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Effect of *Salvia miltiorrhiza* on Aflatoxin B_1-induced Oxidative Stress in Cultured Rat Hepatocytes

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Recent findings have suggested that oxidative damage might contribute to the cytotoxicity and carcinogenicity of aflatoxin B_1 (AFB_1). *Salvia miltiorrhiza* (Sm), a herbal plant that has been used extensively in traditional Chinese medicine for treating cardiovascular and liver diseases, is believed to have some antioxidative capabilities. In this study, the protective effect of Sm against AFB_1-induced cytotoxicity was investigated in cultured primary rat hepatocytes. AFB_1-induced cytotoxicity and lipid peroxidation (LPO) were estimated by determination of lactate dehydrogenase (LDH) leakage and thiobarbituric acid reactive substances (TBARS) formation, respectively. Intracellular reactive oxygen species (ROS) formation was measured using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). In addition, changes of intracellular glutathione (GSH) content were also studied. Results showed that Sm was able to suppress the LDH leakage induced by AFB_1 in a dose-dependent manner. A dose-dependent inhibitory effect of Sm on AFB_1-induced LPO was also found in hepatocytes treated with Sm. It was further observed that Sm produced an inhibitory effect on ROS formation caused by AFB_1. Concomitantly, the GSH content in Sm-treated groups increased substantially compared to those without Sm treatment. These findings suggest that Sm can inhibit the cytotoxicity of AFB_1 through decreasing ROS formation, inhibiting LPO and preventing GSH depletion. The major component of the aqueous extract of Sm was identified by using high performance liquid chromatography (HPLC), proton magnetic resonance (^{1}H-NMR) and mass spectrum (MS). Analytical results suggested that D(+)β3,4-dihydroxyphenol lactic acid (DA) is the main compound of the aqueous extract of Sm.

Keywords: Salvia miltiorrhiza, aflatoxin B_1, primary hepatocyte, cytotoxicity, reactive oxygen species, D(+)β3,4-dihydroxyphenol lactic acid

Aflatoxin B_1 (AFB_1) is produced by certain strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and it is among the most common mycotoxins to which humans are exposed.[1] Experimental studies have shown that AFB_1 is a potent mutagen and hepatocarcinogen in several animal species.[2] Epidemiological evidence has also established a strong association between human exposure to AFB_1 from contaminated food and a high incidence of hepatocellular carcinoma.[3] It is therefore of major public health concern world

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wide, especially in regions with high primary liver cancer incidences such as Asia and Africa.

It is well established that AFB_1 is metabolized by the hepatic cytochrome P450 system to produce a highly reactive intermediate AFB-8,9-epoxide (AFBO). The subsequent covalent binding of AFBO with DNA to form AFB_1-DNA adduct is regarded as a critical step in hepatocarcinogenesis.[4] On the other hand, oxidative damage and its role in AFB_1-induced carcinogenesis have attracted much attention in recent years. Amstad *et al.*[5] discovered that AFB_1 was able to induce chromasomal damage through the release of free oxygen radicals in human lymphocytes. In another study, Kodama *et al.*[6] observed that AFB_1 produced free radicals *in vitro* in both enzymatic and non-enzymatic conditions. Our earlier studies also showed that AFB_1 was capable of inducing the generation of reactive oxygen species (ROS)[7] and lipid peroxidation (LPO)[8] in cultured rat hepatocytes. Furthermore, AFB_1 can cause oxidative DNA damage as measured by the formation of 8-hydroxydeoxyguanosine (8-OHdG) in rat hepatic DNA.[9] All these findings suggest that oxidative damage might contribute to the cytotoxic and carcinogenic effect of AFB_1.

