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원 저

丹蔘추출액이 H_2O_2에 의해 유발된 인간의 장관상피세포의 손상에 미치는 영향

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Beneficial Effect of *Salviae Miltiorrhizae Radix(SR)* on H_2O_2-induced Cell Death in Intestinal Epithelial Cells

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목적 : 반응성산소기들은 장관에서 여러 종류의 질병의 발생과 관련을 가지고 있는 것으로 알려져 있어, 이들에 의한 세포손상을 방지하는 약물의 개발은 시급한 실정이다. 본 연구에서는 항산화작용을 가진 약재로 보고된 단삼추출액이 장관상피세포에서 H_2O_2에 의한 세포손상을 방지할 수 있는 지를 조사하고자 하였다.

방법 : 장관상피세포로는 사람의 소장상피세포에서 유래한 배양세포주인 Caco-2세포를 이용하였고, 세포손상 정도는 trypan blue exclusion assay를 통해 평가하였고, 지질의 과산화는 그 산물인 malondialdehyde의 량을 측정하여 산정하였다.

결과 : H_2O_2는 처리 시간 및 농도에 비례하여 세포손상을 유발하였으며, 이러한 효과는 단삼추출액에 의해 농도의존적으로 방지되었다. H_2O_2에 의한 세포소상은 H_2O_2제거제인 catalase와 철착염제인 deferoxamine에 의해 방지되었으나 항산화제인 N,N-diphenyl-p-phenylenediamine(DPPD)에 의해 영향을 받지 않았다. H_2O_2는 지질의 과산화를 증가시켰으며, 이러한 효과는 단삼추출액과 DPPD에 의해 억제되었다. 단삼추출액은 H_2O_2에 의한 세포내 ATP고갈을 방지하였다. H_2O_2는 DNA손상을 일으켰으며, 이러한 효과는 단삼추출액, catalase 및 deferoxamine에 의해 방지되었으나, DPPD에 의해서는 변화되지 않았다.

결론 : 이상의 결과를 종합하면 단삼추출액은 장관상피세포에서 H_2O_2에 의한 세포손상을 방지하며, 이러한 효과는 항산화작용이 아닌 다른 작용기전에 기인할 것으로 생각된다. 또한 본 연구의 결과는 H_2O_2가 장관상피세포에서 지질의 과산화를 유발하여 세포손상을 일으키지 않음을 가리킨다.

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Key Words: *Salviae Miltiorrhizae Radix*, Danshen, H_2O_2, intestinal epithelial cell

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Introduction

Reactive oxygen species(ROS) contribute to gastrointestinal injury in various pathological conditions

such as ischemia-reperfusion injury[1-3], certain types of drug-induced gastroenteropathy[4-7], necrotising enterocolitis[8], experimental colitis[9-12], and inflammatory bowel diseases[13-16]. However, the precise mechanism by which ROS induce cell death remains unclear.

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 macromolecules, including polyunsaturated fatty acids, DNA, and proteins[17]. Although lipid peroxidation has been considered as one of the best-known manifestations of ROS-induced cell injury, whether lipid peroxidation is involved in the pathogenesis of ROS-induced cell death remains controversial[17,18]. Some studies have demonstrated that ROS-induced injury is not directly linked with lipid peroxidation in renal tubular cells[19,20], hepatocytes[18], and cultured pulmonary artery endothelial cells[21,22].

Salviae Miltiorrhizae Radix(SR)(丹蔘) is bitter in taste and slightly cold in nature. The therapeutic action is related to the channels of the heart, pericardium and liver[23].

SR is beneficial to promoting blood circulation, relieving restlessness and tranquilizing the mind, subduing edema and relieving pains. SR is also generally used to resist inflammation and enhance immunity, reduce exudation of inflammation, to inhibit migration of white blood cell and enzymic release of lysosome, ect[24].

Since magnesium lithospermate B, a compound isolated from SR, has been reported to act as a scavenger of hydroxyl radicals[25], SR may exert the protective effect against ROS-induced cell death through its antioxidant action.

