

Vitamin C attenuates the toxic effect of aristolochic acid on renal tubular cells via decreasing oxidative stress-mediated cell death pathways

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Abstract. Aristolochic acid (AA) is a component of Chinese medicinal herbs, including asarum and aristolochia and has been used in Traditional Chinese Medicine for a long time. Recent studies found that AA has a cytotoxic effect resulting in nephropathy. These studies indicated that AA-induced cytotoxicity is associated with increases in oxidative stress and caspase-3 activation. The present study further demonstrated that AA mainly elevates the H₂O₂ ratio, leading to increases in oxidative stress. Furthermore, the results indicated that AA induces cell death can via caspase-dependent and -independent pathways. It is desirable to identify means of inhibiting AA-induced renal damage; therefore, the present study applied an anti-oxidative nutrient, vitamin C, to test whether it can be employed to reduce AA-induced cell cytotoxicity. The results showed that vitamin C decreased AA-induced H₂O₂ levels, caspase-3 activity and cytotoxicity in renal tubular cells. In conclusion, the present study was the first to demonstrate that AA-induced increases of the H₂O₂ ratio resulted in renal tubular cell death via caspase-dependent and -independent pathways, and that vitamin C can decrease AA-induced increases in H₂O₂ levels and caspase-3 activity to attenuate AA-induced cell cytotoxicity.

Introduction

Aristolochic acid (AA) is contained in the Chinese medicinal herbs asarum and aristolochia (1-3). Numerous studies have demonstrated that AA can cause nephropathy (4-6). In traditional Chinese medicine, asarum and aristolochia are usual components of complex Chinese remedies applied to treat arthritic pain, cough and gastrointestinal symptoms (7-9). Therefore, AA-induced nephropathy is widely discussed in China (10,11). Previous studies showed that AA can induce renal tubular cell death and fibrosis, leading to nephropathy (12-14). As shown in numerous studies, AA-induced tubular cell injury was associated with apoptosis (12,15-17). However, certain studies reported that AA induced cell death through necrosis (18-20).

Apoptosis can be induced via a caspase-dependent or caspase-independent pathway (21,22). AA-induced renal tubular cell death was reported to proceed via the caspase-dependent apoptotic pathway by numerous studies (13,23,24). In general, AA mainly induces caspase-3 activation, resulting in tubular cell apoptosis (25-27). The present study further investigated the cytotoxic effects of AA on renal tubular cells; dose- and time-dependency were examined and apoptotic pathways were tested by using a caspase inhibitor.

Although AA-induced renal damage has been reported in numerous studies (4-6), the mechanisms of AA-induced renal tubular cell death have remained to be clarified. Previous studies reported increases of oxidative stress in AA-treated renal tubular cells and suggested that the increase of reactive oxygen species (ROS) is a possible mechanism for AA-induced renal damage (16,27,28). However, it has remained elusive which ROS is elevated by AA treatment. O₂⁻ and H₂O₂ belong to ROS families which are common in cells (29,30). Thus, O₂⁻ and H₂O₂ levels in renal cells following treatment with AA were examined in the present study.

Based on the observation that the cytotoxicity of AA is mediated via the induction of oxidative stress (16,27,28),

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N-acetyl cysteine (NAC) and glutathione (GSH), anti-oxidative agents, were studied to prevent cell death through AA treatment (16). Vitamin C is a common nutrient with anti-oxidative properties (31-33). In the present study, it was hypothesized that vitamin C is able to inhibit AA-induced cytotoxicity. Thus, AA-treated renal tubular cells were co-treated with vitamin C, and its effects on cell viability, caspase-3 activation and H_2O_2 levels were tested.

The present study indicated that co-administration of vitamin C may be employed to reduce the nephrotoxic effects of the Chinese medicinal herbs asarum and aristolochia.

Materials and methods

Materials. Luminol, lucigenin, aristolochic acid, vitamin C, tubulin antibody and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). TGF- β was obtained from R&D Systems (Minneapolis, MN, USA; cat. no. AB-100-NA). An MTT assay kit was purchased from Bio-Basic Canada Inc. (Markham, ON, Canada). Caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) was purchased from BD Pharmingen (Franklin Lakes, NJ, USA). Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, L-glutamine and penicillin/streptomycin were obtained from Gibco-BRL (Invitrogen Life Technologies, Carlsbad, CA, USA). Caspase-3 and cleaved caspase-3 antibodies (cat. no. 9662) were purchased from Cell Signaling Technology, Inc. (Beverly, MD, USA).

