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Original Article

Cytoprotective Activity of *Glycyrrhizae radix* Extract Against Arsenite-induced Cytotoxicity

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Licorice, *Glycyrrhizae radix*, is one of the herbal medicines in East Asia that has been commonly used for treating various diseases, including stomach disorders. This study investigated the effect of licorice on arsenite (As)-induced cytotoxicity in H4IIE cells, a rat hepatocyte-derived cell line. Cell viability was significantly diminished in As-treated H4IIE cells in a time and concentration-dependent manner. Furthermore, results from flow cytometric assay and DNA laddering in H4IIE cells showed that As treatment induced apoptotic cell death by activating caspase-3. Licorice (0.1 and 1.0 mg ml^{-1}) treatment significantly inhibited cell death and the activity of caspase-3 in response to As exposure. These results demonstrate that licorice induced a cytoprotective effect against As-induced cell death by inhibition of caspase-3.

Keywords: apoptosis – arsenite – cytotoxicity – licorice

Introduction

Licorice, *Glycyrrhizae radix*, is one of the oriental herbal medicines that has been most frequently prescribed for the treatment of various diseases including stomach disorders (1). It has also been described in a medical classic as an agent with the ability to 'improve the tone of the "*middle-jiao*" (中焦; the digestive system) and replenish "*qi*" (氣), to remove "heat" (熱) and toxic substances, to moisturize the lungs and arrest coughing and to relieve spasms and pain' (2). In addition, it is widely used as a flavoring adjuvant in drug preparations and an ingredient of cigarettes for its taste and property to reduce irritation (2,3).

Although there is a lack of well-controlled experiments investigating the effect of licorice extract on cytotoxicity, glycyrrhizin, a major active component of licorice roots (4), prevented development of hepatocellular carcinoma in chronic hepatitis C patients (5), protected aflatoxin-induced cytotoxicity (6), and inhibited replication of the SARS (severe acute respiratory syndrome) associated virus in patients (7) and of the HIV in peripheral blood mononuclear cells (8). In addition, we showed that *G. radix* and its component, liquiritigenin, could inhibit cadmium (Cd)-induced toxicity in a rat-derived hepatocyte cell line (3). Liquiritigenin was also reported to have hepatoprotective effects against acetaminophen-induced acute injuries in rats (9).

Arsenic (As) as a toxic metal affects the functions of mitochondrial enzymes and subsequently impairs tissue respiration, which is believed to cause its cytotoxicity (10,11). Chronic exposure to arsenic can cause severe jaundice, liver cirrhosis (12,13), ascites and cancer (14,15). In Bangladesh and India, about 100 million people are at risk of As poisoning because of drinking groundwater contaminated with As (16). Although homeopathic arsenic remedy was reported to alleviate As poisoning in humans (17,18), attempts to remove As have mostly been unsuccessful.

Arsenic is present in both pentavalent arsenate and trivalent arsenite. Trivalent arsenite is known to be more toxic than pentavalent arsenate (19,20).

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Recently, Szymczyk *et al.* (21) reported that sodium arsenite ($NaAsO_2$, As) triggered the production of H_2O_2 and induced apoptosis via the activation of caspase-3, which is dependent on the production of H_2O_2 in preosteoclastic cells. Arsenic trioxide (As_2O_3) has also been shown to induce apoptosis in other types of cells (22,23). Activation of caspase-3 is a critical step in apoptosis and caspase-3 is believed to be involved in the mediation of As-induced cell death. However, there are few studies that evaluate the effects of useful herbal medicines against As-induced toxicity. The purpose of this research was to investigate whether licorice could prevent As-induced apoptotic cell death via caspase-3.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from BioWhittaker (Walkersville, MD, USA) and Life Technologies (Gaithersburg, MD, USA). Arsenite, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) and other reagents in the molecular studies were supplied from Sigma Chemical (St. Louis, MO, USA). Licorice water extract was prepared by boiling 600 g of *G. radix* (Wolsung, Daegu, Korea) in 5 l of water for 3 h, filtering through a 0.2 μm syringe filter (Nalgene, New York, NY, USA), and storing at −20°C until use. The amount of licorice water extract was estimated by the dried weight of lyophilized water extract of *G. radix*. The yield of lyophilized licorice from *G. radix* was 13%.

