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RAPID COMMUNICATION

Mistletoe alkali inhibits peroxidation in rat liver and kidney

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Abstract

AIM: To explore the antioxidant and free radical scavenger properties of mistletoe alkali (MA).

METHODS: The antioxidant effect of mistletoe alkali on the oxidative stress induced by carbon tetrachloride (CCl_4) in rats was investigated. The rats were divided into four groups (n = 8): CCl_4-treated group (1 mL/kg body weight), MA -treated group (90 mg/kg), CCl_4+MA-treated group and normal control group. After 4 wk of treatment, the level of malondialdehyde (MDA), a lipid peroxidation product (LPO) was measured in serum and homogenates of liver and kidney. Also, the level of glutathione (GSH), and activities of glutathione reductase (GR), glutathione peroxidase (GSPx), superoxide dismutase (SOD), and glutathione-S-transferase (GST) in liver and kidney were determined. Scavenging effects on hydroxyl free radicals produced *in vitro* by Fenton reaction were studied by ESR methods using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap reagent and H_2O_2/UV as the $OH^{\cdot}$ source. Urinary 8-hydroxydeoxyguanosine (8-OHdG) was determined by competitive ELISA.

RESULTS: In CCl_4-treated group, the level of LPO in serum of liver and kidney was significantly increased compared to controls. The levels of GSH and enzyme activities of SOD, GSPx and GR in liver and kidney were significantly decreased in comparison with controls. In CCl_4+MA-treated group, the changes in the levels of LPO in serum of liver and kidney were not statistically significant compared to controls. The levels of SOD, GSPx and GR in liver and kidney were significantly increased in comparison with controls. There was a significant difference in urinary excretion of 8-OHdG between the CCl_4-treated and MA-treated groups.

CONCLUSION: Oxidative stress may be a major mechanism for the toxicity of CCl_4. MA has a protective effect against CCl_4 toxicity by inhibiting the oxidative damage and stimulating GST activities. Thus, clinical application of MA should be considered in cases with carbon tetrachloride-induced injury.

Key words: Mistletoe alkali; Inhibition of peroxidation; Free radical; Liver and kidney

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INTRODUCTION

Recently, great attention has been focused on the role of the antioxidative defense system in oxidative stress. Endogeneous antioxidants in medicinal herbs may play an important role in antioxidative defense against oxidative damage, possibly protecting the biological functions of cells[1]. There is increasing interest in the protective biological function of natural antioxidants contained in Chinese medicinal herbs, which are candidates for the prevention of oxidative damage[2,3].

Viscum coloratum (Komar.) Nakai has long been categorized as a traditional Chinese medicine in China, USA and other countries. A number of lipid-soluble antioxidants have been isolated from Viscum coloratum (Komar.) Nakai, such as mistletoe alkali, thionins, glucoprotein, polysaccharose[4,5]. In the present study, we investigated the antioxidant activities of mistletoe alkali using a rat model of carbon tetrachloride (CCl_4)-induced hepatotoxicity and nephrotoxic nephritis, which could assess *in vivo* antioxidant activities of Chinese medicinal herbs.

Lipid peroxidation is one of the reactions induced by oxidative stress and is especially active in tissues rich in polyunsaturated fatty acid. There have been few comparative studies on the antioxidant ability of traditional Chinese medicine to modify the susceptibility of organs or tissues to oxidative stress and to alter the cellular antioxidant defense system[6,7]. We have recently found that some compositions of Viscum coloratum (Komar.) Nakai can act effectively *in vitro* as antioxidants and peroxyl radical scavengers. Mistletoe alkali is one of these antioxidant compositions.

As the liver is the main target organ of CCl_4, and the

kidney is the main site of CCl_4 accumulation, the present study was to investigate the oxidative status of both organs in rats simultaneously exposed to CCl_4. To determine the ability of mistletoe alkali to act as antioxidants *in vivo*, we fed rats with mistletoe alkali (90 mg/kg per day) and examined the metabolism of this compound as well as its effects on oxidative stress *in vitro*.

MATERIALS AND METHODS

Mistletoe alkali preparation

Viscum coloratum (Komar.) Nakai was immersed into the acidity aqueous solution and smashed to particles, and then the alkaloid was taken out by precipitation with alkaline. In brief, Viscum coloratum (Komar.) Nakai was smashed to particles and soaked in the acidity aqua for 48 h, the liquid was added to the alkali to precipitate and remove infusible part and the alkali was added again to precipitate its total alkali. The acidity aqua of Viscum coloratum (Komar.) Nakai was proceeded to precipitate the different alkalinity with the aqua.