Cell culture. The rat kidney tubular epithelial cell line NRK-52E was obtained from the Bioresource Collection and Research Center (Shin Chu, Taiwan). NRK-52E cells were cultured in a DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 0.1 mM non-essential amino acids and maintained at 37°C in a humidified atmosphere containing 5% CO_2 .

Cell survival assay. The cell survival rate of NRK-52E cells was analyzed using the MTT assay according to the manufacturer's instructions. Briefly, NRK-52E cells were seeded into 96-well plates at a density of 8×10^3 cells/well, and incubated for 24 h in 100 μ l culture medium. The suitable concentration and optimum exposure time of AA were determined as 5, 10, 20 and 100 μ M at 6 h time intervals. At the 6 h time intervals, the control group and experimental groups were examined with the MTT assay kit. Cells were incubated with MTT solution at 37°C for 3 h and the survival rate was then measured at 570 nm absorbance using a Multiskan™ FC Microplate Photometer (Molecular Devices, Sunnyvale, CA, USA). The cell survival rate was calculated using the following formula: $A_{570} \text{ experimental group} / A_{570} \text{ control group} \times 100\%$ (34).

Observation of cell morphology and apoptotic features. Cell morphology was observed under a phase-contrast microscope. Apoptotic features were observed by Hoechst 33342 (cat. no. 23491-52-3; Sigma-Aldrich) nuclear staining (35-37). In brief, cells were treated with Hoechst 33342 (10 μ g/ml) for 10 min. DNA fragmentation and nuclear condensation were observed under an Olympus DP71 fluorescence microscope

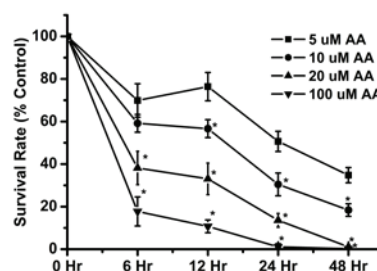


Figure 1. AA induces cell cytotoxicity in a dose- and time-dependent manner. NRK-52E cells were treated with 5, 10, 20 and 100 μ M AA, respectively. Survival rates were measured at the indicated time-points by an MTT assay. Values are expressed as the mean \pm standard deviation from three independent experiments. * $P < 0.05$, vs. the 5 μ M AA-treated group. AA, aristolochic acid.

(excitation, 352 nm; emission, 450 nm; Olympus Corporation, Tokyo, Japan).

Caspase inhibition assay. Z-VAD-FMK is a general caspase inhibitor. In the present study, NRK-52E cells were pre-treated with 20 μ M Z-VAD-FMK prior to AA treatment (0, 5, 10, 20 or 100 μ M). The survival rates of NRK-52E cells were determined using an MTT assay as described above (38).

Determination of H_2O_2 and O_2^- ratios. H_2O_2 and O_2^- ratios were determined by using the lucigenin-amplified chemiluminescence method (39,40). In brief, the culture supernatant (200 μ l) was treated with 0.2 mmol/l of luminol solution (100 μ l) and subsequently evaluated using a chemiluminescence analyzing system (CLA-FSI; Tohoko Electronic Industrial Co., Ltd., Sendai, Japan) for the determination of H_2O_2 levels. Samples (200 μ l) were treated with 0.1 mmol/l lucigenin solution (500 μ l) and were then measured using the CLA-FSI chemiluminescence analyzing system for the determination of O_2^- levels. The ratios of H_2O_2 and O_2^- were calculated as (experimental group counts/control group counts) $\times 100\%$.

Western blot analysis. Western blot analysis was performed as in a previous study (34). Briefly, cells were lysed with radioimmunoprecipitation assay buffer (cat. no. 20-188; EMD Millipore, Billerica, MA, USA). After centrifugation at 16,000 $\times g$ for 10 min at 4°C, proteins were obtained and their concentration was determined using the Bradford assay (cat. no. 23200; Thermo Fischer Scientific, Inc., Waltham, MA, USA). Equal amounts of samples were separated by 13.3% SDS-PAGE (Bio-Rad Rad Laboratories, Inc., Hercules, CA, USA), and then transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked with 5% milk for 2 h at room temperature. After washing with phosphate-buffered saline (PBS), samples were incubated with the primary antibodies for 4 h overnight. Next, samples were washed with PBS and treated with anti-rabbit-HRP secondary antibodies (cat. no. NA934; Amersham Biosciences Inc., Beverly, MD, USA) for 1 h at room temperature. Finally, samples were visualized by using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Waltham, MA, USA) and quantified using a densitometer (41,42).