Salvia miltiorrhiza (Sm) is a herbal plant and its extract and tablets are currently used in traditional Chinese medicine to treat diseases such as chronic hepatitis and coronary heart diseases.[10] It is believed that some of the clinical effects of Sm could be, to some extent, related to its antioxidant activity.[11] A recent study has shown that Sm could scavenge oxygen free radicals generated from ischemia–reperfusion injury in the myocardium as effectively as superoxide dismutase (SOD).[12]

On the other hand, so far no work has been conducted to characterize the anti-cytotoxic potential of Sm. Since oxidative damage is one of the mechanisms contributing to AFB_1-induced cytotoxicity and Sm has been shown to possess antioxidant activities, there is thus a theoretical basis for Sm to reduce the cytotoxicity of AFB_1. In this paper, we present the data on the effect of Sm against AFB_1-induced cytotoxicity in primary rat hepatocytes and on the identification of the main compound that exerts its activities.

MATERIALS AND METHODS

Chemicals

AFB_1, dimethyl sulfoxide (DMSO), Williams' Medium E, reduced glutathione (GSH), sodium dodecyl sulphate (SDS), *o*-phthalaldehyde (OPT) and other common chemicals were purchased from Sigma (St. Louis, MO, USA). 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR, USA). Thiobarbituric acid (TBA) was from Merk (Darmstadt, Germany) and fetal bovine serum (FBS) was obtained from Life Technologies (Faisley, Scotland). The sodium salt of D(+)β3,4-dihydroxyphenyl lactic acid (DA) that was used as the standard was kindly provided by Prof. Zhou Zhu (Shanghai Medical University, P.R. China).

Preparation of Herbal Extract

Sm in root form was purchased from a Chinese herbal medicine company in the Republic of Singapore. The dried root was cut into 0.5 cm long pieces before use. Aqueous extract was prepared as following: 250 g of the dry pieces of Sm were put into 1500 ml of distilled water and heated at 100°C for 8 h. The suspension was then filtered through gauze. The filtrate was centrifuged (2000*g* × 10 min) and the supernatant was filtered again through 0.22 μm membrane filter to sterilize. The sterile extract was used in all experiments. The concentration used in the experiment was based on the dry weight of the extract.

Liver Perfusion and Primary Rat Hepatocyte Culture

Male Fischer (F344) rats (250–300 g body weight) were obtained from the Animal Centre at the

National University of Singapore. Liver perfusion was performed according to the method described by Shen *et al.*[8] Yields of $3–4 \times 10^8$ cells/liver were routinely obtained. All cells were finally resuspended in complete Williams' Medium E with 10% FBS and plated into a 75 cm^2 flask at a density of 0.8×10^6 cells/ml in 10 ml of the medium. After incubation (5% CO_2, 95% air, 37°C) for 2 h, the flasks were washed with Hepes buffer (140 mM NaCl, 6.7 mM KCl, 1 mM $CaCl_2$ and 2.4 mM Hepes, pH 7.4) to remove the unattached dead cells. The hepatocytes were then incubated with serum-free Williams' Medium E under various treatments.

Treatments

AFB_1 was dissolved in DMSO and further diluted with PBS to the required concentration. In Sm-treated groups, 0.01–0.1 mg/ml Sm (final concentration) was added into the medium together with AFB_1 (1 μM) and incubated for 24 h. At the end of incubation, 50 μl of medium was taken from each flask for the determination of lactate dehydrogenase (LDH) activity. Hepatocytes were collected with a cell scraper and washed twice with PBS. The cell homogenate was then prepared for the measurement of TBA reactive substances (TBARS) and GSH.

For the evaluation of ROS formation, after incubation with completed Williams' Medium E with 10% FBS for 2 h, primary hepatocytes were collected using a cell scraper and washed with PBS twice and finally resuspended at 1×10^5/ml in PBS for subsequent fluorescence analysis. AFB_1 and Sm were added simultaneously with DCFH-DA at the beginning of the fluorescence test and incubated for up to 4 h.