This study was therefore undertaken to determine whether *Salviae Miltiorrhizae Radix*(SR), an oriental medicinal herb, protects against ROS-induced cell death in intestinal epithelial cells using the human-derived cultured intestinal epithelial cell line Caco-2. This cell model has been extensively employing in studies to characterize intestinal transport function[26,27].

Materials and Methods

1. SR extract preparation

2kg of crushed crude drug was extracted with methyl alcohol under reflux for 4hr three times and the total extractive was evaporated under reduced pressure to give 168g.

2. Culture of Caco-2 cells

Caco-2 cells were obtained from the American Type Culture Collection(Rockville, MD) and maintained by serial passages in 75-cm^2 culture flasks(Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium/Ham's F12(DMEM/F12, Sigma Chemical Co.) containing 10% fetal bovine serum at 37℃ in 95% air/5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on 24-well tissue culture plates in DMEM/F12 medium containing 10% fetal bovine serum. All experiments started 3-4days after plating when a confluent monolayer culture was achieved. Cells were treated with hydrogen peroxide(H_2O_2) in HBSS without serum in the presence or absence of SR.

3. Measurement of cell death

Cell death 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 for 120min 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.

4. Measurement of ATP content

ATP levels in Caco-2 cells were measured by a luciferin-luciferase assay. After an exposure to oxidant stress, cells were solubilized with 500㎕ of 0.5% Triton X-100 and acidified with 100㎕ of 0.6M perchloric acid and placed on ice. The cell suspension was then diluted with 10mM potassium phosphate buffer containing 4mM $MgSO_4$(pH 7.4) and 100㎕ of 20mg/ml luciferin-luciferase was added to 10㎕ of diluted sample. Light emission was recorded at 20s with a luminometer(MicroLumat LB96P, Berthold, Germany). Protein content was determined on an aliquot of cell suspension.

5. Measurement of lipid peroxidation

Lipid peroxidation was estimated by measuring the tissue content of malondialdehyde(MDA) according to the method of Uchiyama and Mihara[28]. Cells were homogenized in ice-cold 1.15% KCl(5%wt/vol). A 0.5ml aliquat of homogenate was mixed with 3ml of 1% phosphoric acid and 1ml of 0.6% thiobarbituric acid. The mixture was heated for 45min on a boiling water bath. After addition of 4ml of n-butanol the contents were vigorously vortexed and centrifuged at 2,000g for 20min. The absorbance of the upper, organic layer was measured at 535 and 520nm with a diode array spectrophotometer(Hewlett Packard, 8452A), and compared with freshly prepared malondialdehyde tetraethylacetal standards. MDA values were expressed as pmoles per mg protein. Protein was measured by the method of Bradford[29].

6. Measurement of DNA single-strand breaks

DNA strand break was measured by the DNA precipitation assay [30]. Confluent cells grown in 24-wells were labelled in the presence of 0.25㎍ Ci/ml [3H]methylthymidine for 24hr. The cells were thoroughly washed with HBSS, and treated with H_2O_2 in the presence or absence of SR. After treatment, the cells were washed with HBSS and lysed in effendorf tube with 0.5ml of lysis buffer (10mM Tris/HCl, 10mM EDTA, 50mM NaOH, 2% SDS, pH 12.4), followed by addition of 0.5ml of 0.12M KCl. The lysate was incubated for 10min at 65℃, followed by a 5min cooling-and-precipitation period on ice. A DNA-protein K-SDS precipitate was formed under these conditions, from which low-molecular-mass brCaco-2en DNA was released. This DNA was recovered in the supernatant from a 10min centrifugation at 200g, 10℃, and transferred into a liquid scintillation vial containing 1ml of 200mM HCl. The precipitation pellet (intact double-stranded DNA) was solubilized in 1ml of water at 65℃. The tube was rinsed with 1ml of water, and 8ml of scintillation fluid was added to each vial. The amount of double-stranded DNA remaining was calculated for each sample by dividing the d.p.m. value of the pellet by the total d.p.m. value of the pellet plus supernatant and multiplying by 100. The extent of DNA damage was expressed as the ratio of single stranded DNA to total stranded DNA(double stranded + single stranded).

