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원 저

Effect of *Salviae Radix* on Cell Death and DNA Damage in Renal Proximal Tubular Cells Exposed to H_2O_2

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H_2O_2에 노출된 신장 근위 세뇨관 세포에서의 세포 사망 및 DNA 손상에 대한 丹參의 효과

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목적 : 이전 연구에서 丹參 추출액이 강력한 항산화 작용이 있음을 확인한 바 있어 丹參 추출액이 신장세뇨관 세포에서 oxidant에 의한 세포사망 및 DNA 손상을 방지하는 지를 조사하고 이러한 효과가 지질의 과산화를 억제하는 효과에 기인하는 지를 시험하였다.

방법 : 신장 근위세뇨관 세포 유래 세포주인 opossum kidney (OK) 세포를 이용하여 세포 사망은 trypan blue exclusion 방법을 이용하여 평가하였고, DNA 손상 정도는 double stranded DNA의 파괴를 측정하여 평가하였다. Oxidant 약물 모델로는 H_2O_2를 사용하였다.

결과 : H_2O_2는 적용시간과 농도에 비례하여 세포 사망을 유도하였다. 丹參 추출액은 0.05% 농도에서 H_2O_2에 의한 세포사망 및 DNA 손상을 방지하였다. 이러한 방지효과는 H_2O_2 제거 효소인 catalase와 철 착염제인 deferoxamine에 의해서도 나타났다. 그러나 강력한 항산화제인 DPPD는 H_2O_2에 의한 세포 사망이나 DNA 손상을 방지하지 못하였다. H_2O_2는 세포내 ATP 농도를 감소시켰으며, 이러한 감소는 poly (ADP-ribose) polymerase 억제제인 3-aminobenzamide에 의해 방지되었으나 丹參 추출액에 의해서는 영향을 받지 않았다. 3-aminobenzamide는 H_2O_2에 의한 세포 사망을 방지하였다. H_2O_2는 지질의 과산화를 증가시켰으며, 이러한 변화는 丹參 추출액과 DPPD에 의해 방지되었다.

결론 : OK 세포에서 H_2O_2에 의한 세포사망과 DNA 손상에는 지질의 과산화가 중요한 역할을 하지 않으며, 丹參 추출액의 H_2O_2에 의한 세포 사망과 DNA 손상 방지 효과는 항산화 작용이 아닌 다른 기전에 기인하는 것으로 사료된다. *(J Korean Oriental Med 2001;22(3):21-30)*

Key Words: *Salviae Radix*, H_2O_2, acute renal failure, GFR, tubular reabsorption, lipid peroxidation

INTRODUCTION

Reactive oxygen species produced by stimulated neutrophils or macrophages induces cell injury and lysis in surrounding target cells *in vivo*[1-3] and *in vitro*[4-7]. They have been shown to participate in the pathogenesis of

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several different renal diseases including inflammatory lesions such as *glomerulonephritis* and *interstitial* nephritis, ischemic reperfusion injury, toxic nephropathies, and possibly in the progression of chronic renal failure[8-11].

While proteins, lipids, carbohydrates, DNA and RNA can all be targets of oxidant-induced injury[12,13], damage to a small percentage of DNA molecules may bear greater consequence than damage to other cellular components. Low concentrations of H_2O_2, less than 100 μM and well within the range reached in the proximity of stimulated leukocytes, induce DNA strand breaks in various target cells[14-16].

Previous studies showed that *Salviae Radix* (SR) extract exerts the protective effect against reactive oxygen species in the kidney tissues of rabbits17). Therefore, the present study was carried out to determine whether SR prevents cell death and DNA damage induced by H_2O_2 in opossum kidney (OK) cells, an established kidney proximal tubular cell line.

MATERIALS AND METHODS

Extraction and preparation of *Slaviae Radix*

2 kg 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 168 g. 50 g of methyl alcohol extract was suspended into water and extracted with n-hexane to remove fats. The remaining water suspension was extracted with ethyl acetate to obtain 6.8 g.

Culture of opossum kidney cells

OK cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained by serial passages in 75-㎠ 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 37oC 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 3-4 days after plating when a confluent monolayer culture was achieved. Cells were treated with H_2O_2 in Hank' s balanced salt solution (HBSS) (Sigma Co. USA) without serum.