Determination of LDH Activity

LDH activity in the medium was measured using an Abbott VP Biochemical Analyzer with the test kit (Abbott laboratory, Chicago, USA). Total LDH activity in cells was determined after thorough breakdown of hepatocytes using sonication. The percentage of LDH leakage from cells was used as the index of cell injury, which is calculated according to the following formula: % LDH leakage = (LDH activity present in the medium after incubation/total LDH activity at the beginning of the incubation) ×100.

Measurement of TBARS

The cell homogenate for TBARS measurement was obtained by resuspending hepatocytes in PBS with 1% SDS. TBARS concentration in hepatocyte homogenate was determined using the colorimetric method described by Shen *et al.*[8] Protein concentration was determined by Lowry's method[13] using bovine serum albumin as standard. TBARS concentration was expressed as nmol/mg protein.

Analysis of DCF Fluorescence in Cultured Hepatocytes

DCFH-DA was dissolved in ethanol at the concentration of 2 mM and kept at −70°C in dark as the stock. The reaction was carried out in a fluorescence cuvette (Sigma, St. Louis, MO, USA). The reaction mixture contained 3×10^5 hepatocytes, 2 μM DCFH-DA, 1 μM AFB_1 and/or 0.01–0.1 mg/ml Sm in 3 ml PBS. The reaction was initiated by the addition of DCFH-DA into the mixture and incubated at 37°C for up to 4 h. The fluorescence intensity was measured using a Perkin-Elmer spectrofluoremeter LS-5B with excitation wavelength at 485 nm and emission wavelength at 530 nm.

Determination of GSH

After treatments, hepatocytes were washed twice with PBS and resuspended in 1 ml PBS. The cell homogenate for the measurement of GSH was obtained by sonication. Determination of GSH

was performed by a modification of the method of Hissin and Hilf.[14] Briefly, 0.75 ml of the cell homogenate was mixed with 0.2 ml 25% H_3PO_3, which was used as a protein precipitant. After centrifugation (59,000*g* × 5 min, 4°C), the supernatant was diluted 10 times with 0.1 M sodium phosphate–5 mM EDTA buffer (pH 8.0). The final reaction mixture (2.0 ml) contained 100 µl of the diluted supernatant, 1.8 ml of phosphate–EDTA buffer and 100 µl of OPT solution (1 mg/ml). After thoroughly mixing and incubation at room temperature in the dark for 15 min, the solution was then transferred to a quartz cuvette. Fluorescence intensity at 420 nm was determined with the excitation at 350 nm. The GSH content in hepatocytes was expressed as nmol/10^6 cells.

Separation and Identification of Major Component

Chromatography of the aqueous extract of Sm and fraction collection of the major component was performed on HPLC system.[15] This consisted of a Gilson programmable gradient HPLC system (Model 302), a slave pump (Model 302), a dynamic mixture (Model 811), an auto-injector (Model 231-401) and a ASPEC system used as fraction collector (Gilson, Villiers-le-Bel, France). A Waters photodiode array detector (Model 996) was used for peak purity test and Millennium 2010 software was used for peak identification and integration (Milford, MA, USA). The chromatographic separation was performed on a guard and analytical cartridges system (Parti-Sphere 2 C_{18}, 5 µm, 100 × 4.6 mm ID; Whatman). The solid phase extraction (SPE) used for sample cleaning prior to HPLC fraction collection was a 12 ml tube column containing 2 g of C_{18} packing material (Supelco, USA). After freeze drying, the crude extract was thoroughly dissolved in 2 ml of water and allowed to be percolated through the preconditioned SPE column. The column was eluted with 2 ml water twice and the eluate was collected and then centrifuged at 1500*g* for 2 min. The fraction collection was started at 5 min after each injection of 200 µl of this eluate onto the HPLC column. The two mobile phases used were: (A) 0.25% (v/v) acetic acid in water and (B) methanol–water–acetic acid (70 : 30 : 0.25, v/v). The flow rate was set at 1.0 ml/min with HPLC gradient elution profile as follows: 0–10 min, A–B (95 : 5); 15–19 min, A–B (50 : 50) and 20–30 min, A–B (95 : 5). For peak purity test and quantification analysis, the sample injection volume was 20 µl and the gradient elution profile was: 0–3 min, A–B (70 : 30); 6–20 min, A–B (30 : 70) and 21–25 min, A–B (70 : 30).

