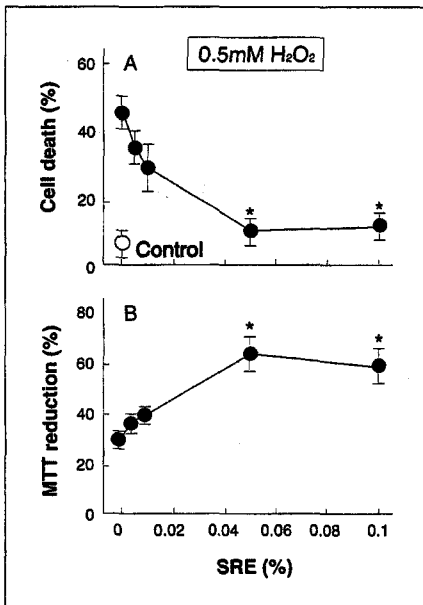
Fig. 1. Effect of *Scutellariae Radix* extract (SRE) on H_2O_2-induced cell death in human glioma cells. Cells were exposed to 0.5 mM H_2O_2 for 120 min the presence or absence of various concentrations of SRE. Cell death was evaluated by a trypan blue exclusion assay (A) and a MTT reduction assay (B). Data are mean ±SE of four experiments. *p<0.05 compared with H_2O_2 alone.
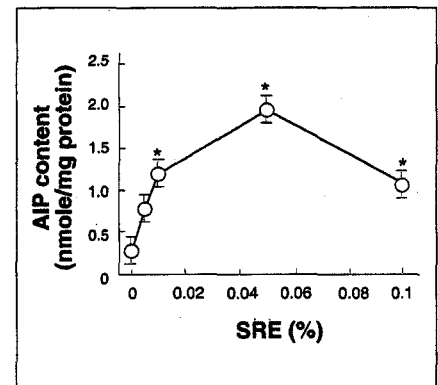

Fig. 2. Effect of *Scutellariae Radix* extract (SRE) on t-buytlhydroperoxide (tBHP)-induced cell death in human glioma cells. Cells were exposed to 2 mM tBHP for 120 min the presence or absence of various concentrations of SRE. Cell death was evaluated by a trypan blue exclusion assay. Data are mean ±SE of six experiments. *p<0.05 compared with H_2O_2 alone.

Fig. 3. Effect of *Scutellariae Radix* extract (SRE) on H_2O_2-induced cell deple tion of ATP in human glioma cells. Cells were exposed to 0.5 mM H_2O_2 for 120 min the presence or absence of various concentrations of SRE. Data are mean ±SE of six experiments. *p<0.05 compar ed with H_2O_2 alone.

Effect of SRE on H_2O_2-induced depletion of ATP

ATP depletion has been demonstrat ed to be early response of cells to oxidative stress,[27,28] which has been proposed to be involved in the patho genesis of oxidant-induced cell death[11]. Thus, it was examined whether SRE prevents H_2O_2-induced ATP depletion. H_2O_2 at 0.5 mM) reduced ATP level to approximately 6% of control (0.30 ± 0.14 vs. 5.06 ± 0.15 nmole/mg protein in control). The H_2O_2-induced ATP depletion was prevented by 0.005-0.05% SRE

in a dose-dependent fasion (Fig. 3). However, when SRE concentration was increased to 0.1%, the ATP level was lower than that in 0.05%. This may due to a toxic effect of SRE at high concentrations.

Effect of SRE on PARP activity

ROS-induced DNA damage can lead to activation of the nuclear enzyme PARP, which may be involv ed in the pathogenesis of cell injury caused by ROS in neuronal and non- neuronal cell systems.[12] Thus, inhibi- tion of PARP can provide the protec- tive effect against ROS-induced cell death.[15,16] In the present study, we examined the effect of SRE on PA RP activity and compared with a well-known flavonoid, quercetin, and an iron chelator, phenanthroline.