7. Chemicals

Hydrogen peroxide (H_2O_2), deferoxamine, and catalase were purchased from Sigma Chemical(St. Louis, MO, USA). N,N' -diphenyl-p-phenylenediamine(DPPD) was obtained from Aldrich Chemical(Milwaukee WI, USA). All other chemicals were of the highest commercial grade available.

8. Statistical analysis

The data are expressed as mean±SE and the difference between two groups was evaluated using Student' s t-test. A probability level of 0.05 was used to establish significance.

Results

1. H_2O_2 cytotoxicity in Caco-2 cells

Fig. 1 shows effect of various concentrations of H_2O_2 on cell death in the presence or absence of 0.05% SR. When cells were exposed to various concentrations (0.05-1.0mM) of H_2O_2 for 120 min, cell death was increased in a dose-dependent manner. The significant cell death was observed at 0.1mM H_2O_2 (12.09±1.32 vs. 3.29±0.54% in control). The H_2O_2-induced cell death was attenuated by the presence of SR.

In order to determine the time course of H_2O_2-induced cell injury, cells were exposed to 0.5mM H_2O_2, and the cell death was determined at various time points (0-180 min). A significant cell death was present 30min after exposure of cells to H_2O_2, with cell death increasing up to 180min(Fig. 2). However, the cell death was significantly prevented by addition of 0.05% SR.

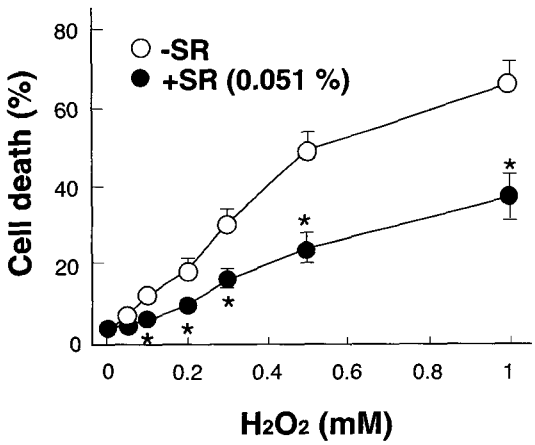

Fig. 1. Concentration-dependency of H_2O_2 effect on cell death in Caco-2 cells. Cells were incubated for 120min in medium containing various concentrations of H_2O_2 in the presence or absence of 0.05% *Salviae Miltiorrhizae Radix*(SR). Cell death was measured by a trypan blue exclusion assay. Data are mean±SE of five experiments. *P<0.05 compared with -SR.

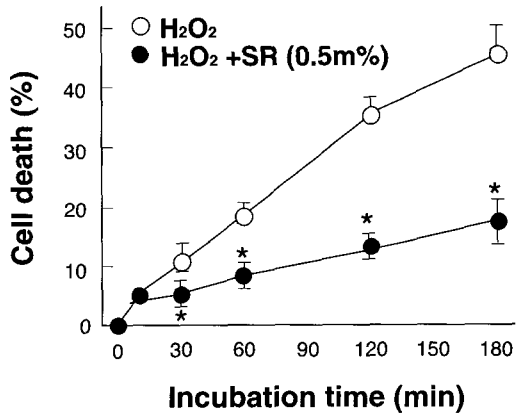

Fig. 2. Time course of H_2O_2 effect on cell death in Caco-2 cells. Cells were incubated for various times in medium containing 0.5mM H_2O_2 in the presence or absence of 0.1% *Salviae Miltiorrhizae Radix*(SR). Cell death was measured by a trypan blue exclusion assay. Data are mean±SE of four experiments. *P<0.05 compared with H_2O_2 alone.

2. Dose-dependency of SR protection against H_2O_2-induced cell death

In order to determine dose-dependency of the protective effect of SR, cells were treated with 0.5mM H_2O_2 in the presence of various concentrations of SR. The results are depicted in Fig. 3. Treatment of H_2O_2 alone caused 47.94±5.13% cell death, which was prevented by SR in a dose-dependent fashion with a significant protection at 0.005% (29.28±1.893%). When SR concentrations were increased up to 0.05 and 0.1%, the cell death was decreased 11.37±1.46 and 9.84±1.52%, respectively. SR did not exert any effect in control cells untreated with H_2O_2 even when cells were treated with 0.1% SR.