CCl_4-induced hepatotoxicity and nephrotoxic nephritis

Female Sprague Dawley rats (200-250 g) were maintained in a 12-h light/dark cycle at 22℃ with free access to food and water. Vitamin E was used as positive control. In each experiment, the animals were randomly assigned into groups (n = 8). In the pretreatment groups, the animals were treated intragastrically with the drug preparation at a desired daily dose for 6 d. Mistletoe alkali and vitamin E were dissolved or suspended in olive oil. Twenty-four hours after the last dosing, the animals were administered intragastrically with CCl_4 (11% (v/v) in olive oil at a dose of 1 mL/kg. Control animals were given the appropriate vehicle, and all treatments were conducted between 900 and 1000 h to minimize variations in animal response due to circadian rhythm. The animals were killed 24 h after CCl_4 treatment. After 2 wk of mistletoe alkali treatment, the rats were starved for 12 h and killed by anesthetization with diethyl ether. Blood was taken from the abdominal aorta and plasma was separated. The kidneys, liver, stomach, large and small intestines were removed and washed with cold PBS. Plasma and tissue homogenates were stored at -80℃. Protein concentrations were determined with bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Chemicals

Vitamin E, 5,5-dimethyl pyrrole-N-oxide (DMPO), thiobarbituric acid (TBA) were bought from the Sigma Company (USA). "8-OhdG Check" kit was purchased from Fukuroi (Japan) for the control of aging. All other chemicals were of analytical grade.

Hydroxyl radical scavenging assay by electron spin resonance trapping method

Hydroxyl radical (· OH) scavenging activity of mistletoe alkali was determined by spin trapping ESR using DMPO as a spin trap reagent as previously described[8]. Both Fenton reaction and H_2O_2/ UV system were used as the · OH source. Comparative study on the · OH scavenging activity of mistletoe alkali was carried out in the presence of liver and kidney homogenates and serum using the Fenton system as · OH generator. Briefly, rat liver and kidney homogenates (0.923 mg protein) were incubated with an aliquot of mistletoe alkali at 37℃ for 30 min. The homogenates (100 mL) were mixed with 160 mL of distilled water, 30 mL of 100 mol/L H_2O_2 and 30 mL of 200 mol/L DMPO. Then, 30 mL of 2 mmol/L $FeSO_4$ was finally added to the mixture to initiate the Fenton reaction. DMPO-OH adduct formation was measured in a silica flat cell 2 min after the addition of $FeSO_4$ using a JEOL JESTE 200 electron spin resonance (ESR) spectrometer (X-Band Microwave Unit). The spectrometer settings were as follows: microwave power: 8 mW; microwave frequency: 9.20 GHz; modulation amplitude: 0.1 mT; time constant: 0.03 s; sweep time: 30 s; center fields: 332.6/322.6 mT.

TBARS formation determination

Briefly, an aliquot of liver and kidney homogenates was mixed with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% TBA, then the volume was adjusted to 4.0 mL with distilled water. After boiled at 95℃ for 60 min, the reaction solution was extracted with 1.0 mL of distilled water and 5.0 mL of n- butanol and pyridine (15:1 v/v). The absorbance at 532 nm of the organic layer was determined after centrifugation. The whole procedure was performed as previously described[9].

GSH-Px activity determination

Briefly, an aliquot of liver and kidney homogenates (4 mg wet tissue) in 0.05 mol/L phosphate buffer containing 1.15% (w/v) KCl was mixed in a quartz cuvette with 935 mL of the coupling solution prepared by dissolving 33.6 mg disodium EDTA, 6.5 mg NaN3, 30.7 mg of GSH, 16.7 mg NADPH and 100 units of GSH reductase in 100 mL of 50 mmol/L Tris-HCl (pH 7.6). Kinetic decay of NADPH fluorescence (Ex. 355 nm/Em. 465 nm) was measured after the addition of 25 mL of 1 mmol/L H_2O_2 as substrate using a Hitachi model 650-60 fluorescence spectrophotometer. The whole procedure was carried out as previously described[10].

SOD activity measurement

One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of epinephrine auto-oxidation to adrenochrome. For Mn SOD activity measurement 1 mmol/L KCN was added to the reaction mixture to inhibit CuZn SOD activity which was approximated by subtracting Mn SOD activity from total SOD activity as previously described[11]. Protein content was assayed by the Bradford method (Bio-Rad, Hercules, Calif., USA).