After fraction collection, the proton magnetic resonance spectra of the major compound was analyzed in CD_3OD on a Bruker ADVANCE DRX 500 MHz and a Bruker ACF300 MHz FT-NMR spectrometers (Faellanden, Switzerland) using $SiMe_4$ as reference. The ESI-MS spectrum was recorded on a TSQ 7000 FINNIGAN-MAT Mass Spectrometer.

Statistical Analysis

All data are presented as means ± SD of six samples from at least two independent experiments. Results were analyzed for statistical significance using Student's *t*-test. Difference at a *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of Sm and DA on AFB_1-induced Cell Injury

The inhibitory effect of Sm and DA on AFB_1-induced cell injury is summarized in Figure 1. In AFB_1-treated hepatocytes, a significant increase of LDH activity in the medium was observed at 12 h after the incubation (data not shown). At 24 h, the percentage of LDH leakage reached

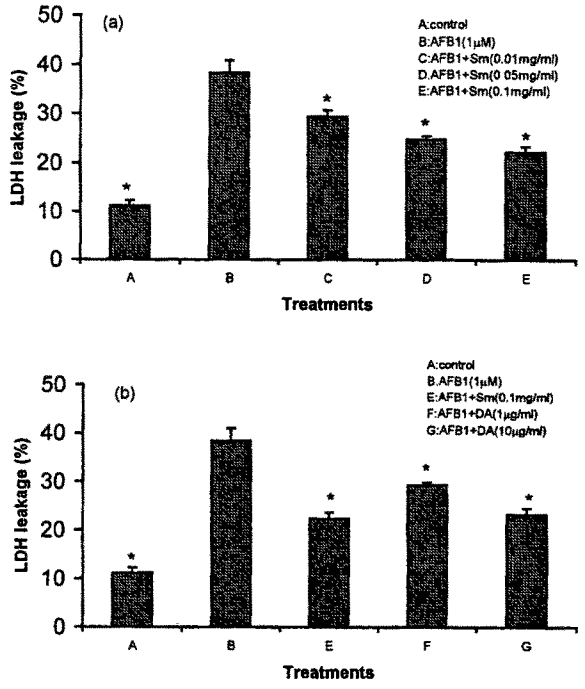

FIGURE 1 Effect of Sm (0.01–0.1 mg/ml) (a) and DA (1 μg/ml and 10 μg/ml) (b) on LDH leakage induced by AFB_1 in cultured rat hepatocytes. Sm or DA was administered at the same time with 1 μM AFB_1 and the incubation was terminated after 24 h treatment. Data are presented as means ± SD ($n = 6$) and analyzed using Student's t-test. * indicates significant difference from the group receiving AFB_1 only (group B) ($p < 0.05$).

about 40%, suggesting the presence of cell injury caused by AFB_1 ($p < 0.05$). Meanwhile, LDH release in the control group remained at a relatively low level. The addition of Sm (0.01–0.1 mg/ml) alone did not cause any change of LDH leakage (data not shown). However, Sm significantly inhibited the LDH release caused by AFB_1 ($p < 0.05$), which indicates that Sm is able to suppress the hepatotoxic effect caused by AFB_1. The inhibitory effect was also found to be dose-dependent within the concentration range used (0.01–0.1 mg/ml). Nevertheless, the results also show that with up to 0.1 mg/ml of Sm, the AFB_1-treated hepatocytes still had higher LDH release than the control cells, suggesting that SM is unable to completely prevent the increase of LDH release induced by AFB_1.