3. Effects of antioxidants on H_2O_2-induced cell death

To evaluate whether H_2O_2 causes cell death through lipid peroxidation in Caco-2 cells, effects of other well-known antioxidants on H_2O_2-induced cell death were examined and compared with that of SR. The results are

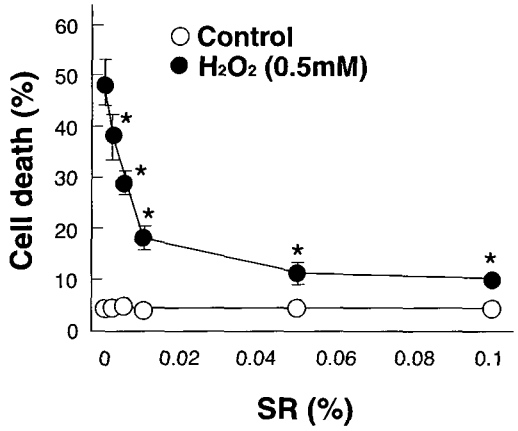

Fig. 3. Dose-dependency of *Salviae Miltiorrhizae Radix*(SR) effect on H_2O_2-induced cell death in Caco-2 cells. Cells were incubated for 120min in medium containing 0.5mM H_2O_2 in the presence or absence of various concentrations of SR. Cell death was measured by a trypan blue exclusion assay. Data are mean±SE of five experiments. *P<0.05 compared with H_2O_2 alone.

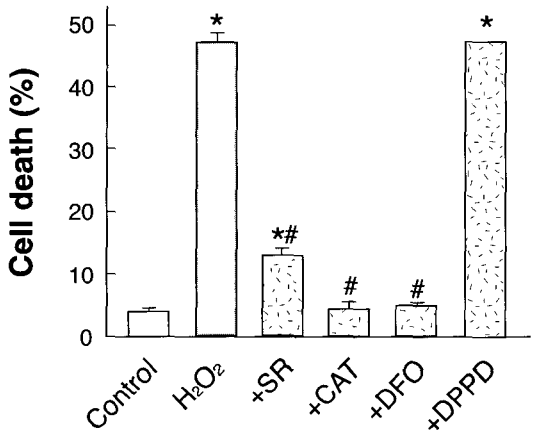

Fig. 4. Effects of *Salviae Miltiorrhizae Radix*(SR), hydrogen peroxide scavenger, and antioxidants on H_2O_2-induced cell death in Caco-2 cells. Cells were incubated for 120min in medium containing 0.5mM H_2O_2 in the presence or absence of 0.05% SR, 500units/ml catalase(CAT), 2mM deferoxamine(DFO), and 0.02mM N,N' -diphenylphenylene diamine(DPPD). Cell death was measured by a trypan blue exclusion assay. Data are mean±SE of five experiments. *P<0.05 compared with the control; #P<0.05 compared with H_2O_2 alone.

summarized in Fig. 4.

As expected, catalase(500 units/ml), a hydrogen peroxide scavenge, prevented completely cell death induced by 0.5mM H_2O_2. A similar protection was observed with deferoxamine(5mM), an iron chelator, suggesting involvement of an iron-dependent mechanism in the H_2O_2-induced cell death. By contrast, a potent antioxidant DPPD at 20μM did not affect the cell death induced by H_2O_2, indicating that the H_2O_2-induced cell death is not associated with lipid peroxidation.

4. Effects of SR and antioxidant on H_2O_2-induced lipid peroxidation

The failure of DPPD effect on the H_2O_2-induced cell death may be attributed to its inability to block H_2O_2-induced lipid peroxidation in Caco-2 cells. To test this possibility, we examined changes in lipid peroxidation in cells treated with H_2O_2 in the presence or absence of SR and DPPD. Exposure of cells to 0.5mM H_2O_2 caused an increase in lipid peroxidation, which could be prevented by 0.05% SR and 20μM DPPD(Fig. 5).