Determination of uninary 8-OHdG by competitive ELISA

Urine samples were centrifuged at 10 000 r/min for 15 min, and the supernatant was used for determination of 8-OHdG by competitive ELISA using the "8-OHdG Check" kit. The determination range was 0.64-2000 μg/

Table 1 LPO, GSH- Px, SOD in rat liver (n = 32, mean ± SD)

Groups	Dosage (mg/kg)	LPO (nmoL/mg protein)	GSH-Px (nmoL/mg protein)	SOD (nmoL/mg protein)
Normal control	-	11.142 ± 0.916[b]	18.713 ± 3. 587[b]	31.537 ± 2.848[b]
CCl_4 + MA group	90	10.326 ± 1.213[b]	20.403 ± 2.388[c]	39.901 ± 2.667[c]
CCl_4 + vit E group	400	11.221 ± 2.182[b]	16.353 ± 2.962[b]	27.736 ± 3.646[b]
CCl_4 group	-	17.271 ± 1.108	13.342 ± 3.636	21.639 ± 3.991

[b]$P < 0.01$, [c]$P < 0.05$ *vs* CCl_4 group.

Table 2 LPO, GSH- Px, SOD in rat kidney (n = 32, mean ± SD)

Groups	dosage (mg/kg)	LPO (nmol/mg protein)	GSH-Px (μ/g mL)	SOD (μ/mg protein)
Normal control	-	1.268 ± 0.99[b]	7.174 ± 0.576[c]	39.573 ± 7.322[c]
CCl_4 + MA group	90	1.973 ± 0.512[b]	7.431 ± 0.483[c]	37.887 ± 6.979[b]
CCl_4 + Vit E group	400	1.312 ± 0.418[b]	6.698 ± 0.746[b]	39.399 ± 8. 684[c]
CCl_4 group	-	3.826 ± 0.289	4.576 ± 0.611	22.639 ± 5.342

[b]$P < 0.01$, [c]$P < 0.05$ *vs* CCl_4 group.

Table 3 MDA, SOD, GSH- Px in blood plasma (n = 32, mean ± SD)

Groups	Dosage (g/kg)	MDA (mol/L)	GSH-Px (μ/mL)	SOD (μ/mL)
Norma control	-	14.341 ± 2.01	21.17 ± 3.82[b]	19.01 ± 2.36[b]
CCl_4 + MA group	90	12.88 ± 3.98[b]	22.67 ± 3.42[b]	23.62 ± 3.17[c]
CCl_4 + Vit E group	400	14.91 ± 2.79	18.77 ± 1.98[b]	17.63 ± 1.88[b]
CCl_4 group	-	14.98 ± 3.63	16.11 ± 2.41	14.88 ± 3.15

[b]$P < 0.01$, [c]$P < 0.05$ *vs* CCl_4 group.

Table 4 Effects of mistletoe alkali on Fenton and H_2O_2/UV-mediated DMPO-OH formation

Drug concentration (μg/ mL)	Sample size (n)	Opposite high peak of ESR (mean ± SD)	Clearance rate (%)
0	20	38.71 ± 3.62	_
2	20	34.16 ± 3.75	11.67
10	20	29.42 ± 2.99	47.82
50	20	22.65 ± 4.83	67.67
100	20	14.38 ± 2.76	91.28

mL and the specificity of the monoclonal antibody used in the competitive ELISA kit was established.

Statistical analysis

Significance was tested by the paired t-test or analysis of variance. Results were presented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

MDA and LPO formation in liver and kidney homogenate and plasma was considerably increased in the model group. However, in the mistletoe alkali-treated groups, no significant increase in MDA and LPO formation was observed (Table 1, Table 2 and Table 3). Results showed that hepatotoxicity and nephrotoxic nephritis in rats were induced by CCl_4 with formation of lipid peroxide. However, mistletoe alkali was effective in reducing malonyl dialdehyde (MDA) content in the tissues, and had good scavenging effects on hydroxyl free radicals.

The effective protection of liver and kidney against oxidative damage induced by mistletoe alkali was further demonstrated by determining GPx and SOD activity in the Liver and kidney homogenate and plasma. GPx and SOD activity decreased markedly in the liver and kidney homogenate and plasma in model group, but activity loss was prevented by mistletoe alkali in the pretreatment groups. GPx and SOD activity increased slightly compared to normal group. The data in the present study suggested that mistletoe alkali could be a potential herbal medicine for improving GPx and SOD activity in liver and kidney tissue and plasma. The enzymes level is shown in Table 1, Table 2 and Table 3. The scavenging activity was steadily increased with the increase of drug density, suggesting that the free radical scavenging activity was related with mistletoe alkali density.

