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Free radical scavenging and inhibition of lipid peroxidation by magnesium lithospermate B[1]

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KEY WORDS *Salvia miltiorrhiza*; magnesium lithospermate B; hydroxyl radical; superoxides; lipid peroxidation

ABSTRACT

AIM: To study the effect of magnesium lithospermate B (MLB) on the lipid peroxidation and on its free radical scavenging activity. **METHODS**: MLB was incubated in rat tissue homogenate or in a free radical generating system. MLB induced inhibition of lipid peroxidation and its scavenging activity on superoxide anions and hydroxyl radicals was studied using colorimetric estimation. **RESULTS**: MLB inhibited the lipid peroxidation induced by either an auto-oxidant or Fe^{2+}/VitC *in vitro*, in the liver homogenate, the inhibitory rate of MLB (10 mg/L) being 69.2 % and 57.7 %, respectively. MLB (25 and 50 mg/kg) decreased the amount of thiobarbituric acid reactive substances (TBARS) in rat serum, liver, kidney, and heart. However, it did not inhibit the lipid peroxidation of brain homogenate *ex vivo*. MLB scavenged superoxide anions generated from xanthine/xanthine oxidase system and iron-dependent hydroxyl radicals. **CONCLUSION**: MLB is an inhibitor of lipid peroxidation and scavenge superoxide anions and hydroxyl radicals both *in vitro* and *ex vivo*.

INTRODUCTION

It has been demonstrated that flavonoids and other phenolic compounds of plant origin have antioxidative properties[1-4]. Magnesium lithospermate B(MLB) is a biologically active component isolated from Radix Salviae Miltiorrhizae aqueous extract[5], which has aromatic hydroxyl groups. Some researches indicated its hypertension property[6], ameliorated cephaloridine-induced renal injury[7], carbon tetrochloride-induced hepatitis[8], and as an antioxidant-based protector of rabbit cardiocytes[9]. Recently, we found that MLB inhibited lipid peroxidation in rat liver homogenate. In this study, its free radical scavenging property and its inhibitory effect on lipid peroxidation were investigated.

MATERIALS AND METHODS

Rat tissue homogenate lipid peroxidation assay[10] Sprague-Dawley (SD) rats (either sex, 250 - 300 g, SPF grade, 152th of BK Co, from the Department of Experimental Animals, Shanghai Institute of Materia Medica) were decapitated, and the tissues (liver, kidney, heart, brain) were rapidly removed, weighed, and were made into 5 % homogenates with normal saline at 40 ℃. Homogenate, 1 mL in each tube, was incubated with variable concentration of MLB at 37 ℃. After a 90-min incubation, 10 % trichloroacetic acid 1 mL and 0.67 % thiobarbituric acid 1 mL were added, and contents were boiled for 15 min, cooled with flowing water, and centrifuged at 2000 × *g* for 10 min. The supernatant fraction was extracted and the amount of thiobarbituric acid reactive substances (TBARS) determined at 532 nm spectrophotometically (752 spectrophotometer, the 3rd analytical instrument factory, Shanghai, China). TBARS were determined by linear regression analysis of a standard aliquot using 1,1,3,3-tetraethoxypropane as a standard.

In another experiment, homogenate, 1 mL in each tube, was incubated with variable concentrations of MLB at 37 ℃ for 5 min, then $FeSO_4$ (5 μmol/L) and VitC (0.1 mmol/L)[11] were added and contents were incubated for another 90 min, the following steps were done as described above.

Measurement of TBARS in tissues after ad-

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ministration of MLB *Ex vivo* 10 SD rats were anesthetized, then MLB (25 mg·kg^{-1} and 50 mg·kg^{-1} was injected iv and 5 min after its administration, blood, liver, kidney, heart, and brain were isolated, and determination of TBARS was done as above. Serum (0.2 mL) was put into 0.1 mol/L HCl (2 mL)[10], mixed and shaken, the samples were extracted with methanol-butanol (15:85, vol/vol) (4 mL) and centrifuged at 2000 × *g* for 10 min. The absorbance of the methenol-butanol phase was determined at 532 nm.

Superoxide anion scavenging activity Superoxide anion was generated in a xanthine/xanthine oxidase system and was measured by the nitroblue tetrazolium (NBT) reduction method[12]. Xanthine oxidase (0.05 kU/L) was added to the reaction mixture prepared in phosphate buffer (50 mmol/L, pH 7.4) containing xanthine (0.1 mmol/L), NBT (600 mmol/L) and MLB . Following incubation at 25 ℃ for 10 min, absorbance was read at 560 nm. Percent scavenging of superoxide anion was calculated by making a comparison between the results with or without (control experiments) MLB.

Deoxyribose degradation by iron-dependent hydroxyl radical The method of Gutteridge was conducted with minor modifications[13]. In brief, the reaction was carried out in sodium phosphate buffer (30 mmol/L, pH 7.4) containing deoxyribose (9 mmol/L), H_2O_2 (0.1 mmol/L), NaCl (40 mmol/L), Fe^{2+} (30 μmol/L) and MLB or vehicle in a final volume of 1 mL. The reaction mixture was incubated at 37 ℃ for 1 h and thiobarbituric acid reactivity was developed by adding TBA reagent (1 %, wt/vol in NaOH 50 mmol/L) 0.5 mL and TCA (2.8 %, wt/vol) 0.5 mL followed by heating at 1000 ℃ for 10 min. When the mixture was cooled, the absorbance *A* was measured at 532 nm against appropriate blanks.