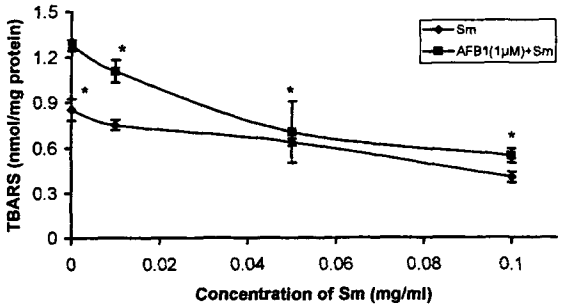

FIGURE 2 Effect of Sm (0.01–0.1 mg/ml) on AFB_1-induced TBARS generation in cultured rat hepatocytes. Sm was administered simultaneously with 1 μM AFB_1 and the treatment was terminated after 24 h. Data are presented as mean ± SD ($n = 6$) and analyzed using Student's t-test. * indicates significant difference compared to the group treated with AFB_1 ($p < 0.05$).

Figure 1(b) shows that in a separate experiment, when cells were treated with 1 μg DA, at which level the amount of DA is equivalent to that found in 0.1 mg Sm, there was also a decrease of LDH leakage. However, the LDH leakage was significantly higher than that of the whole extract ($p < 0.05$). This finding suggests that in addition to DA, other compounds in Sm could have also contributed to the protective effect.

Effect of Sm on AFB_1-induced Lipid Peroxidation

Figure 2 illustrates the effect of Sm on AFB_1-induced LPO as measured by TBARS generation. After 24 h incubation, a significant increase of TBARS was noted ($p < 0.05$) in AFB_1-treated cells. It was further observed that all three doses of Sm were able to reduce the increase of TBARS generation induced by AFB_1 in a dose-dependent manner ($p < 0.05$). In the group treated with 0.1 mg/ml Sm, the TBARS content was only about 50% of the value in cells treated with AFB_1 alone.

Effect of Sm on AFB_1-induced ROS Formation

Figure 3 shows the effect of Sm on DCF fluorescence intensity in AFB_1-treated hepatocytes for

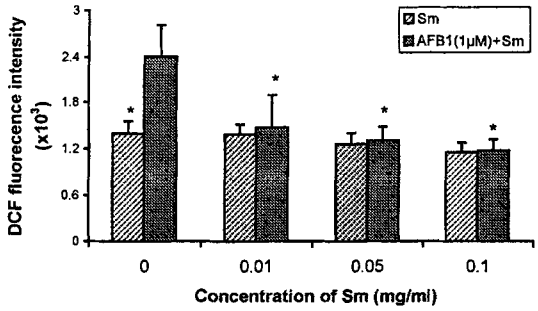

FIGURE 3 Effect of Sm (0.01–0.1 mg/ml) on AFB_1-induced ROS formation in cultured rat hepatocytes. The reaction mixture contained 1×10^5 cells, 2 μm DCFH-DA, 1 μM AFB_1 and/or Sm in 3 ml PBS. The fluorescence intensity was measured after the reaction mixture was incubated for 4 h. Data are presented as mean ± SD ($n = 6$) and analyzed using Student's t-test. * indicates significant difference from the AFB_1 group ($p < 0.05$).

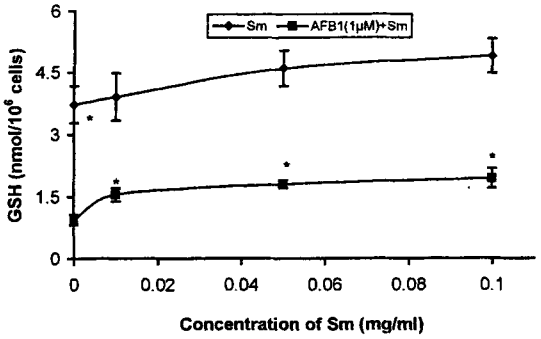

FIGURE 4 Effect of Sm (0.01–0.1 mg/ml) on GSH depletion induced by AFB_1 in cultured rat hepatocytes. Sm was added into the medium at the same time with 1 μM AFB_1 and the incubation was terminated after 24 h treatment. Data are presented as mean ± SD ($n = 6$) and analyzed using Student's t-test. * indicates significant difference compared with the group receiving only AFB_1 ($p < 0.05$).