Cell viability

Cells were grown to confluence in 24-well dishes, incubated in the HBSS containing H_2O_2 for 120 min at 37oC 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.

Measurement of ATP content

ATP levels were measured on OK cells with a luciferin-luciferase assay. After an exposure to oxidant stress, 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 ㎎/㎖ luciferin-luciferase was added to 10 μl of diluted sample. Light emission was recorded at 20 s with a luminometer (MicroLumat LB96P, Berthold, Germany). Protein content was determined on a portion of the cell sample, and ATP was expressed as nmoles per ㎎ cell protein.

Measurement of DNA single-strand breaks

DNA strand break was measured by the DNA precipitation assay[18]. Confluent cells grown in 24-wells were labelled in the presence of 0.25 μCi/㎖

[^{3}H]methylthymidine for 24 hr. The cells were thoroughly washed with HBSS, and treated with mercury chloride in the presence or absence of SR. After treatment, the cells were washed with HBSS and lysed in effendorf tube with 0.5 *ml* of lysis buffer (10 mM Tris/HCl, 10 mM EDTA, 50 mM NaOH, 2% SDS, pH 12.4), followed by addition of 0.5 *ml* of 0.12 M KCl. The lysate was incubated for 10 min at 65oC, followed by a 5 min cooling-and-precipitation period on ice. A DNA-protein K-SDS precipitate was formed under these conditions, from which low-molecular-mass broken DNA was released. This DNA was recovered in the supernatant from a 10 min centrifugation at 200 g, 10oC, and transferred into a liquid scintillation vial containing 1 *ml* of 200 mM HCl. The precipitation pellet (intact double-stranded DNA) was solubilized in 1 *ml* of water at 65oC. The tube was rinsed with 1 *ml* of water, and 8 *ml* 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).

Lipid peroxidation assay

The grown cells in the 6-well dishes were exposed to the various concentrations of H_2O_2 in HBSS (4 *ml*/well) for 1 hour. Cells were lysed by addition of 0.1% sodium deoxycholate. Lipid peroxidation was estimated by measuring the tissue content of malondialdehyde (MDA) according to the method of Uchiyama and Mihara19). Briefly, cell lysates (4 *ml*) were mixed with 0.5 *ml* of 10% phosphoric acid and 1 *ml* of 0.6% thiobarbituric acid aqueous solution. The mixture was heated for 45 min on a boiling water bath. After addition of 4 *ml* of n-butanol, the contents were vigorously vortexed and centrifuged at 2,000 g for 20 min. The absorbance of the upper, organic layer was measured at 535 and 520 nm with diode array spectrophotometer (Hewlett Packard, 8452A), and was compared to results obtained using freshly prepared malondialdehyde tetraethylacetal standards. MDA values were expressed pmoles per ㎎ protein. Protein was measured by the method of Bradford[20)].

Chemicals

[^{3}H]methylthymidine was purchased from the Amersham International (Amersham, UK). Catalase, deferoxamine, 3-aminobenzamide were purchased from Sigma Chemical (St. Louis, MO, USA). N,N'-diphenyl-p-phenylenediamine (DPPD) was purchased from Aldrich Chemical (Milwaukee WI, USA). All other chemicals were of the highest commercial grade available.

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

Time course of *Slaviae Radix* effect on H2O2-induced cell death

Exposure of cells to 0.5 mM H_2O_2 for various time periods caused an increase in cell death in a time-dependent fashion (Fig. 1). When cells were treated for 10, 30, 60, 120, and 180 min with H_2O_2, the loss of cell viability was 15.30 ± 1.80, 16.44 ± 2.50, 18.74 ± 1.40, 33.04 ± 1.60, and 33.04 ± 1.18%, respectively. These values were significantly higher than those of the control cells incubated for 60 or 120 min (6.53 ± 2.64 and 7.32 ± 2.51%, respectively). However, exposure

of cells to H_2O_2 in the presence of 0.05% SR produced a significant reduction in cell death for all time periods as compared with H_2O_2 alone.