H_2O_2 produced the activation of PARP enzyme, which was inhibited by 0.05% SRE, 0.2 mM quercetin, and 0.2 mM phenanthroline, with a similar protective potency (Fig. 4).

Comparison of effects of SRE, quercetin, and antioxi dants on H_2O_2-induced cell death

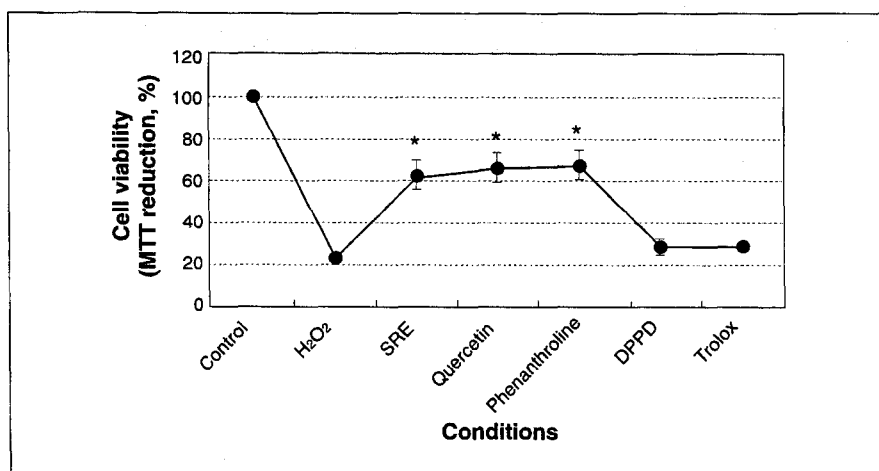Effects of well-known flavonoid and antioxidants and on H_2O_2- induced cell death were compared with that of SRE. The results are summarized in Fig. 6. SRE (0.05) significantly prevented the loss of cell viability induced by 0.5 mM H_2O_2 and its protective effect was similar to that by 0.2 mM quercetin, a flavonoid, and an iron chelator phenanthroline (0.2 mM). By con- trast, a potent antioxidant DPPD (10
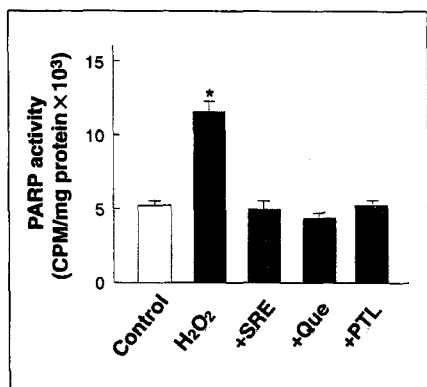
Fig. 4. Effect of *Scutellariae Radix* extract (SRE), quercetin (Que), and phenanthroline (PTL) on H_2O_2-induced activation of poly (ADP-ribose) polymerase (PARP) in human glioma cells. Cells were exposed to 0.5 mM H_2O_2 for 120 min the presence or absence of various concentrations of 0.05% SRE, 0.2 mM Que or 0.2 mM PTL. Data are mean ± SE of four experiments. *p<0.05 compared with H_2O_2 alone.
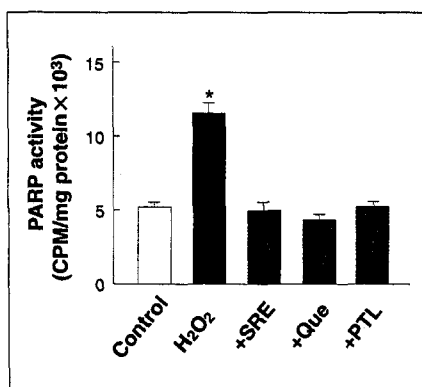
Fig. 5. Effect of *Scutellariae Radix* extract (SRE) on $HgCl_2$-induced cell death in human glioma cells. Cells were exposed to 8 M $HgCl_2$ for 60 min the presence or absence of various concentrations of SRE. Cell death was evaluated by a trypan blue exclusion assay. Data are mean ± SE of four experiments. *p<0.05 compared with H_2O_2 alone.