Cell Culture

The rat-derived hepatocyte cell line, H4IIE, was obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM containing 10% FCS, 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin at 37°C in humidified atmosphere with 5% CO_2. H4IIE cells (1×10^6 cells ml^{-1}) were plated in a 100 mm plastic dish for 2–3 days (i.e. at 80% confluency), serum-starved for 12 h and cultured for the indicated time periods in the medium with or without As.

MTT Cell Viability Assay

H4IIE cells were plated at a density of 5×10^4 cells per well in 96-well plates to determine cytotoxicity induced by heavy metals. Cells were exposed to As at concentrations of 50–800 μM at 37°C under 5% CO_2. Cells were serum-starved for 12 h treated with licorice for 12 h and exposed to As and licorice for another 12 h. After cell incubation, viable cells were stained with MTT (0.5 mg ml^{-1}, 4 h). The media were then removed and formazan crystals produced in viable cells were dissolved by adding 200 μl of dimethylsulfoxide (DMSO). Absorbance was measured at 570 nm using a Titertek Multiskan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL, USA). Cell viability was defined relative to untreated control cells [i.e. viability (% control) = 100 × (absorbance of treated sample)/(absorbance of control)].

Flow Cytometric Analysis

Detection of apoptotic and/or necrotic cells was assessed with the green fluorescent dye FITC annexin V entering apoptotic cells and the red fluorescent dye PI not entering (Molecular Probes, Eugene, OR, USA). Briefly, cells were harvested, washed in cold phosphate buffered saline (PBS), and adjusted to the density of $\sim 1 \times 10^6$ cells ml^{-1} in PBS. Cells were incubated on ice with PI (0.1 μg ml^{-1}) and FITC annexin V for 20 min. The stained cells were analyzed by a flow cytometer (PAS system, Partec, Münster, Germany).

DNA Fragmentation Assay

H4IIE cells were pre-treated with licorice for 12 h, and then incubated with licorice with or without 400 uM of As for the next 12 h. After washing the cells with PBS, they were lysed in a solution containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA and 1% NP-40. The supernatants were incubated with 1% SDS and 2.5 mg ml^{-1} RNase A for 2 h at 37°C, and then incubated with 0.5 mg ml^{-1} proteinase K at 42°C. DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1), and precipitated with 0.3 M sodium acetate and 2.5 volumes of ethanol. DNA samples (20 μg) were electrophoretically separated in 1.5% agarose gel containing 0.1 mg ml^{-1} ethidium bromide.

Cleaved Caspase-3 Assay

Caspase-3 is a key mediator of apoptosis and responsible for the proteolytic cleavage of many key proteins such as PARP. Activation of caspase-3 requires proteolytic processing of procaspase-3 into activated p17 and p12 fragments. To measure caspase-3 cleavage, H4IIE cells were pre-treated with licorice for 12 h, and then incubated with licorice with or without 400 μM of As for the next 12 h. The cells were washed with PBS and 0.5 ml of ice-cold cell lysis buffer (Cell signaling, MA, USA), after which 1 mM PMSF was added to each plate (100 mm) on ice. After 5 min, cells were scraped off the plate, sonicated on ice and microcentrifuged for 10 min at 4°C. The supernatant was collected and applied to cleaved caspase-3 sandwich ELISA kit (Cell signaling, MA, USA) according to the manufacturer's instructions.

Statistical Analysis

One way analysis of variance (ANOVA) procedures were used to assess significant differences among the treatment groups. For each significant effect of treatment, the Tukey test was used for comparisons of multiple group means. The criterion for statistical significance was set at $P<0.05$ or $P<0.01$.

Results

Cytotoxicity Induced by As in H4IIE

To assess whether As reduces cell viability in a concentration-dependent manner, H4IIE cells were maintained in serum free medium for 12 h and then incubated with various concentrations (50 ~ 800 μM) of As for 18 h. As shown in Fig. 1, a significant increase in cell death was observed upon exposure to 800 μM As (27% of control value). To investigate the time-dependent effect of As on cell viability, H4IIE cells were also maintained with serum free medium for 12 h and then incubated with 200 and 400 μM of As for 6, 12, 18 and 24 h. Results showed that cell viability was reduced to 23% of the control group following exposure to 400 μM As for 24 h. In addition, cytotoxicity was observed after As treatment (200 and 400 μM) for 12, 18 and 24 h. (Fig. 2).

