Protective actions of salvianolic acid A on hepatocyte injured by peroxidation in vitro

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INTRODUCTION
Salvianolic radix, one of the most commonly used traditional Chinese herbs, was widely studied about its actions against liver injury and fibrosis, and was one of the focuses of recent research⁴⁻⁵. Salvianolic acid-A (SA-A) was an aqueous soluble component of Salvianolic radix. In our previous work⁶, SA-A was found to have protective effects against liver injury and fibrosis induced by carbon tetrachloride (CCl₄) in rats. In order to investigate the effect of SA-A on peroxidation in hepatocytes, we induced the injured hepatocyte model by CCl₄ fumigation in vitro, treated the cell model with SA-A or aqueous soluble vitamin E (Vitamin E), the latter served as the control drug, and observed the influences of the drugs on the functions of the hepatocytes injured by peroxidation.

MATERIALS AND METHODS

Animals
Wistar rats, male, specific pathogens free (SPF), weighing 140g-160g, were provided by the Experimental Center of Animals, Shanghai University of Traditional Chinese Medicine.

Drug
SA-A, molecular formula C₂₆H₂₂O₁₀, molecular structure as Figure 1, weight 494, was extracted and identified by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Vitamin E was purchased from Hoffman Co. USA.

Isolation and culture of hepatocytes
According to a modified method⁷, hepatocytes were isolated and primarily cultured from rats. In brief, after anesthesia with ether, the rat liver was perfused in situ with Ca²⁺ and Mg²⁺ - free Hank's solution via the portal vein for 5 min, followed by perfusion with Hank's solution containing 0.5 g/L collagenase for 20 min. The liver was then excised and minced with forceps to remove Glisson’s capsule and the liver cells dispersed. The liver cell suspension was filtrated with double layers of gauze and was adjusted to 2 ×10¹⁰/L. Then 5 mL cell suspension was added onto the top of 20 mL 492 g/L Ficoll, and centrifuged at 50 × g, 4°C, 5 min to purify the hepatocytes. The cell recovery was about 1 × 10¹⁰ cells per liver, the purity was more than 95% identified by the cell typical appearance via phase contrast microscope, and the viability was more than 90% assessed by trypan blue exclusion. Hepatocytes were suspended with M199.
containing 5% (v/v) NBS to adjust their density to 5 × 10^6 cells/L, seeded on plastic dishes (Nunc, Denmark) and primarily cultured at 37 °C in a humidified atmosphere of 50 mL/L CO_2 and 950 mL/L air.

**Induction of hepatocyte peroxidative injured model**
According to David method[4], the peroxidative injury of hepatocytes was induced by CCl_4 fumigation. Briefly, after 48 h of isolation and culture, the cells were placed in sealed box, to which 1 mL/L CCl_4 was added, and the cells were fumigated with CCl_4 at 37 °C for 24 h.

**Drug treatment**
Normal hepatocytes in dishes were divided into the following groups: the normal, the control, vitamin E (2 × 10^{-3} mol/L)[5] and SA-A treated groups at different concentrations (10^{-4} mol/L-10^{-8} mol/L). All but the normal group, were incubated with vitamin E or SA-A at different concentrations and fumigated with CCl_4 spontaneously for 24 h, then the culture medium was collected respectively and stored at -70 °C until assay.

**Biochemical index assay**
The ALT and AST in culture medium were assayed with Bacon’s method[6]. Superoxide dismutase (SOD), catalase (CAT), lactase dehydrogenase (LDH), glutathione peroxidase (GSH-PX), glutathione (GSH) were measured following the protocols provided by the manufacturer (Jianchueng Biochemical Technological Institute, Nanjing).

**Statistics**
Chi- square test and q test.

**RESULTS**

**The cell morphological changes**
After 48 h of isolation and culture, primary hepatocytes gathered, attached and grew very well. At 24 h after fumigation with CCl_4, the hepatocytes partially shrank, their plasma membrane became rough and organelles swollen. When the cell models were incubated with SA-A or Vitamin E, the plasma membrane became smoother and the organelles less smother than those of controlled model cells.

**Effects of SA-A or Vitamin E on ALT, AST and LDH activity in hepatocytes injured by peroxidation**
The activities of ALT and AST both increased, while the former increase more obviously. LDH activity enhanced approximately 20 folds. SA-A inhibited these pathological increase in dosage dependent manner, and among all concentrations tested the 10^{-4} mol/L SA-A had the best effect on the cell structure and enzymes. The effect of SA-A was better than that of Vitamin E, but 10^{-3} mol/L-10^{-5} mol/L SA-A was not effective compared with the control (Table 1).

**Effects of SA-A or Vitamin E on ALT, AST and LDH activity in hepatocytes injured by peroxidation**

MDA content in the control nearly doubled that of the normal, and the activities of SOD and CAT increased remarkably. SA-A decreased these pathological changes, and 10^{-4} mol/L SA-A had a significant inhibitory action. Vitamin E also decreased the MDA content markedly, but had no obvious influence on SOD activity (Table 2).

**DISCUSSION**
In this study, 24 h after fumigation of hepatocytes with CCl_4, the ALT, AST and LDH all increased remarkably, the rate of elevation was in order of LDH, ALT and AST. It is suggested that the
hepatocytes were acutely injured, cell membrane integrity was broken and the enzymes in cell plasma leaked out. However, after the hepatocytes injured by peroxidation which were incubated with SA-A, the pathological increases of ALT, AST and LDH reduced markedly. It is indicated that SA-A had a potential effect against hepatocyte injury.

The free radicals and its triggered lipid peroxidation were involved in the main mechanisms by which carbon tetrachloride injured hepatocytes. MDA was one of main lipid peroxidatic products, its elevated levels could reflect the degrees of lipid peroxidatic injury in hepatocytes. GSH, a peroxide scavenger with a lower molecular weight, could eliminate superoxide anion and hydrogen peroxide. The content of GSH reflected the ability against peroxidation[7].

In this study, GSH in hepatocytes of the model group was reduced remarkably, suggesting that the potency of antioxidation in injured cells was decreased. There were many other markers that could reflect lipid peroxidation, e.g. SOD, a scavenger of peroxide anion radicals, which could inhibit the initiation of lipid peroxidation by free radicals; GHS-PX, which could particularly catalyze the reductive action of GSH to \( \text{H}_2\text{O}_2 \) to protect the integrity of plasma membrane and functions; CAT etc. All the above-mentioned enzymes increased in the model cells. This may result from acute compensation after injury, and peroxidatic reaction stimulated by \( \text{CCl}_4 \) in hepatocytes. SA-A markedly inhibits the increase of MDA level and the decrease of GSH, also reduced the activities of GSH-PX, CAT, SOD in different extents. Among these results, SA-A had better effect than vitamin E, which is a widely recognized antioxidation drug. It is indicated that SA-A had potential action against lipid peroxidation, this effect perhaps is the main mechanism of protection on liver injury. The results are also in accordance with the other reports[8] and our previous work[2].

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