were similar to or higher than the concentrations that have effectively prevented oxidant-induced cell injury and lipid peroxidation in brain tissues.[29-31]

Effect of SRE on $HgCl_2$-induced cell death

In order to determine if SRE is able to prevent toxic agent-induced cell death, the effect of SRE on $HgCl_2$-induced cell death was investigated. When cells were treated with 8 M $HgCl_2$, cell death increased 86.18 ± 1.66% from 3.47 ± 1.42% of control. However, exposure of cells to $HgCl_2$ in the presence of SRE produced a dose-dependent decrease in cell death (Fig. 5).

IV. DISCUSSION

ROS are generated during metabolism of a cell even under normal conditions. The mitochondrialelec tron transport chain is the principal site of cellular production of ROS such as superoxide and H_2O_2 with approximately 2-5% of the O_2 consumed in state 4 respiration.[32,33] ROS have been implicated in the pathogenesis of a number of neurode generative disorders.[1-4] Therefore, the targeted development of new antioxi dant drugs has been required.

In the present study, SRE prevent ed the loss of cell viability induced by the oxidant H_2O_2 in human glioma cells. Likewise, SRE prevented ATP depletion induced by the oxidant.

Fig. 6. Effects of *Scutellariae Radix* extract (SRE), antioxidants, and quercetin on H_2O_2-induced cell death in human glioma cells. Cells were treated with 0.5 mM H_2O_2 for 120 min in the presence or absence of various agents. Cell viability was measured by a MTT assay. Data are mean ± SE of six experiments. * p<0.05 compared with H_2O_2 alone.

μM) and a water-soluble vitamin E analogue, Trolox (1 mM)), did not affect the loss of cell viability induced by H_2O_2, indicating that lipid peroxidation is not involved in H_2O_2-

induced cell death in human glioma cells and the protective effect of SRE is not resulted from its antioxidant activity. The concentrations of antioxidants used in the present study

Although ATP depletion has been proposed to be involved in the pathogenesis of oxidant-induced cell death,[11] other studies have shown that the oxidant injury is dissociated with ATP depletion.[28,34] Therefore, it is unclear that protection of H_2O_2-induced cell death by SRE was resulted from the prevention of ATP depletion.

Biological membranes contain a large amount of polyunsaturated fatty acids, which are particularly susceptible to peroxidative attacks by oxidants, resulting in lipid peroxidation.[35,36] Therefore, lipid peroxidation has been used as indirect marker of oxidant-induced cell injury.[6] Previous studies have demonstrated that lipid peroxidation plays a critical role in oxidant-induced cell injury.[37,38] However, the present study showed that the antioxidants DPPD and Trolox did not prevent H_2O_2-induced cell death (Fig. 6). These results suggest that the H_2O_2-induced cell death may be not the result of lipid peroxidation in human glioma cells. These data are consistent with those reported in cultured endothelial cells[10,11] and murine fibroblasts[17] showing no protection of H_2O_2-induced cell death by antioxidants.

H_2O_2 reacts with ferrous ion to produce a more potent oxidant, the hydroxyl radicals, by Fenton chemistry[5]. Thus, the role of iron in cell death by H_2O_2 was demonstrated by utilizing an iron chelator, phenanthro line. These agents almost completely prevented the H_2O_2induced cell death (Fig. 6). These data are consistent with those reported in cultured mouse astrocytes[31], hepatocytes,[39] and renal epithelial cells.[7,37,40] These results indicate that hydroxyl radicals are responsible for the H_2O_2-induced cell death than peroxide itself.