Dose-dependency of *Slaviae Radix* effect on H_2O_2-induced cell death

When cells were exposed to H_2O_2 for 120 min, the cell death was increased from 3.67 ± 0.56% of the control cells to 47.20 ± 1.56 and 64.43 ± 1.81% in cells treated with 0.5 and 1.0 mM H_2O_2, respectively (Fig. 2). To determine whether SR prevents H_2O_2-induced cell death, the cells were treated with H_2O_2 in the presence of various concentrations of SR. SR at 0.01% concentration did not alter effects of 0.5 and 1.0 mM H_2O_2. However, at 0.05% concentration SR produced a significant reduction in H_2O_2-induced cell death (16.43 ± 2.0% and 31.78 ± 2.63% for 0.5 and 1.0 mM H_2O_2, respectively). Interestingly, when SR concentration was increased to 1%, the drug did not affect H_2O_2-cell death. SR did not alter the viability in the control cells over concentrations examined.

Effects of *Slaviae Radix*, H_2O_2 scavenger, iron chelator, and antioxidant on cell death and DNA damage induced by H_2O_2

The protective effect of SR on H_2O_2-induced cell death was compared with that of various drugs that have been well known to prevent H_2O_2 cytotoxicity. The results are summarized in Fig. 3. Exposure of cells to 0.5 mM H_2O_2 caused an increase in cell death from 2.50 ± 0.33% in the control to 22.60 ± 1.30%. SR, a H_2O_2 scavenger catalase, and an iron chelator deferoxamine significantly prevented H_2O_2-induced cell death, but a potent antioxidant DPPD did not affect.

In order to examine if SR exerts the beneficial effect against H_2O_2-induced DNA damage, DNA breaks were measured in cells treated with H_2O_2 in the presence or absence of SR. As shown in Fig. 4, exposure of cells to 0.5 mM H_2O_2 caused a significant increase in DNA damage as evidenced by a decrease in double stranded DNA (37.20 ± 1.67 vs. 88.80 ± 5.04% in the control). These changes were significantly prevented by addition of 0.05% SR (61.90 ± 6.17%). As expected, catalase completely prevented H_2O_2-induced DNA damage.

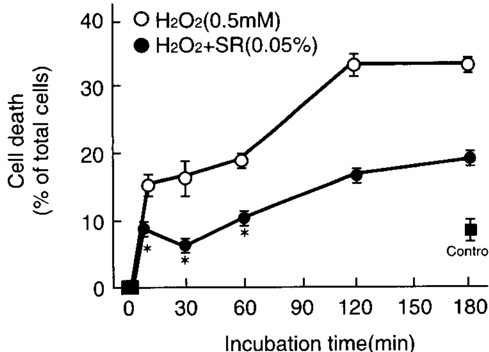

Fig. 1. Time course of *Salviae Radix* (SR) effect on H_2O_2-induced death in possum kidney (OK) cells.
Cells were treated with 0.5 mM H_2O_2 for 10-180 min in the presence or absence of 0.05% SR, and then cell viability was measured by trypan blue exclusion assay. Data are mean ± SE of five experiments. *P<0.05 compared with H_2O_2 alone.

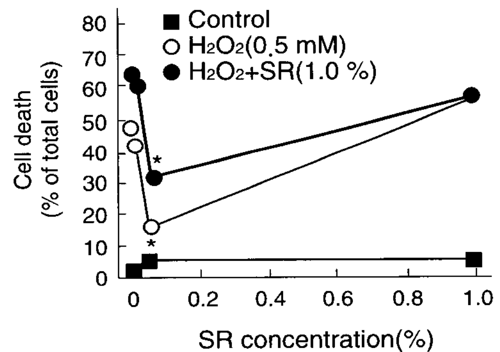

Fig. 2. Dose dependency of *Salviae Radix* (SR) effect on H_2O_2-induced cell death in opossum kidney (OK) cells.
Cells were treated with 0.5 and 1.0 mM H_2O_2 for 120 min in the presence or absence of 0.01, 0.05, and 1.0% SR, and then cell death was measured by the trypan blue exclusion assay. Data are mean ± SE of five experiments. *P<0.05 compared with H_2O_2 alone.