Reagents MLB (purity 95 %) was provided by Department of Biotechnology of this institute. 1,1,3,3-Tetraethoxypropane, xathine, xanthine oxidase were obtained from Sigma Chemical Co (St Louis, MO, USA). 2-Deoxyribose was purchased from Fluka (Buchs, Switzerland). All other chemicals were of AR.

Statistics Data were expressed as $\bar{x} \pm s$ and analyzed using *t* test.

RESULTS

Inhibition of lipid peroxidation in tissue homogenate In auto-oxidation control experiments, the amount of TBARS generated in liver, kidney, heart and brain homogenates was (131 ± 40), (136 ± 38), (81 ± 23), (167 ± 43) nmol/g, respectively. On pre-incubation with MLB (10 mg/L and 30 mg/L) , the TBARS generation was greatly inhibited (Fig 1). The inhibitory rate of MLB 10 mg/L, in the liver, kidney, heart, and brain homogenates was 69.2 %, 70.5 %, 56.3 %, and 49.1 %, respectively.

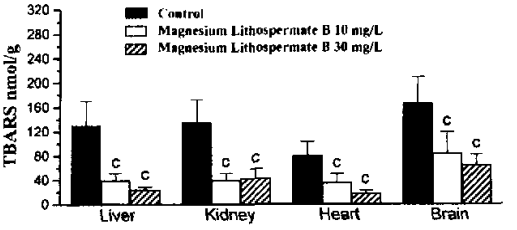

Fig 1. Effect of MLB on the TBARS generation in tissues induced by auto-oxidation *in vitro*. $n = 5$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

In another experiment lipid peroxidation was induced by the Fe^{2+}/VitC system. The amount of TBARS generated in the liver, kidney, heart and brain stimulated by Fe^{2+}/VitC was (278 ± 40), (259 ± 26), (227 ± 51), (321 ± 47) nmol/g protein, respectively, high above the control group ($P < 0.01$) (Fig 2). On pre-incubation with MLB (10 mg/L and 30 mg/L), the amount of TBARS was (117 ± 32), (91 ± 37), (88 ± 52), (178 ± 43) and (76 ± 12), (117 ± 19), (57 ± 25), (152 ± 34) nmol/g protein in the respective tissues thus showing significant inhibition (Fig 2).

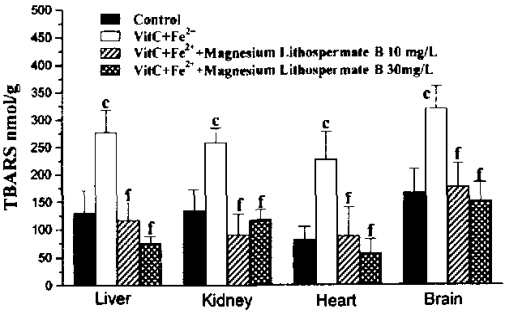

Fig 2. Effect of magnesium lithospermate B on the TBARS formation in tissues induced by Fe^{2+}/Vitc *in vitro*. $n = 5$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* VitC + Fe^{2+} group.

Inhibition of TBARS formation in tissues *ex vivo* The amount of TBARS in serum was (22.5 ± 1.6) μmol/L and in liver, kidney, and heart was (131 ± 55), (119 ± 35), and (84 ± 30) nmol/g. Compared with the control, after administration of MLB (25 mg/kg and 50 mg/kg) the generation of TBARS was greatly inhibited (Tab 1). However, the change of TBARS in brain homogenate did not show any statistical significance (Tab 1).

Tab 1. Effect of magnesium lithospermate B(MLB) on the TBARS generation in tissue induced by auto-oxidation *ex vitro*. $n = 5$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Group	Serum/ μmol/L	Liver/ nmol/g	Liver/ nmol/g	Heart/ nmol/g	Brain/ nmol/g
Control	22.5 ± 1.6	131 ± 55	119 ± 35	84 ± 30	177 ± 58
MLB 25 mg/kg	14.4 ± 2.0^c	73 ± 13^c	75 ± 11^c	56 ± 6^c	141 ± 12
MLB 50 mg/kg	12.1 ± 1.5^c	55 ± 12^c	51 ± 11^c	50 ± 10^c	178 ± 57

Effect of MLB on generation of superoxide anion with NBT reduction in the xanthine/xanthine oxidase system Superoxide anion generated in the xanthine/xanthine oxidase reduces NBT to produce the blue chromogen formazan. Compounds capable of scavenging superoxide anion can inhibit NBT reduction. MLB (10 – 100 mg/L) scavenged superoxide anion in a concentration-dependent manner (Tab 1). The inhibitory rate of MLB (10, 30, and 100 g/mL) was 11.8 %, 24.3 %, 32.2 %, respectively. Control experiments revealed that MLB did not directly reduce NBT (Tab 2).