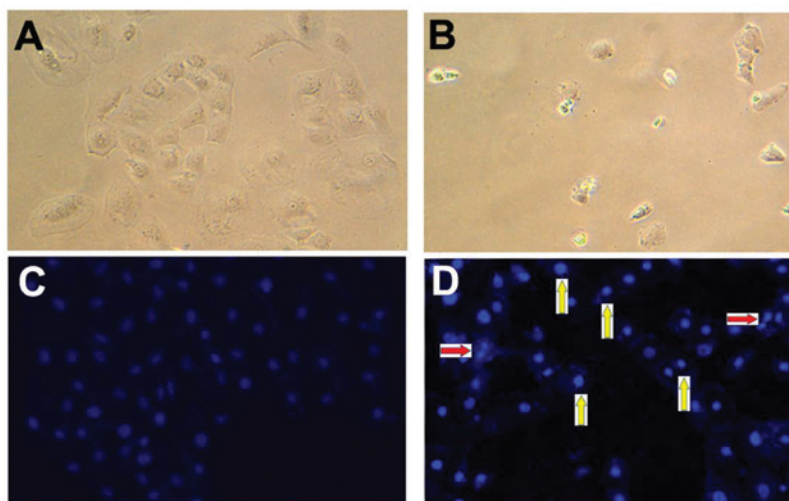


Figure 2. AA induces cell death via the apoptotic pathway. (A and B) Phase-contrast microscopic observation showed that compared with (A) the control group, shrunken cells were present among the (B) Cells treated with 10 μ M AA for 12 h. (C and D) Fluorescent-microscopic observation (magnification, x400) showed that compared with (C) the control group, nuclear condensation (yellow arrow) and DNA fragmentation (red arrow) were present in (D) Cells treated with 10 μ M AA for 12 h. AA, aristolochic acid.

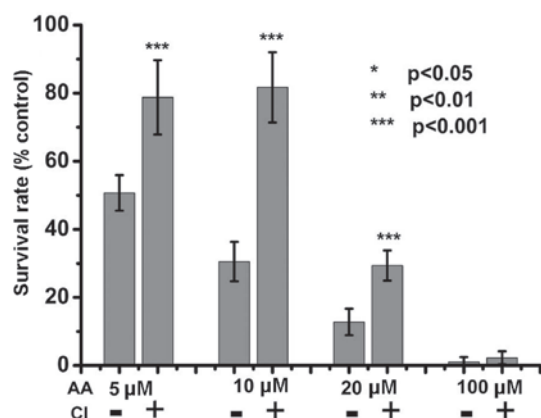


Figure 3. AA-induced cell death involves caspase-dependent and -independent signals. NRK-52E cells were treated with 5, 10, 20 and 100 μ M AA with or without 20 μ M CI. After 24 h, survival rates were determined using the MTT assay. Values are expressed as the mean \pm standard deviation from three independent experiments. AA, aristolochic acid; CI, caspase inhibitor.

Statistical analysis. Student's t-test was utilized for the analysis of data. Values are expressed as the mean \pm standard error. $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

AA induces cytotoxicity in a dose- and time- dependent manner. Previous studies have demonstrated that AA exerts cytotoxic effects in renal tubular cells (16,43,44). Similarly, the results of the present study also indicated that AA can exert cytotoxic effects on renal tubular cells (Fig. 1). The cell survival rate was $<50\%$ by the following treatments: 100 μ M AA (6 h), 20 μ M AA (6 h), 10 μ M AA (24 h) and 5 μ M AA (48 h). The results indicated that high-dose AA treatment exerted a higher cytotoxic effect on renal tubular cells than low-dose AA treatment. In addition, long durations of incubation had a greater