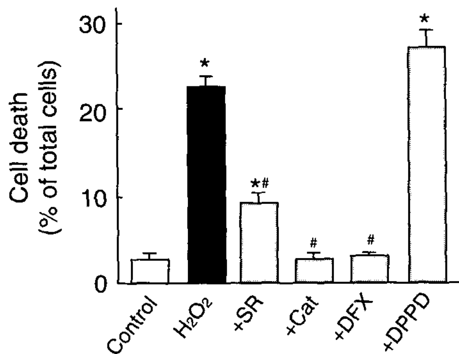

Fig. 3. Effects of *Salviae Radix* (SR), catalase (Cat), deferoxamine (DFX), and DPPD on H_2O_2-induced cell death in opossum kidney (OK) cells.

Cells were treated with 0.5 mM H_2O_2 for 120 min in the presence or absence of 0.05% SR, 500 units/*ml* Cat, 2 mM DFX, and 20 μM DPPD, and then cell death was measured by the 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.

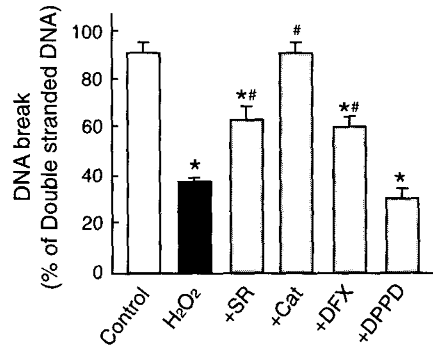

Fig. 4. Effects of *Salviae Radix* (SR), catalase (Cat), DPPD, and deferoxamine (DFX) on H_2O_2-induced DNA damage in opossum kidney (OK) cells.

Cells were treated with 0.5 mM H_2O_2 for 120 min in the presence or absence of 0.05% SR, 500 units/*ml* Cat, 2 mM DFX, and 20 μM DPPD, and then DNA damage was measured. Data are mean ± SE of six experiments. *$P<0.05$ compared with the control; #$P<0.05$ compared with H_2O_2 alone.

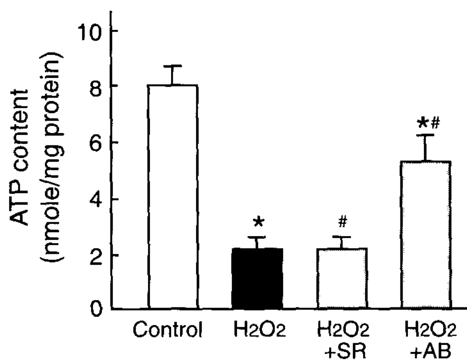

Fig. 5. Effects of *Salviae Radix* (SR) and inhibitor of poly (ADP-ribose) polymerase on H_2O_2-induced depletion of ATP in opossum kidney (OK) cells.

Cells were treated with 0.5 mM H_2O_2 for 120 min in the presence or absence of 0.05% SR and 1.0 mM 3-aminobenzamide (AB), and then ATP content was measured. Data are mean ± SE of four experiments. *$P<0.05$ compared with the control; #$P<0.05$ compared with H_2O_2 alone.

Similar results were obtained by deferoxamine. However, DPPD did not alter H_2O_2-induced DNA damage.

ATP depletion and DNA damage have been known to be early responses of cells to oxidant stress[7,21,22]. Several studies have also shown that depletion of ATP resulting from the activation of poly (ADP-ribose) polymerase is involved in the pathogenesis of oxidant-induced cell death[23,24]. Thus, effect of SR on H_2O_2-induced ATP depletion was examined. The results of Fig. 5 indicate that H_2O_2 caused ATP depletion, which was prevented by an inhibitor of poly (ADP-ribose) polymerase, 3-aminobenzamide, but not by SR. Prevention of ATP depletion by 3-aminobenzamide was associated with a decrease in cell death (Fig. 6). These results suggest that cell death and DNA damage induced by H_2O_2 are dependent on iron-dependent mechanisms, but they are not associated with lipid peroxidation.