Cytoprotective Activity of Licorice on As-induced Cell Toxicity

There was a significant reduction in cell viability in the 400 μM treated group to 40% of the control group. Most importantly, licorice treatment suppressed As-induced cell death. Cell viability after licorice treatment (0.1 and 1.0 mg ml^{-1}) was restored to 92% of the control group (Fig. 3).

Licorice Prevents H4IIE Cells from As-induced Apoptosis by Inhibiting Caspase-3 Cleavage

To investigate whether cell death observed in response to As was apoptosis, cells were analyzed using a flow cytometer. As shown in Fig. 4, As-induced apoptotic cell death showed that a larger apoptotic population of cells was increased in the As-treated group compared with the control group (7.93% versus 0.73%). In contrast, licorice prevented As-induced apoptotic cell death, with 1.0 mg ml^{-1} of licorice reducing the apoptotic population to only 1.73%. Our previous study showed that licorice prevented Cd-induced cell death in H4IIE cells (3). Taken together, these results provided strong evidence that licorice effectively inhibits Cd- or As-induced cytotoxicity.

Moreover, results from an additional DNA experiment with As-exposed cells showed that As induced a typical

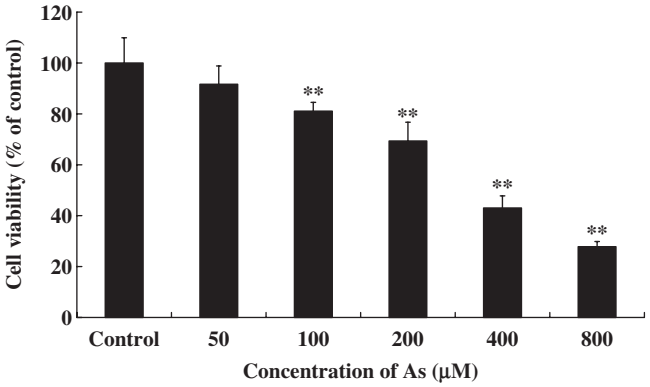

Figure 1. Cytotoxicity induced by As in a concentration dependent manner. H4IIE cells were exposed to various concentrations (50–800 μM) of As for 18 h. Cell viability was assessed by MTT assay. Data represent the mean ± SD of eight separate experiments. **Significant at $P<0.01$ compared with vehicle-treated control.

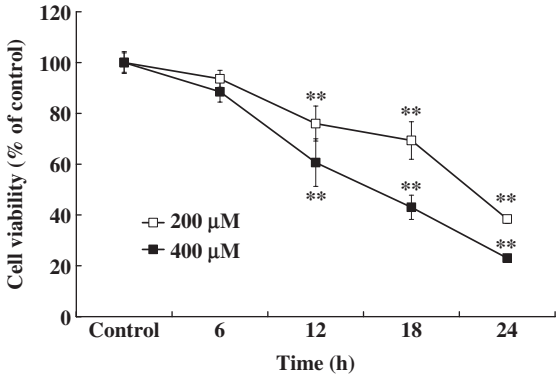

Figure 2. Cytotoxicity induced by As in a time-dependent manner. H4IIE cells were exposed to 200 and 400 μM of As for 0–24 h. Cell viability was assessed by MTT assay. Data represent the mean ± SD of eight separate experiments. **Significant at $P<0.01$ compared with vehicle-treated control.

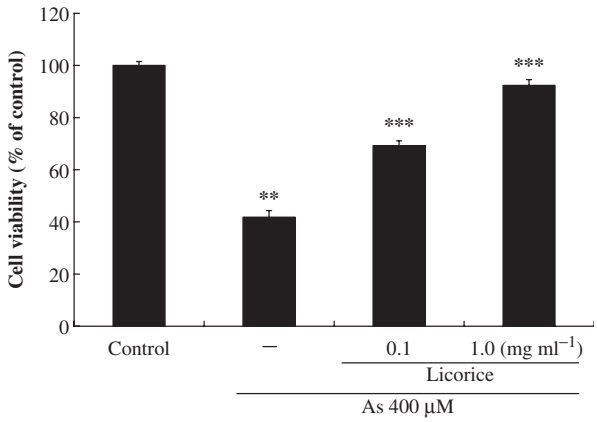

Figure 3. Increase in viability of H4IIE cells exposed to As by licorice. H4IIE cells pre-treated with licorice for 12 h and further incubated with licorice + As (400 μM) for the next 12 h. Cell viability was assessed by MTT assay. Data represent the mean ± SD of eight separate experiments. **Significant at $P<0.01$ compared with vehicle-treated control, ***significant at $P<0.01$ compared with the cells treated with As alone.