5. Effect of SR on H_2O_2-induced ATP deletion

Since ROS have been known to cause decrease in cell ATP content which may lead to cell death, SR may exert the protective effect against H_2O_2-induced cell death by preventing ATP depletion. To test the possibility, cellular ATP content was measured in Caco-2 cells exposed to 0.5mM H_2O_2 in the presence or absence of 0.05% SR. The results in Fig. 6 demonstrate that H_2O_2 significantly decreased the ATP content and SR prevented the ATP depletion.

6. Effect of SR and antioxidants on H_2O_2-induced DNA damage

DNA damage has been reported to play an important role in the oxidant-induced cell death[18,31,32]. Thus, it was

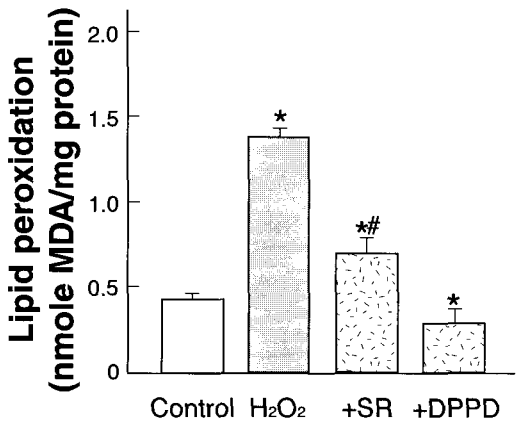

Fig. 5. Effect of *Salviae Miltiorrhizae Radix*(SR) and antioxidant on H_2O_2-induced lipid peroxidation in Caco-2 cells. Cells were incubated for 120min in medium containing 0.5mM H_2O_2 in the presence or absence of 0.05% SR and 0.02mM N,N' -diphenylphenylene diamine(DPPD), and then lipid peroxidation was measured. Data are mean±SE of five experiments. *P<0.05 compared with the control(cont); #P<0.05 compared with H_2O_2 alone.

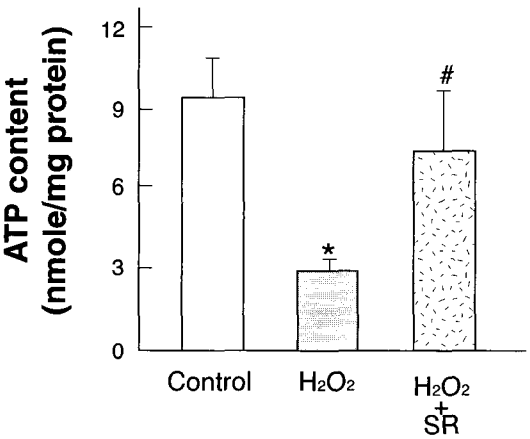

Fig. 6. Effects of *Salviae Miltiorrhizae Radix*(SR) on H_2O_2-induced ATP depletion in Caco-2 cells. Cells were incubated for 120min in medium containing 0.5mM H_2O_2 in the presence or absence of 0.05% SR. Data are mean±SE of four experiments. *P<0.05 compared with the control; #P<0.05 compared with H_2O_2 alone.

examined whether SR exerts the beneficial effect against DNA damage induced by H_2O_2. As shown in Fig. 7, H_2O_2 resulted in a significant increase in DNA damage as evidenced by a decrease in double stranded DNA, which was prevented by SR, catalase, and deferoxamine, but not by DPPD.