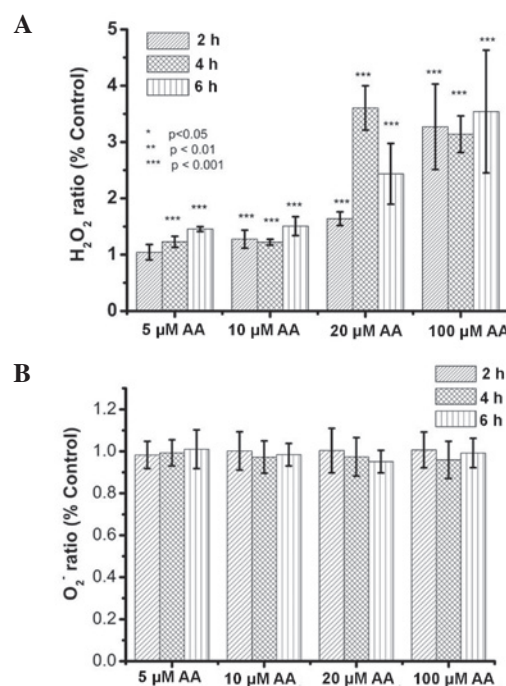


Figure 4. AA induces increases in the H_2O_2 ratio but not in the O_2^- ratio. (A) H_2O_2 ratios in AA-treated cells. (B) O_2^- ratios in AA-treated cells. AA induced increases in H_2O_2 ratios but not O_2^- ratios. Values are expressed as the mean \pm standard deviation from three independent experiments. AA, aristolochic acid.

cytotoxic effect as compared with short incubations with AA. These results suggested that AA induced cytotoxicity in a dose- and time-dependent manner.

AA-induced cell death is associated with the apoptotic pathway. Cell death can be associated with apoptotic or necrotic pathways (45,46). In the present study, cell morphology was observed under a phase-contrast and a fluorescent microscope (Fig. 2). Compared with control cells (Fig. 2A), shrunken types were observed among AA-treated

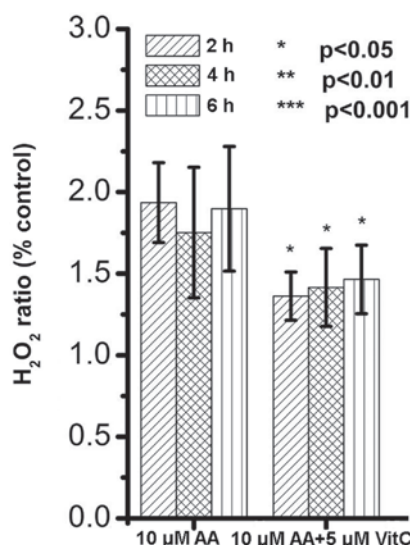


Figure 5. Vitamin C reduces the AA-induced H₂O₂ ratio. H₂O₂ ratios were measured in 10 μM AA-treated cells with or without 5 μM vitamin C. Vitamin C significantly reduced H₂O₂ ratios in AA-treated cells. Values are expressed as the mean ± standard deviation from three independent experiments. AA, aristolochic acid.

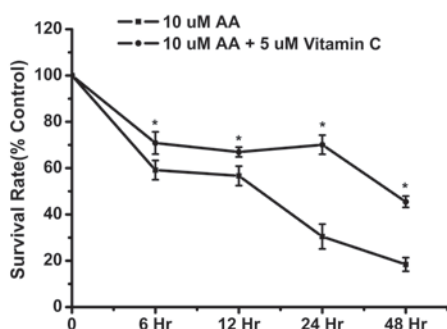


Figure 6. Vitamin C inhibits AA-induced cytotoxicity. NRK-52E cells were treated with 10 μM AA with or without 5 μM vitamin C. Survival rates were measured at the indicated time-points by MTT assay. Values are expressed as the mean ± standard deviation from three independent experiments. *P<0.05, vs. the 10 μM AA-treated group AA, aristolochic acid.

cells (Fig. 2B) under a phase-contrast microscope. In addition, apoptotic characteristics, including nuclear condensation and DNA fragmentation, were determined by Hoechst nuclear staining (35,36). Compared with the control cells (Fig. 2C), nuclear condensation and DNA fragmentation were observed in AA-treated cells under a fluorescent microscope (Fig. 2D). These observations indicated that AA-induced cell death was associated with the apoptotic pathway.