up to 4 h of incubation. The fluorescence intensity in AFB_1-treated group was about two times as that in the control group, suggesting that AFB_1 enhances the intracellular ROS formation in cultured hepatocytes. Sm, on the other hand, demonstrates an inhibitory effect on DCF formation. With the addition of Sm, the DCF fluorescence intensity in AFB_1-treated cells returned to the control level ($p < 0.05$). Meanwhile, Sm itself did not cause obvious changes of ROS formation in the hepatocytes.

Effect of Sm on AFB_1-induced GSH-depletion

In order to further investigate the role of Sm in protecting against cytotoxicity induced by AFB_1, the effect of Sm on GSH content in hepatocytes was examined. Results in Figure 4 show that at a concentration of 0.1 mg/ml, Sm could significantly increase the GSH concentration in the control cells ($p < 0.05$). Meanwhile, AFB_1 decreases the contents of GSH in hepatocytes substantially (to only 25% of the control) ($p < 0.05$). The same figure also shows that there was a dose-dependent inhibition on AFB_1-induced GSH-depletion in hepatocytes treated with Sm. With the addition of Sm, GSH concentration increased substantially comparing to those without Sm treatment ($p < 0.05$). With the treatment of 0.1 mg/ml Sm, the GSH concentration was approximately two times higher than that of cells treated with AFB_1 only. However, it was noted that the AFB_1-treated hepatocytes still have lower GSH content than the control cells under the same Sm concentration ($p < 0.05$), suggesting that Sm could only prevent the decrease of GSH induced by AFB_1 to some extent.

Analysis and Identification of the Main Component of Sm

The chromatograms of a pure DA standard and Sm water extract are shown in Figure 5. Of all the components, DA is one of the main compounds. It accounts for 1.15% of the dry weight of Sm. The major component is further characterized by ^{1}H-NMR and ESI-MS spectroscopy.

DISCUSSION

In recent years, carcinogen-induced oxidative damage and its role in the cytotoxicity and