Tab 2. Effect of MLB on generation of superoxide anion with NBT reduction in the xanthine/xanthine oxidase system. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Groups	A_{560}	Inhibitory rate
Control	0.886 ± 0.020	
MLB 10 mg/L	0.781 ± 0.025^c	11.8 %
MLB 30 mg/L	0.670 ± 0.053^c	24.3 %
MLB 100 mg/L	0.600 ± 0.043^c	32.2 %

Effect of MLB on deoxyribose degradation caused by hydroxyl radical The ability of MLB to scavenge hydroxyl radicals was measured by studying the competition between deoxyribose and MLB for hydroxyl radicals generated in the Fe^{2+}-H_2O_2 system. The hydroxyl radical attacks deoxyribose and sets off a series of reactions that eventually result in TBARS formation. The extent of deoxyribose degraded by hydroxyl radical generated by Fe^{2+}-H_2O_2 was 0.637 ± 0.051. The deoxyribose degradation was not affected by MLB at 10 mg/L, while at 30 mg/L and 100 mg/L, the deoxyribose degradation was 0.504 ± 0.101 and 0.361 ± 0.052 showing an inhibitory rate of 20.9 %, 43.3 %, respectively (Tab 3).

Tab 3. Effect of magnesium lithospermate(MLB) on the degradation of deoxyribose mediated by hydroxyl radical. $n = 8$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Groups	A_{532}	Inhibitory rate
Control	0.637 ± 0.051	
MLB (10 mg/L)	0.643 ± 0.077	0
MLB (30 mg/L)	0.504 ± 0.101^b	20.9 %
MLB (100 mg/L)	0.361 ± 0.052^c	43.3 %

DISCUSSION

Lipid peroxidation, defined as the oxidative deterioration of polyunsaturated fatty acids, is a free radical-mediated phenomenon[14]. Both transition metals and enzymes catalyze this process. In our study MLB 10 and 30 mg/L was quite effective in inhibiting auto-oxidation and Fe^{2+}/VitC induced lipid peroxidation in rat serum and in rat liver, kidney, heart, and brain homogenates in *in vitro* experiment. These inhibitory actions on the TBARS formation might be related to a direct scavenging of free radicals.

In this study, it was also indicated that MLB had no effect on the lipid peroxidation of brain homogenate but did affect other tissue *ex vivo*, which prompts the postulation that it may not be able to permeate though the brain blood barrier. It is suggested that the therapeutic effect of MLB in the traumatic or ischemic injury of brain may be investigated further.

The xanthine-xanthine oxidase system is frequently used as a generator of superoxide anions. Superoxide anions can, directly or indirectly, by forming hydrogen peroxide or reactive hydroxyl radical, damage the biomacromolecules[15]. In this study, MLB scavenged superoxide anions generated in xanthine/xanthine oxidase

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system in a concentration-dependent manner. Thus, MLB may be advantageous in preventing superoxide-induced damages. Hydroxyl radical is a highly potent oxidant that reacts with almost all biomacromolecules found in living cells[16]. Deoxyribose undergoes "site-specific" degradation when the hydroxyl radical is generated in the iron-ascorbate-H_2O_2 system in the absence of edetic acid[12]. A biologically active molecule such as MLB scavenging the hydroxyl radical could prevent the degradation. In our experiment, the deoxyribose degradation by MLB 30 and 100 mg/L was inhibited by 20.9 % and 43.3 %, respectively. This indicates that MLB might be an effective scavenger of hydroxyl radical.

In summary, these results indicated that MLB was an inhibitor of lipid peroxidation and more specially a scavenger of superoxide anions, and hydroxyl radicals. These effects may be correlated with its phenolic structure, which can react with a free radical to form the phenoxyl radicals. These results may indicate Radix Salviae Miltiorrhizae or its active constituents in future antioxidative therapy, however, its *in vivo* antioxidant activity requires further investigation.

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丹酚酸 B 镁盐对自由基的清除作用和对脂质过氧化的抑制作用[1]

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关键词 丹参；丹酚酸 B 镁盐；羟自由基；超氧化物；脂质过氧化

目的：研究丹酚酸 B 镁盐对过氧化脂质生成的影响和对自由基的清除作用. **方法**：不同浓度的丹酚酸 B 镁盐与大鼠组织匀浆或自由基发生系统共浴后，比色法观察它对过氧化脂质生成的抑制作用和对自由基的清除作用. **结果**：丹酚酸 B 镁盐抑制组织匀浆的脂质自氧化或硫酸亚铁和维生素 C 激发的脂质过氧化. 在肝组织匀浆，丹酚酸 B 镁盐 10 mg/L 对两者的抑制率分别是 69.2 %，57.7 %. 丹酚酸 B 镁盐可清除黄嘌呤/黄嘌呤氧化酶系统产生的氧阴离子和 Fe^{2+}-H_2O_2 系统产生的羟自由基. **结论**：丹酚酸 B 镁盐能抑制脂质过氧化并对自由基有一定的清除作用.

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