AA-induced apoptosis involves caspase-dependent and -independent signaling. AA can activate caspase, resulting in cell cytotoxicity (25-27). However, whether AA-induced apoptosis is dependent on caspases has remained elusive. Therefore, in the present study, caspase inhibitor was applied to cells prior to AA treatment in order to investigate the association between caspase activity and AA-induced cytotoxicity. The results showed that blocking of caspase activity significantly

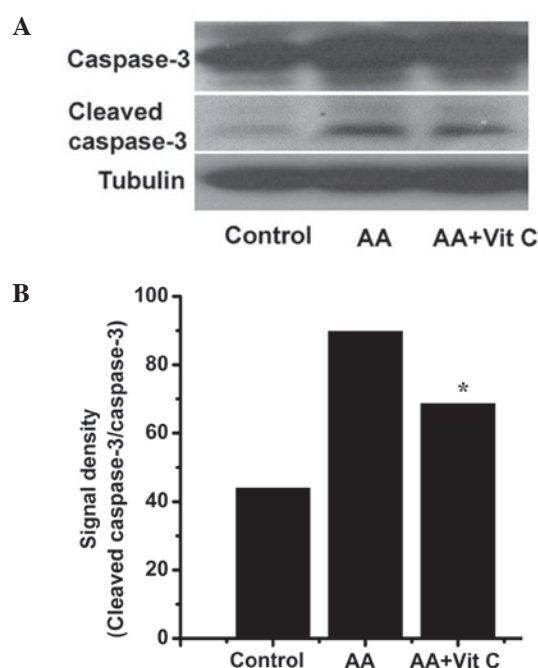


Figure 7. Vitamin C decreases caspase-3 activation by AA. (A) Western blot showing caspase-3, cleaved caspase-3 and tubulin levels at 6 h in the control group (control), 10 μM AA-treated group (AA) and 10 μM AA plus 20 μM vitamin C-treated group (AA + Vit C), respectively. (B) Densitometric analysis of caspase-3 activity ratio (cleaved caspase-3/caspase-3). The data indicated that vitamin C may decrease AA-induced caspase-3 activity. *P<0.05, compared with 10 μM AA-treated group. AA, aristolochic acid.

inhibited the cytotoxic effects of low doses of AA (5-20 μM) AA treatment (Fig. 3); however, blocking of caspase activity did not inhibit the cytotoxicity of AA at a high dose (100 μM). These results indicated that caspase activity is an important factor in low-dose AA-induced cytotoxicity. In addition, caspase-independent apoptosis signaling, which remains to be further investigated, is involved in the cytotoxic effects of high doses of AA.

AA increases H₂O₂ but not O₂⁻ levels in kidney cells. H₂O₂ and O₂⁻, distinct species of the family of ROS, are generally present in cells. Previous studies have demonstrated that AA can induce increases in ROS in renal tubular cells (16,27,28). However, it has remained elusive which ROS are elevated by AA treatment. Therefore, the present study examined H₂O₂ and O₂⁻ levels in kidney cells treated with AA by using the lucigenin-amplified method (39,40). As shown in Fig. 4A, H₂O₂ levels were elevated in renal tubular cells after AA treatment. The results also indicated that AA induced increases in H₂O₂ levels in a dose-dependent manner. By contrast, the O₂⁻ ratio was not significantly altered in renal cells following AA treatment (Fig. 4B). Therefore, the results of the present study indicated that H₂O₂, but not O₂⁻, was among the ROS increased in kidney cells by AA treatment.

Vitamin C reduces AA-induced increases in H₂O₂ levels and inhibits AA-induced cytotoxicity. As AA treatment increased H₂O₂ levels (Fig. 4A), it was further investigated whether H₂O₂ levels were associated with AA-induced cytotoxicity in renal tubular cells. Vitamin C, an anti-oxidative nutrient, was applied in AA-treated renal tubular cells. The results showed

that vitamin C was able to attenuate the increases in H_2O_2 levels in AA-treated renal cells (Fig. 5). Furthermore, the cell survival rate of renal cells was assessed following combined treatment with AA and vitamin C. The results showed that vitamin C can inhibit AA-induced cytotoxicity in renal tubular cells (Fig. 6). These results suggested that H_2O_2 was an important factor in AA-induced toxicity to renal tubular cells. In addition, vitamin C effectively reduced H_2O_2 levels alongside cytotoxicity in AA-treated cells.