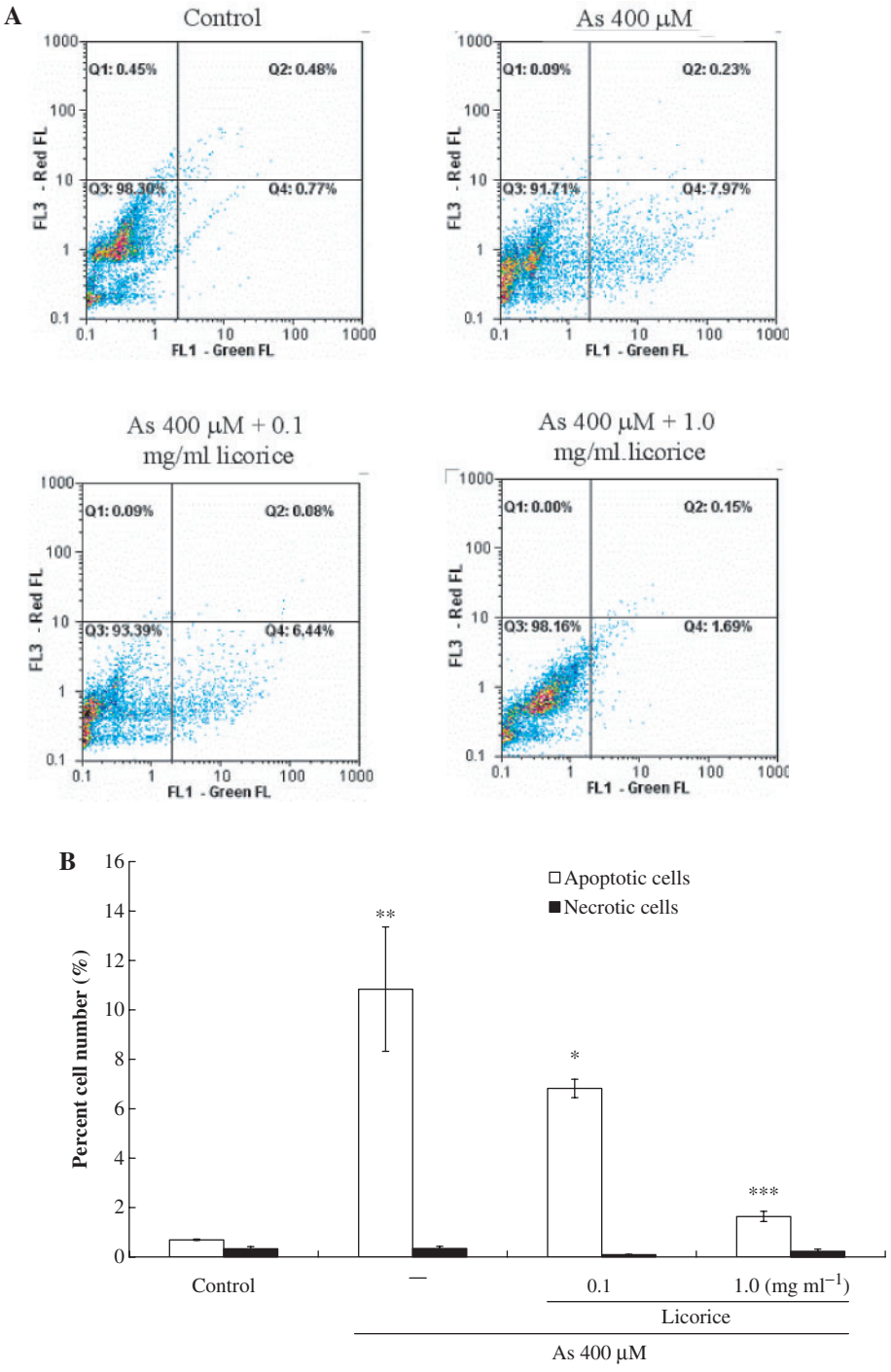

Figure 4. Flow cytometric profile of green versus red fluorescence of H4IIE cells with FITC Annexin V and PI. H4IIE cells were treated with licorice and 400 μM of As for 6 h. The population was separated into three groups: live cells showing only a low level of fluorescence, apoptotic cells showing green fluorescence and necrotic cells showing both red and green fluorescence (**A**), and the percentage of apoptotic and necrotic death cell (**B**) was calculated. Data represent the mean ± SD of six separate experiments. *Significant at $P<0.05$, **significant at $P<0.01$ compared with vehicle-treated control and ***significant at $P<0.01$ compared with the cells treated with As alone.