The DMPO-OH signal produced by the Fenton reaction was reduced by the addition of mistletoe alkali in a concentration dependent manner (Table 4). The 50% inhibitory concentration of mistletoe alkali for DMPOI-OH formation was compared to that for Trolox as a reference radical scavenger. The results indicated that mistletoe alkali not only scavenged ·OH directly but also inhibited ·OH generation in the Fenton reaction. This was further confirmed when the ·OH scavenging activity of mistletoe alkali was determined in the H_2O_2/UV system as ·OH source. In this system, the Trolox equivalence of the mistletoe alkali solution was about 0.1 mmoL/L (data not shown).

There was a significant difference in urinary excretion of 8-OHdG between the control group and 900 mg/kg mistletoe alkali-fed group (7.84 ± 3.72, 3.26 ± 1.92 μmoL/d, $P < 0.05$).

DISCUSSION

There is a growing body of evidence that oxygen-derived free radicals are involved in the pathogenesis of over 50 human diseases[12]. Antioxidant therapy aimed at reducing free radical-mediated tissue damage represents a rational approach in preventing the onset and/or progression of free radical-related tissue damage. In this connection, the measurement of antioxidant activity should form an additional basis for drug screening and selection[13]. In the present study, we examined the antioxidant potential of a traditional Chinese medicine, Viscum coloratum (Komar.) Nakai.

Recently, rapid progress has been made in research of Viscum coloratum (Komar.) Nakai. The preparation of Oujisheng has already been applied extensively in

treatment of cancer[14]. China has a long history in research of Viscum coloratum (Komar.) Nakai. In 1934, Jingli Tong discovered that the Chinese Viscum coloratum (Komar.) Nakai (Huangguo Viscum coloratum (Komar.) Nakai) can lower blood pressure[15].

The involvement of free radical-mediated oxidative process in the development of CCl_4 hepatotoxicity is well established[16]. It was reported that impairment of hepatic antioxidant status is associated with a substantial hepatocellular damage induced by CCl_4[17]. GSH is a crucial determinant of tissue susceptibility to oxidative damage, and the depletion of hepatic GSH content has been shown to be associated with an enhanced toxicity to chemicals including CCl_4[18]. The liver protection afforded by pretreatment with NAC, a precursor for GSH synthesis, and vitamin E, an inhibitor of lipid peroxidation, indicates that both the sustained hepatic GSH content and the lipid peroxidation inhibition are important factors involved in protecting against CCl_4 hepatotoxicity. Changes in the sensitivity of tissue homogenates to *in vitro* oxidative challenge have been used as an indicator of altered tissue susceptibility to free radical-induced oxidative injury *in vivo*[19]. Our results, indicating an increase in sensitivity of tissue homogenates to ferric ion-induced lipid peroxidation following CCl_4 treatment, have validated the application of "forced peroxidation" assays in measuring alterations in tissue after oxidative challenge.

To our knowledge, there is no report indicating that the use of MA *in vivo* studies counteracts CCl_4 toxicity. Mistletoe alkali pretreatment at a daily dose of 900 mg/kg body weight for 6 d significantly enhanced hepatic antioxidant status in CCl_4-treated rats, with a concomitant reduction of hepatocellular damage, suggesting that the involvement of GSH-enhancing action can protect liver against damage.

8-OHdG, a DNA base-modified product generated by reactive oxygen species, is mutation prone and a good marker for oxidative damage. It has been hypothesized that oxidative damage can occur in DNA during the peroxidative breakdown of membrane polyunsaturated fatty acids. Lipid peroxidation can mediate 8-OHdG formation *in vitro* and may play a role in carcinogenesis by inducing 8-OHdG generation *in vitro*. In this study, the amount of 8-OHdG in the urine of rats fed with mistletoe alkali was significantly lower than that in the control rats, suggesting that mistletoe alkali plays a role in the prevention of oxidative DNA damage *in vivo*.

In conclusion, increased level of free radicals is associated with decreased antioxidant status in liver and kidney of rats treated with CCl_4. This finding is similar with other investigations[20,21]. Mistletoe alkali strongly inhibits lipid peroxidation and thereby decreases liver and kidney damage.

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