Vitamin C decreases AA-induced caspase-3 activation to attenuate AA-induced cytotoxicity. The results of the present study demonstrated that vitamin C decreased AA-induced increases in H_2O_2 levels to inhibit AA-induced cytotoxicity (Figs. 5 and 6). In addition, previous studies (25-27) and the results of the present study (Fig. 3) indicated that caspase-3 activity was an important factor associated with AA-induced cytotoxicity. Therefore, the present study further investigated whether vitamin C can decrease caspase-3 activity to attenuate AA-induced cytotoxicity. For this, caspase-3 (inactivated caspase-3) and cleaved caspase-3 (activated caspase-3) levels were determined by western blot analysis (Fig. 7A) and the caspase-3 activity ratio (cleaved caspase-3/caspase-3) was obtained by densitometric analysis (Fig. 7B). Compared with the control group, the caspase-3 activity ratio was obviously increased in the AA-treated group (43.9 vs. 89.8%). Of note, compared with that in the AA-treated group, the caspase-3 activity ratio was decreased to 68.6% in the AA plus vitamin C-treated group. These results indicated that vitamin C decreased AA-induced caspase-3 activation. Regarding the association between AA-induced cytotoxicity and vitamin C (Figs. 5-7), the results suggested that vitamin C can attenuate AA-induced cytotoxicity, at least in part via decreasing AA-induced H_2O_2 levels and caspase-3 activity.

Discussion

Numerous studies have demonstrated that AA can induce renal tubular cell death (4-6) and kidney fibrosis (14,47), resulting in nephropathy. Regarding AA-induced cell death, the apoptotic pathway was widely identified in AA-treated cells (12,15-17); however, necrosis was also reported by certain studies on AA-treated cells (18-20). DNA fragmentation and nuclear condensation were observed by fluorescent microscopic observation. Therefore, the results of the present study indicated that AA-induced cytotoxicity was mediated via the apoptotic pathway. Previous studies have shown that AA can activate caspase-3 activity leading to apoptosis (25-27). However, whether AA-induced cytotoxicity depends on caspase-3 activation had remained elusive. The results of the present study showed that AA induced caspase-3 activation in accordance with the results of previous studies (25-27). Furthermore, the present study found that the cytotoxicity of low doses of AA was attenuated by a caspase inhibitor, whereas that of high doses of AA was not inhibited. These data suggested that the cytotoxicity of AA at low doses is mediated via caspase activation signaling, while the cytotoxicity of AA at high doses involves caspase-dependent as well as -independent signaling, which remains to be further elucidated in the future.

Previous studies have demonstrated that AA induces increases of ROS levels, resulting in renal injury (16,27,28). However, these studies did not determine which type of ROS is generated by AA treatment. O_2^- and H_2O_2 are ROS which are common in cells, and O_2^- can be converted into H_2O_2 by superoxide dismutase (48,49). Furthermore, H_2O_2 can be converted into H_2O by GSH and GSH peroxidase (50,51). In the present study, O_2^- and H_2O_2 levels were determined using the lucigenin-amplified method (39,40). The results showed that AA treatment induced increases of H_2O_2 levels but not of O_2^- levels. From these results it can therefore be deduced that AA may be able to decrease GSH levels or GSH peroxidase activities. This would also explain why NAC, which is required for GSH synthesis, or GSH pre-treatment can inhibit AA-induced cell death (16,27). A major function of SOD is converting O_2 to H_2O_2 . The present study demonstrated that AA is not able to influence O_2 levels. These results suggested that AA may not be associated with SOD activity.

Because AA is a component of various Traditional Chinese Medicinal plants (7,52-56), it is important to discover means of preventing AA-induced renal damage. Though studies have developed a monoclonal antibody against AA-induced cytotoxicity (3,57,58), most approaches are directed against oxidative stress to suppress AA-induced cytotoxicity. Based on the above findings, elucidating whether vitamin C inhibits AA-induced cytotoxicity in animal models will require further study. To date, anti-oxidant agents including Tiron, NAC, GSH, bone morphogenetic protein 7 and darbepoetin have been studied for their ability to reduce AA-induced cytotoxicity (13,27,59-61). However, these substances are not easily available. Vitamin C has anti-oxidative activities and is contained in numerous vegetables and fruits (31-33). The present study therefore assessed whether vitamin C can suppress AA-induced cytotoxicity to kidney cells. The results demonstrated that vitamin C markedly attenuated increases of H_2O_2 levels, caspase-3 activation and cytotoxicity following AA treatment. These results therefore suggested that supplementation of vitamin C may be beneficial for reducing AA-induced renal damage when using Traditional Chinese Medicines.

In conclusion, the present study demonstrated that: 1) AA activates caspase-3 activity and induces increases of H_2O_2 levels, resulting in renal tubular cell death; 2) AA-induced cell death is mediated via caspase-dependent and -independent pathways, depending on the dose; and 3) Vitamin C can decrease AA-induced increases in H_2O_2 levels and caspase-3 activity to attenuate AA-induced cytotoxicity.

Acknowledgements

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