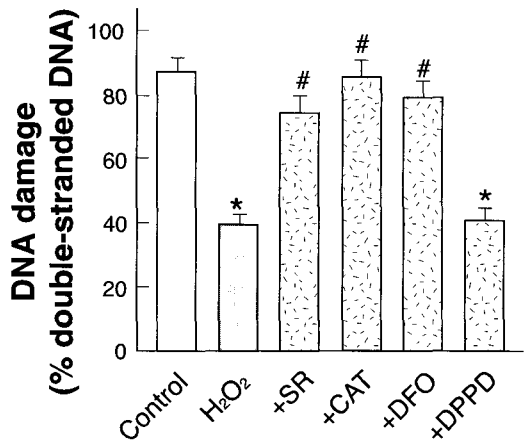

Fig. 7. Effects of *Salviae Miltiorrhizae Radix*(SR), hydrogen peroxide scavenger, and antioxidants on H_2O_2-induced DNA damage in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5mM H_2O_2 in the presence or absence of 0.05% SR, 500 units/ml catalase(CAT), 2mM deferoxamine(DFO), and 0.02mM N,N' -diphenylphenylene diamine(DPPD). Data are mean±SE of five experiments. *P<0.05 compared with the control; #P<0.05 compared with H_2O_2 alone.

Discussion

Since ROS have been implicated in the pathogenesis of stress- and chemically-induced gastrointestinal injury[33], a potent antioxidant may serve as a possible preventive intervention for gastrointestinal injury. Therefore, the search for natural antioxidants and other preparations of plant origin to achieve this objective has been intensified.

As I mentioned before, *Salviae Miltiorrhizae Radix*(SR) appears to have a very powerful effect on warfarin, which is used to improve circulation, coronary vasodilatation and protect against myocardial ischemia. It has been associated with decreasing blood pressure, inhibiting platelet aggregation, and coronary artery vasodilation. The SR has several roots that are in the shape of a slightly curved long cylinder, and some of which have branches and fine fibrous roots. The root is 10-20 cm in length and 0.3-1 cm in diameter. Its surface is rough, brownish-red in colour with longitudinal wrinkles. The outer skin of the maternal root is loose, usually purple brown, with scales easy to be stripped. The root is hard and fragile. Its cross section is loose with fissures or looks smooth and dense; the bark looks brownish-red, the xylem greyish-yellow or purple brown and the fibrovascular bundle, yellowish-white in radial arrangement. The root has a faint smell and a slight bitter and puckery taste. The therapeutic action is related to the channels of the heart, pericardium and liver. SR regulates the channels and relieves pain, nourishes heart and quiets spirit[23].

SR has been used to cure stasis due to blood-heat, irregular menstruation, dysmenorrhea, amenorrhea, mass in the abdomen, postpartum lochiorrhea and other syndromes. It can also be administered for treatment of angina pectoris in coronary heart disease, hypochondriac pain due to stagnation of the liver-qi, and stomachache due to blood stasis and sluggish flow of qi. The drug is used to cure impairment of ying (the vessels in which blood and qi are lodged) during the course of febrile diseases, vexation and insomnia. Liquor infusion of red sage root can cure neurasthenia. It is used to subdue swelling and relieve pain in carbuncles, sores and other skin and external diseases, swelling and pain of the joints and muscles due to arthritis of heat type, etc. The drug can be administered for the treatment of thromboangiitis obliterans and hypertension. But SR is not suitable for patients with only deficiency but no stasis, or with deficiency accompanied by cold, or with tendency of bleeding.

In this study, H_2O_2 increased cell death in a time- and dose-dependent manner in Caco-2 cells as measured by a trypan blue exclusion assay(Figs. 1 and 2). Such changes were prevented by SR and its effect was dose-dependent(Fig. 4). As expected, the H_2O_2 increased cell death was completely prevented by 500 units/ ml catalase, a scavenger enzyme of hydrogen peroxide(Fig. 4).

H_2O_2 can be converted to hydroxyl radical by Haber-Weiss reaction in the presence of ferrous iron. The hydroxyl radical is the most potent oxidant because its ability to initiate lipid peroxidation[18]. Indeed, iron appears to be the critical in the cytotoxic effect of H_2O_2 in Caco-2 cells as an iron chelator deferoxamine was markedly protective(Fig. 4). The source of iron, how it becomes biologically available, and the mechanism of action of deferoxamine on this system remain unknown. Gannon et al[34] have presented evidence that, in oxygen radical-mediated cell death, the source of iron is the target cell itself. They demonstrated that stimulated neutrophils were cytotoxic for endothelial cells in an iron dependent manner. Pretreatment of the neutrophils with deferoxamine did not protect against the cytotoxicity. However, pretreatment of the endothelial cells with deferoxamine was significantly protective in a time and concentration dependent fashion.