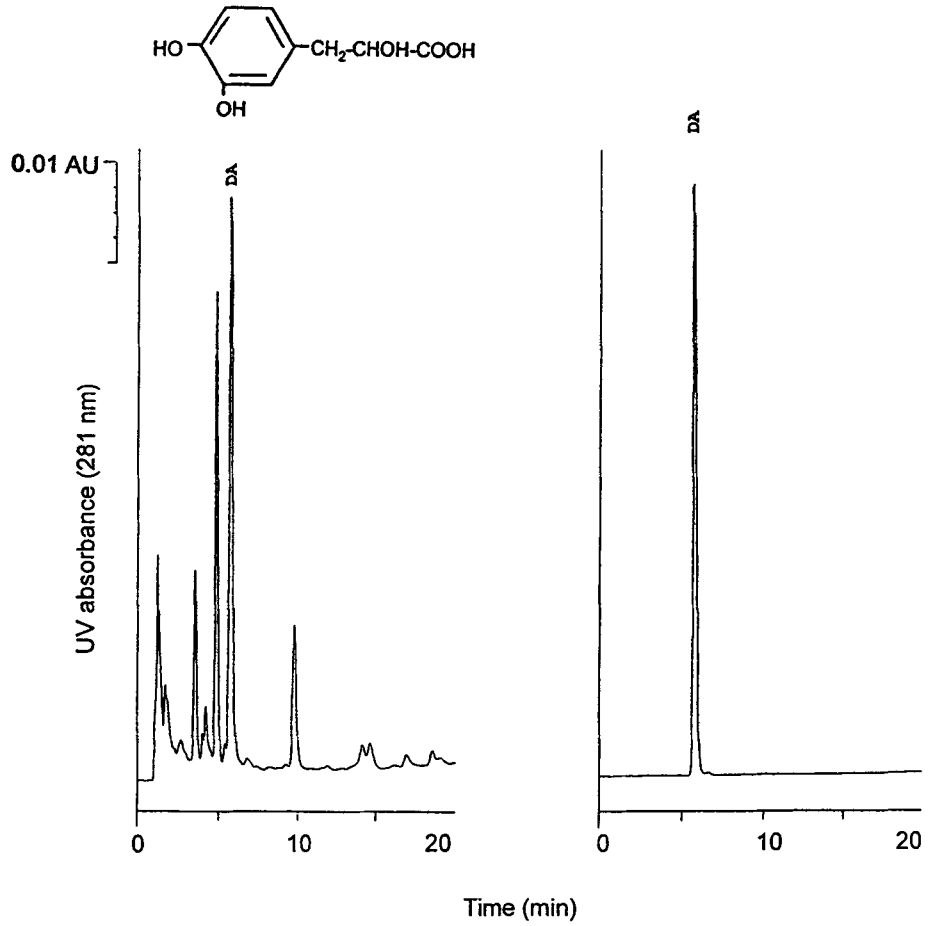

FIGURE 5 Chromatograms of a diluted sample (1:5) of Sm extract and a pure standard containing the sodium salt of DA at a concentration of 25 μg/ml analyzed by the present HPLC method. The injection volume was 5 μl; UV absorbance detected at 281 nm.

carcinogenesis have attracted much attention. Oxidative damage usually refers to the impairment of cellular components such as enzymes, nucleic acids, membrane lipids and proteins by ROS.[16] In the present study, DCFH-DA was used as a probe of ROS formation. This method has been widely used to measure intracellular ROS formation.[17] Our results show that Sm could reduce intracellular ROS level in AFB_1-treated cells (Figure 3). This may be due to its scavenging effect on oxygen free radicals. Several previous studies have found that the aqueous extract of Sm has a scavenging effect on superoxide radical and hydroxyl radical in cell-free systems.[18–20] These oxygen free radicals, as shown in our earlier study, are involved in AFB_1-induced cell injury in cultured hepatocytes.[7] Although the exact mechanism of cellular damage caused by AFB_1 has not been fully elucidated, the enhanced level of ROS and oxidative damage caused by AFB_1 may contribute to its cytotoxic and carcinogenic effects. One of the important sources of ROS generation is believed to be the metabolic processing of AFB_1 by cytochrome P450.[7] Therefore, through scavenging of oxygen free radicals induced by AFB_1, Sm could reduce AFB_1-caused oxidative damage, as shown by our observations on LDH leakage and LPO (Figures 1 and 2).

ROS are highly toxic species capable of reacting with biologically important molecules

leading eventually to cell death and tissue injury.[21] Although the precise biochemical mechanism by which ROS cause cell death have not been fully characterized, of the many reactions described, the ability of hydroxyl radicals to initiate the chain reaction of lipid peroxidation (LPO) in cell membranes may be among the most important.[22] The chain reaction of LPO yields several types of secondary free radicals and a large number of reactive compounds, resulting in the destruction of cellular membranes and other cytotoxic responses.[23] As LPO is one of the main manifestations of oxidative damage initiated by ROS, any factor interfering with the formation of ROS will affect LPO and cell injury.[24] As seen in Figures 3 and 4, Sm decreased the ROS formation and GSH-depletion induced by AFB_1. This may explain why Sm could decrease LPO and LDH in AFB_1-treated cells (Figures 1 and 2). Although Sm can completely inhibit the LPO increase induced by AFB_1 (Figure 2), it did not offer a complete protection against AFB_1-induced LDH leakage in primary hepatocytes. A possible explanation is that LPO associated with AFB_1 toxicity may only be one of the events that lead to hepatocellular injury. Direct covalent binding between activated metabolites of AFB_1 and cellular macromolecules such as DNA, RNA and protein has been proposed as a major event in its hepatotoxicity.[4]