Effects of *Slaviae Radix* on H2O2-induced lipid peroxidation

In the last series of experiments, effect of SR on H_2O_2-induced lipid peroxidation was examined. As shown Fig. 7, H_2O_2 increased lipid peroxidation which was completely prevented by 0.05% SR. Lipid peroxidation was 0.53 ± 0.06, 1.29 ± 0.06, and 0.63

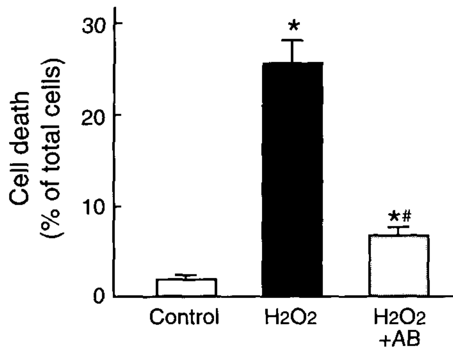

Fig. 6. Effects of inhibitor of poly (ADP-ribose) polymerase on H_2O_2-induced cell death in opossum kidney (OK) cells.

Cells were treated with 0.5 mM H_2O_2 for 120 min in the presence or absence of 1.0 mM 3-aminobenzamide (AB), and then cell death was measured by the trypan blue exclusion assay. Data are mean ± SE of four experiments. *P<0.05 compared with the control; #P<0.05 compared with H_2O_2 alone.

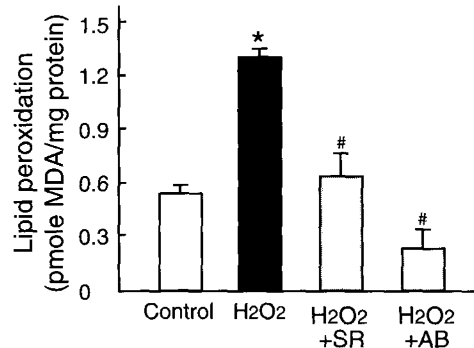

Fig. 7. Effect of *Salviae Radix* (SR) and DPPD on H_2O_2-induced lipid peroxidation in opossum kidney (OK) cells.

Cells were treated with 0.5 mM H_2O_2 for 120 min in the presence or absence of 0.05% SR and 20 μM DPPD, and then lipid peroxidation was measured. Data are mean ± SE of three experiments. *P<0.05 compared with the control; #P<0.05 compared with H_2O_2 alone.

± 0.13 pmole MDA/㎎ protein in the control, H_2O_2-treated, and H_2O_2 treated cells in the presence of SR, respectively. As expected, the lipid peroxidation was prevented by a potent antioxidant DPPD (0.21 ± 0.12 pmole MDA/㎎ protein).

DISCUSSION

When oxidant generation increases or when antioxidant defense mechanism are decreased, oxidant injury results from the shift in the oxidant-antioxidant balance. Early responses of cells to oxidant stress include activation of the glutathione redox cycle, DNA damage, ATP depletion, and oxidation of susceptible amino acids in proteins and lipids[21,25], while disruption of the cytoskeleton, blebbing, cell retraction, gap formation, and eventual cell lysis are later events[26-28]. Although multiple metabolic effects of oxidant injury have been elucidated, the events eventually leading to irreversible injury and cell death are not well understood.

In the present study, SR at 0.05% concentration significantly prevented H_2O_2-induced cell death. SR at concentration higher (1%) than 0.05% had no effect. The precise mechanism of no effect of SR at the higher concentration remains to be defined. Since the previous studies demonstrated that SR prevents oxidant-induced cell injury by scavenging action of reactive oxygen species[17], the protective effect of SR against the H_2O_2-induced cell death could be resulted from its antioxidant action. To test the possibility, the effect of SR was compared with that of H_2O_2 scavenger enzyme and antioxidants. In the present study, a H_2O_2 scavenger enzyme catalase and an iron chelator deferoxamine completely prevented the H_2O_2-induced cell death. This suggests that H_2O_2 induces the cell death by an iron-dependent mechanism.

Lipid peroxidation has been considered to be an evidence for oxidant-induced cell injury. Nevertheless, the role that lipid peroxidation plays as an early and critical event in the pathogenesis of oxidant-induced lethal cell injury has been controversial[29-31]. Lipid