DNA laddering effect indicating apoptotic cell death. In addition, 1.0 mg ml^{-1} of licorice decreased the As-induced DNA laddering (Fig. 5). Activated/cleaved caspase-3 is a key protein during apoptosis. As shown in Fig. 6, 400 uM As induced cleavage of caspase-3 protein. Similar to the apoptosis data, licorice significantly reduced As-induced caspase-3 cleavage. Our results indicate that licorice may play a potent role in inhibiting As-induced apoptosis in H4IIE cells.

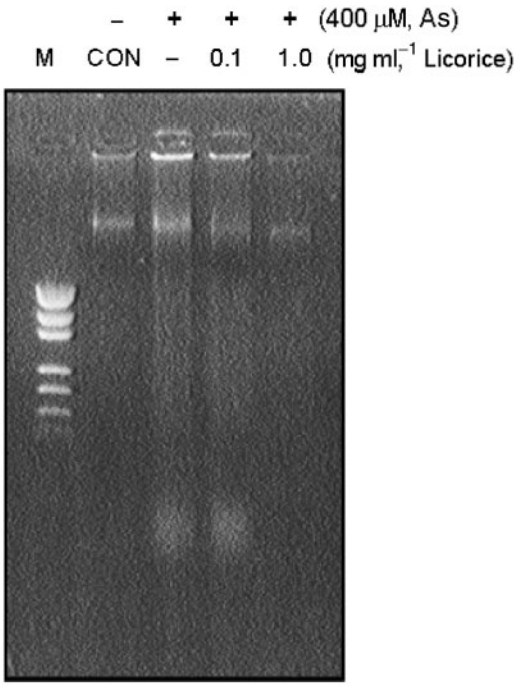

Figure 5. DNA Laddering of H4IIE cells exposed to As with/without licorice. H4IIE cells that had been pre-treated with licorice for 12 h were further incubated with licorice + As (400 μM) for another 12 h.

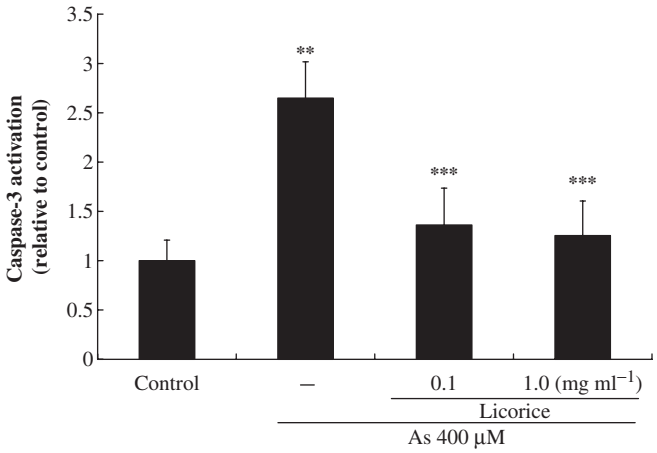

Figure 6. Inhibition of As-stimulated cleavage of caspase-3 by treatment of H4IIE cells with licorice. H4IIE cells were pre-treated with licorice for 12 h, and then incubated with licorice with or without 400 μM of As for the next 12 h. The cells were lysed and the total cell lysate was applied to cleaved caspase-3 sandwich ELISA kit (Cell signaling, MA, USA) according to the manufacturer's instructions. Data represent the mean ± SD of three separate experiments. **Significant at $P<0.01$ compared with vehicle-treated control, ***significant at $P<0.01$ compared with the cells treated with As alone.

Discussion

Licorice, *G. radix*, is very common in most of the polyherbal preparations in oriental medicine. For example, 70 of 110 prescriptions in one Chinese medicine text book (傷寒論) include *G. radix*. Licorice extract is believed to have life-enhancing properties, as well as curing and detoxifying effects against injury or swelling. In the United States and European countries, *G. radix* products are currently used as flavoring or sweetening agents for tobaccos, chewing gums, candies or beverages and are consumed in large quantities at approximately 1.5 kg per person per year (1).