Although lipid peroxidation of cell membrane has been considered to be an evidence for oxidant-induced cell injury[18], the role that lipid peroxidation plays as a critical event in the pathogenesis of oxidant-induced cell injury has not been clearly established[35-37]. Lipid peroxidation can be a result or an epiphenomenon of cell death rather than a cause of cell injury[18]. To determine whether H_2O_2 leads to cell death via a lipid peroxidation-dependent mechanism, Caco-2 cells were treated with H_2O_2 in the presence of antioxidants. DPPD

have been reported to effectively prevent oxidant-induced cell injury in renal proximal tubular cells[38] and renal cortical slices[19]. If H_2O_2-induced cell injury was caused by lipid peroxidation, both the cell death and lipid peroxidation should be prevented by DPPD. In the present study, however, despite H_2O_2-induced lipid peroxidation was completely blocked by DPPD(Fig. 5), the cell death was not prevented(Fig. 4). These results suggest that the H_2O_2-induced cell death is not mediated by lipid peroxidation in Caco-2 cells. The H_2O_2-induced lipid peroxidation may be a result of the cell injury rather than a mechanism by which the cell death is induced[18]. Although the results of the present study showed that SR inhibits H_2O_2-induced lipid peroxidation, therefore, its protective effect against H_2O_2-induced cell death may be mediated by a mechanism independent of antioxidative action.

DNA is an important cellular and molecular target of oxidant stress. Oxidant stress results in DNA damage by induction of single-strand breaks, by base modification, or by the induction of apoptosis[18,31,39,40]. However, whether DNA damage leads to cell killing is controversial. Various investigators reported that DNA damage plays a central role in cell death[18,31,32], whereas DNA damage is not the primary mediators of cell death following oxidative stress in renal epithelial cells[41] and heptocytes[42]. The present study demonstrated that SR, catalase, and deferoxamine prevented the H_2O_2-induced DNA damage(Fig 7). Similarly to cell death, however, DPPD did not prevent the H_2O_2-induced DNA damage. This may suggest that DNA damage is linked to cell death in Caco-2 cells .

The possible mechanism of SR protective effect is not clear from the results of the present study. In the present study, the H_2O_2-induced cell injury was dependent of catalase and deferoxamine rather than DPPD. Therefore, SR may act as a H_2O_2 scavenger and/or as an iron chelator. Although the precise mechanism remains to be explored, the results of the present study provide extensive information on the underlying mechanism of ROS-induced cell death, and suggest that SR may be developed as a useful drug for treatment and prevention of gastrointestinal injuries mediated by ROS.

CONCLUSION

This study was undertaken to determine whether *Salviae Miltiorrhizae Radix*(SR) extract exerts beneficial effect against cell injury induced by reactive oxygen species(ROS) in human intestinal epithelial cells. Effects of SR on cell injury were examined using Caco-2 cells, cultured human intestinal cell line.

1. H_2O_2 increased in the cell death in a time- and dose-dependent fashion.
2. SR prevented H_2O_2-induced cell death and its effect was dose-dependent over concentration range of 0.005-0.1%.
3. The H_2O_2-induced cell death was prevented by catalase, the hydrogen peroxide scavenger, and deferoxamine, the iron chelator, but not by a potent antioxidant DPPD.
4. H_2O_2 increased lipid peroxidation, which was inhibited by SR and DPPD.
5. SR restored H_2O_2-induced ATP depletion.
6. H_2O_2 caused DNA damage in a dose-dependent manner, which was prevented by SR, catalase, and deferoxamine, but not DPPD.

These results indicate that SR exerts the protective effect against the H_2O_2-induced cell injury through a mechanism other than antioxidant action. The present study suggests that SR may be developed as a therapeutic drug for the treatment of human gastrointestinal diseases mediated by ROS.

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