peroxidation can be a result of the cell injury or an epiphenomenon of the cell death rather than as the cause of the cell injury[32]. To determine if H_2O_2 induces the cell death in OK cells via a lipid peroxidation-dependent mechanism, cells were treated with H_2O_2 in the presence of an antioxidant. DPPD have been reported to prevent oxidant-induced cell injury in renal proximal tubular cells[33] and renal cortical slices[34] with its high potency. If the H_2O_2-induced cell death is resulted from lipid peroxidation, the cell death would be prevented by DPPD. Recently, Kim and Kim[34] reported in rabbit renal cortical tissues that the LDH release induced by H_2O_2 is prevented by DPPD, suggesting that lipid peroxidation plays an important role in oxidant-induced cell death in renal tubular cells. In the present study, however, despite the H_2O_2-induced lipid peroxidation was completely prevented by DPPD (Fig. 7), the H_2O_2-induced cell death was not affected by the antioxidant (Fig. 3). These results suggest that the H_2O_2-induced cell death is not mediated by lipid peroxidation in OK cells. Thus, the lipid peroxidation induced by H_2O_2 may be a result or an epiphenomenon than a cause of the cell injury[32]. The results obtained from the present study with DPPD are at variance with those in rabbit renal cortical slices that DPPD prevents completely oxidant-induced cell death[34]. This discrepancy may be attributed to difference in animal species. Several investigators have shown species difference in the role of lipid peroxidation in oxidant-induced cell death[30,35]. The results of the present study may be, in part, in agreement with those of Andreoli and Mallett[36] who reported in LLC-PK1 cells, another proximal tubular cell line, that antioxidants 2-Mac and Trolox do not inhibit the cell death in cells treated with H_2O_2 for 24 hr. Thus, the present study suggests that the beneficial effect of SR is attributed to mechanisms other than its antioxidant 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[32,37-39]. The present study demonstrated that the H_2O_2-induced DNA damage was prevented by SR, catalase, and deferoxamine, but not DPPD (Fig. 4). The effects of these drugs on DNA damage were of the same as those of cell death. This may suggest that the cell death is associated with DNA damge.

Several studies have shown that depletion of ATP resulting from the activation of poly (ADP-ribose) polymerase is involved in the pathogenesis of oxidant-induced cell death[23,24]. However, other studies reported that the oxidant injury is not attributed to the depletion of ATP itself[21,29,35,36]. In the present study, H_2O_2 decreased cellular ATP content, and both ATP depletion and cell death were prevented by 3-aminobenzamide (Figs. 5 and 6). These results suggest that the H_2O_2-induced cell death in OK cells may be attributed to the depletion of ATP resulting from the activation of poly (ADP-ribose) polymerase. However, SR fails to reverse the H_2O_2-induced ATP depletion, suggesting that the protective effect of SR is not associated with inhibition of poly (ADP-ribose) polymerase. In addition, these data suggest that the cell death and DNA damage induced by H_2O_2 are dissociated with ATP depletion. These results are consistent with those of Andreoli and Mallett[36] who reported in LLC-PK1 cells that ATP depletion and DNA damage are not the primary mediators of early cytotoxicity following oxidant stress.

In summary, H_2O_2-induced cell death and DNA damage by a mechanism independent on lipid peroxidation. SR prevented the H_2O_2-induced cell death and DNA damage, and its effect is not associated with prevention of lipid peroxidation.

CONCLUSION

This study was undertaken to determine if *Salviae Radix* (SR) exerts protective effect against cell death and DNA damage induced by oxidant in renal proximal tubular cells. The cell death was evaluated by trypan blue exclusion and DNA damage was estimated by measuring double stranded DNA breaks in opossum kidney (OK) cells, an established proximal tubular cell line. H_2O_2 was used as a model oxidant. H_2O_2-induced cell death in time- and dose-dependent manner. SR at 0.05% concentration exerted the beneficial effect against cell death and DNA damage induced by H_2O_2. A H_2O_2 scavenger catalase and an iron chelator deferoxamine also prevented cell death and DNA damage induced by H_2O_2. However, a potent antioxidant DPPD did not affect H_2O_2-induced cell injury. H_2O_2 decreased ATP levels, which was prevented by an inhibitor of poly (ADP-ribose) polymerase 3-aminobenzamide, but not by SR. 3-Aminobenzamide prevented the H_2O_2-induced cell death. H_2O_2 increased lipid peroxidation and its effect was completely prevented by SR and DPPD. These results indicate that cell death and DNA damage induced by H_2O_2 are not associated with lipid peroxidation in OK cells. SR exerts the protective effect against the H_2O_2-induced cell injury by mechanisms other than its antioxidant action. The precise mechanism of the protective effect of SR remains to be explored in OK cells.

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