Glycyrrhizae radix prevents carcinogenesis induced by toxicants and hormones (24). It suppressed estradiol-induced expression of c-Fos/Jun in the uterine corpus, and prevented endometrial carcinogenesis (25). *Glycyrrhizae radix* comprises flavonoids and pentacyclic triterpene saponins as the major constituents, which include liquiritin, liquiritigenin, isoliquiritigenin, liquiritin apioside, glycyrrhizin and glycyrrhizic acid. Glycyrrhizin is reported to be one of the most important constituents with a content variation from minimum 4% to maximum 13% of the dried root weight (1). Glycyrrhizin and 3-glycyrrhetinic monodesmoside suppressed lactate dehydrogenase leakage and malonedialdehyde release in rats subjected to ischemia-reperfusion (26), showing the anti-oxidative function of these compounds. We also reported that licorice and liquiritigenin prevented Cd-induced apoptosis, and Cd-induced non-apoptotic cell death, irrespective of sulfhydryl deficiency, whereas liquiritin, isoliquiritigenin or glycyrrhizin did not prevent Cd-induced cell death (3,27).

In this study, we focused on the cytoprotective effects of licorice against As-induced cytotoxicity. We revealed that licorice was capable of blocking As-induced apoptotic cell death, and that, in particular, licorice pre-treatment prevented the subsequent As-induced cytotoxicity. Incubation of cells with licorice alone at the concentration of 1.0 mg ml^{-1} for 12–24 h increased cell proliferation (4), suggesting that the cellular protein(s) responsible for cell survival is (are) activated by the components in licorice, which may also be associated with its cytoprotective effect against As-induced cell injury.

A previous study from our laboratory showed that licorice prevented Cd-induced toxicity in H4IIE cells (3). Thus, we are interested in whether or not licorice protects against As toxicity in H4IIE cells. In the present study, licorice significantly inhibited As-induced cell death. As-induced either apoptosis at lower concentration or necrosis at high concentration in mouse embryonic fibroblasts (28), human osteogenic sarcoma cell line U-2OS (29) and human leukemia HL-60 cells (30). Previously, Son *et al.* (31) determined that the sulfur amino acid deprivation (SAAD) potentiated the cytotoxicity of As by 20-fold in H4IIE cells. Although SAAD potentiated cytotoxicity of H4IIE cells with IC_{50} value of 19 uM, the IC_{50} value of As in H4IIE cells incubated in control medium was 401 uM. It is consistent with our results showing about 50% of cytotoxicity by 400 uM of As in H4IIE cells.

To determine whether As induces apoptotic cell death, we analyzed cell death using a flow cytometer. Incubation with As produced apoptotic cell death, but this As-induced apoptotic cell death was significantly prevented by 1.0 mg ml^{-1} of licorice. These results present strong evidence that licorice's potent blocking of cell death is due to its inhibitory effect on As-mediated apoptotic process. As-induced apoptosis was confirmed by data that licorice inhibits As-induced DNA laddering.

In the apoptotic process, there are two different types of cell death: caspase-dependent and caspase-independent pathways. In As-induced apoptosis, involvement of caspase-3 activation has been well known in both *in vivo* and *in vitro* experimental systems (32,33). In this study, we found that the cytoprotective effect of licorice against As-induced cell death was mediated by the inhibition of apoptisis. Licorice inhibited caspase-3 activation (Fig. 6) and reduced the population of apoptotic cells (Fig. 4) in a dose dependent manner. In Fig. 6, caspase-3 activation was inhibited by both 0.1 and 1.0 mg ml^{-1} of licorice to a similar extent. However, the net cell viability by 1.0 mg ml^{-1} of licorice was much higher than that by 0.1 mg ml^{-1} of licorice (Fig. 3) indicating the role of caspase-independent apoptotic pathways in this cell line. Apoptosis inducing factor (AIF) and endonuclease G (EndoG) are apoptogenic factors that mediate caspase-independent cell death (34,35). Omi/HtrA2 also induces caspase-independent apoptosis by inhibiting IAPs (inhibitors of apoptosis) for activating caspase-9 and -3 (35). To confirm apoptosis through the caspase-independent pathways including EndoG-induced DNA degradation, further studies are needed. In summary, we demonstrated that licorice blocked As-induced cell death by inhibiting the apoptotic process, and suggested that licorice is a potent preventer of As-induced cytotoxicity.

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