Flavonoids are found in almost every plant, which act as pharmacological active constituents in many herbal medicines. They have multiple biological activities including vasodilatory,[41] anticancer, antiinflammatory, antibacterial, antiallergic, and antiviral effects.[42,43] SRE has been reported to contain numerous flavonoids[19]. Although SRE and flavonoids have been reported to have antioxidant activity,[20,44] the underlying mechanism by which SRE exerts antioxidant activity remains to be defined. In the present study, Since the cell death was not prevented by the antioxidants DPPD and Trolox, lipid peroxidation is not responsible for the H_2O_2-induced cell death. Thus, the protective effect of SRE may be due to mechanisms other than inhibition of lipid peroxidation. One possible mechanism for antioxidant action of SRE is reduction of formation of hydroxyl radicals by removal of Fe^{2+}. It has been reported that flavonoids enhanced autooxidation of Fe^{2+} ion, resulting in the inhibition of the formation of ROS.[45] The results of the present study that the iron chelator phenanthroline prevented the H_2O_2-induced cell

death may support this hypothesis.

ROS have been known to cause DNA damage[46] and PARP activation.[47,48] Since PARP catalyzes the transfer of ADP-ribose from NAD to protein with the concomitant release of nicotinamide, the activation of this enzyme results in depletion of NAD and a consequent reduction in ATP, which may be involved in the pathogenesis of oxidant-induced injury. PARP has been demonstrated to be involved in the cell death caused by H_2O_2 in neuronal cells[15,16] and various nonneuronal cell systems.[49,50] Recently, Lee et al.[26] demonstrated that PARP is activated as a result of oxidant stress by H_2O_2 and that the PARP inhibitor 3-AB prevents the cell death and stimulation of PARP activity caused by H_2O_2 in human glioma cells. The present study showed that H_2O_2-induced activation of PARP was inhibited by SRE, quercetin, and phenanthroline (Fig. 4). Based on these data, we suggest that SRE inhibits the PARP activation via reduction in an iron-dependent hydroxy radical formation. Therefore, these results again suggest that the protective effect of SRE may be attributed to interaction of iron and falvonoids of SRE.

SRE could exert the antioxidant activity by directly scavenging H_2O_2 itself. However, SRE prevented the cell death induced by an organic hydroperoxide tBHP and $HgCl_2$, similarly to its effect on H_2O_2-induced cell death (Figs. 2 and 5).

In summary, H_2O_2 induces the loss of cell viability and ATP depletion in human glioma cells. Such changes were significantly prevented by SRE. The cell death induced by an organic hydroperoxide tBHP and $HgCl_2$ was also prevented by SRE. The H_2O_2-induced cell death was not altered by the antioxidants DPPD and Trolox, whereas it was effectively prevented by a well-known flavonoid quercetin and an iron chelator phenantroline. The activation of PARP enzyme was prevented by SRE, quercetin, and phenanthroline. Therefore the protective effect of SRE is unlikely due to its inhibition of lipid peroxidation, but rather may be due to interaction with iron and flavonoids of SRE.

V. CONCLUSION

This study was undertaken to determine if Scutellariae Radix extract (SRE) exerts protective effect against H_2O_2-induced cell death in human glioma cells. The cell viability was evaluated by a trypan blue exclusion assay and a MTT reduction assay in A172 cells, a human glioma cell line. SRE prevented the cell death and ATP depletion induced by H_2O_2 in a dose-dependent manner. The H_2O_2-induced cell death was effectively prevented by a well-known flavonoid quercetin and an iron chelator phenanthroline, but not altered by the antioxidants DPPD and Trolox. H_2O_2 induced the activation of PARP enzyme, which was inh

ibited by SRE, quercetin, and phenanthroline. SRE prevented the cell death induced by an organic hydroperoxide tBHP and $HgCl_2$.

These results indicate that SRE exerts the protective effect against H_2O_2-induced cell death and its effect may be due to reduction of an iron-dependent hydroxyl radical formation by interaction of iron and flavonoids of SRE.

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