GSH is an essential tripeptide that is found in all mammalian cells with especially high level in the liver.[25] It has a critical role in protection against chemical injury, by serving as a substrate for glutathione peroxidase (GSH-Px) and glutathione transferase (GST). GSH-Px utilizes GSH as a reductant to reduce toxic peroxides, and GST catalyzes the reaction of GSH with metabolites of xenobiotics to form non-toxic conjugates.[26] It is likely that there are three processes by which AFB_1 could deplete the intracellular GSH. One is that GST catalyzes the reaction of GSH with metabolites of AFB_1, which is an important pathway in the detoxification of AFB_1.[27] The other is that, the elevated level of ROS in AFB_1-treated cells could lead to higher GSH utilization by GSH-Px. Another possibility that could lead to the decrease of GSH in AFB_1-treated group is the efflux of GSH from cells as the permeability of cell membranes increase. Our data show that Sm could significantly suppress the increase of ROS formation induced by AFB_1. This is believed to play a role in its prevention against AFB_1-induced GSH depletion. Based on our data, there is a close correlation ($r = 0.988$) between ROS reduction and the recovery of GSH depletion in Sm-treated hepatocytes (data not shown). Another possible mechanism that could have contributed to the increase of GSH content in Sm-treated groups is that Sm may stimulate GSH synthesis. As shown in Figure 4, Sm is able to enhance GSH content in control cells without affecting the ROS level (Figure 3). Furthermore, since Sm could increase the stability of cell membrane, this may help to prevent the GSH leakage in AFB_1-treated cells. As GSH plays a critical role in the detoxification of AFB_1 as well as in the defence against oxidative damage, it is conceivable that the increase of intracellular GSH concentration induced by Sm may partially account for its protective effect against AFB_1 toxicity. By minimising GSH depletion, Sm can enhance the protection against oxidative damage caused by ROS and strengthen the detoxification of AFB_1, thus explaining the fact that the LDH leakage in Sm-treated group was lower than that of AFB_1 control (Figure 1).

Sm is a herbal medicine that has been used with no known ill effect for centuries in traditional Chinese medicine. A recent study showed that the herbal extract of *Salvia* significantly decreased lipid peroxidation in cultured lung cells exposed to iron or ozone.[28] At present, it is unknown what constituents might be responsible for the antioxidative potential of Sm extract. Previous studies have shown that the aqueous extract of Sm contains a mixture of natural phenolic compounds.[29] Our results suggested that DA is one of the major components of Sm extract (Figure 5). The chemical structure of DA may also explain

its antioxidant properties: (1) DA possesses an unsaturated ring, which is a common feature in antioxidants. (2) Similar to other antioxidants such as vitamin E and butylated hydroxytoluene, the existence of phenolic hydroxyl groups on its structure (Figure 5) is closely related to its antioxidant activity. Furthermore, two earlier studies have shown that DA could protect the mitochondrial membrane from ischemia–reperfusion injury and LPO[30] and effectively scavenge superoxide anion radical and hydroxyl radical that were generated from xanthine/xanthine oxidase and H_2O_2/Fe^{2+} systems.[31] The overall data and the result from our study tend to suggest that this phenolic compound could be the active ingredient of Sm responsible for its antioxidant activity, and at least a part of the pharmacological bases, for using Sm for the clinical treatment of certain disease.

In summary, our study shows that Sm can partially inhibit the cytotoxicity of AFB_1 on cultured rat hepatocytes through reducing ROS formation, inhibiting LPO and preventing GSH depletion. Further investigations are ongoing in our laboratory to elucidate the mechanism of this protective action and to further identify other effective components that could be responsible for these activities.

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