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Inhibitory effects of Keishi-bukuryo-gan on peroxidation of erythrocyte ghost by active oxygen

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Abstract

It was found that Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan) has inhibitory effects on lipid peroxidation of erythrocyte ghost by active oxygen, hydroxyl radical and superoxide anion, derived from Fe^{2+} -hydrogen peroxide system and xanthine-xanthine oxidase system. These effects were similar to those of catalase, mannitol, superoxide dismutase or dl-α-tocopherol as a scavenger or an antioxidant. This drug inhibited the peroxidation of erythrocyte ghost by active oxygen in a concentration-dependent manner.

Key words hydroxyl radical, Keishi-bukuryo-gan, peroxidation, erythrocyte ghost, superoxide anion.

Abbreviations ADP, adenosin-5'-diphosphate monopotassium salt; EDTA, ethylenediaminetetraacetic acid, disodium salt; IC_{50}, 50 % inhibitory concentration; LPO, lipid peroxidation; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid; XOD, xanthine oxidase.

Introduction

Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan) is a representative drug for blood stagnation (Oketsu), and an effective drug for chronic hepatitis or gynecological disorders.[1] These pharmacological effects are based on improvements of erythrocyte deformity [2] and microvascular-circulation.[3] We found that it has inhibitory effects on lipid peroxidations (LPOs) of lecithin liposome, induced by active oxygens.[4] Such active oxygens were derived from the Fe^{2+} -hydrogen peroxide (H_2O_2) system and xanthine-xanthine oxidase (XOD) system.

In this paper, we investigated whether this drug has inhibitory effects on LPOs of erythrocyte ghost, one of cell membrane, induced by the same oxidation system.

Materials and Methods

Animals : Male ddY mice weighing 25-30 g were purchased from the Shizuoka Laboratory Animal Center. They were housed in an animal room at room temperature : about 25°C, humidity ; at about 60 %, relative humidity.

Reagents : The composition of Keishi-bukuryo-gan is shown in Table I. The dried herbs were boiled gently with 500 ml of water for 60 min in a glass flask, filtered and the resultant decoction was lyophilized. The used herbs were obtained from Takasago-Yakugyo Co. (Osaka, Japan), and drugs listed in the Pharmacopoeia of Japan. The yield was 9 %. Ethylenediaminetetraacetic acid, disodium salt (EDTA), ferrous ammoniumsulfate, ferric cholide ($FeCl_3$), H_2O_2, mannitol, thiobarbituric acid (TBA), dl-α-tocopherol and xanthine were obtained from Wako Pure Chemicals Co. (Osaka, Japan). Catalase (EC 1. 11. 1. 6, from bovine liver), superoxide dismutase (SOD : EC 1. 15. 1. 1, from bovine erythrocyte) and xanthine oxidase (XOD : EC 1. 2. 3. 2, from butter milk, Grade I) were obtained from Sigma Co. (St. Louis. Mo, U.S.A.). Adenosine-5-diphosphate monopotassium salt (ADP) was obtained from Oriental

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Yeast Co. (Tokyo, Japan). Other reagents were of analytical grade.

Preparation of erythrocyte ghost : White erythrocyte ghost was prepared according to the method of Dodge *et al.*.[5] In brief, erythrocyte were hemolyzed and washed with phosphate buffer (pH 7.4) and glucose. The final concentration of white erythrocyte ghost obtained was 500 μg/ml. The protein content of white erythrocyte ghost was determined using bovine serum albumin as a standard according to the method of Lowry *et al.*.[6]

LPO of erythrocyte ghost catalyzed by Fe^{2+}-H_2O_2 *system* : 1 ml of reaction mixture contained erythrocyte ghost (100 μg / ml protein), 100 μM ferrous ammonium sulfate, 100 μM H_2O_2 and test material in Tris-HCl buffer (pH 7.4) was incubated at 37°C for 10 min. The incubated mixture was added to 1 ml of 10% trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 10 min. 1 ml of supernatant was added 2.5 ml of 0.67% TBA and heated at 100°C for 60 min. The solution was added 5 ml of n-butanol-pyridine mixture (15 : 1). The mixture was centrifuged at 3000 rpm for 15 min. The absorbance at 532 nm of TBA-reactive product in supernatant was determined as malondialdehyde (MDA). Inhibitory ratio of test material was evaluated by the following equation (A) :

$$\text{Inhibitory ratio}(\%) = \frac{\text{MDA formed in the absence of test material} - \text{MDA formed in the presence of test material}}{\text{MDA formed in the absence of test material}} \times 100$$

LPO of erythrocyte ghost catalyzed by xanthine-XOD system : Following the method of Burge and Aust,[7] 1 ml of reaction mixture contained erythrocyte ghost, 0.33 mM xanthine, 1.7 mM ADP-0.1 mM $FeCl_3$, 0.11 mM EDTA-0.1 mM $FeCl_3$, 0.1 U/ml XOD and test material in 50 mM Tris-HCl buffer (pH 7.4) was incubated at 37°C for 10 min. To the incubated mixture was added 2.0 ml of TBA reagent which contained 0.37% TBA, 15 % TCA, 0.04 % butylated hydroxytoluene and 2 % ethanol. This mixture was heated at 100°C for 15 min. The solution was centrifuged at 3000 rpm for 10 min. The absorbance of supernatant was determined as MDA. Inhibitory ratio of test material was evaluated by equation (A).

Statistical analysis : Values were expressed as the mean±standard error of five experiments. Statistical analyses were carried out using Student's *t*-test.

Results

LPO of erythrocyte ghost catalyzed by Fe^{2+}-H_2O_2

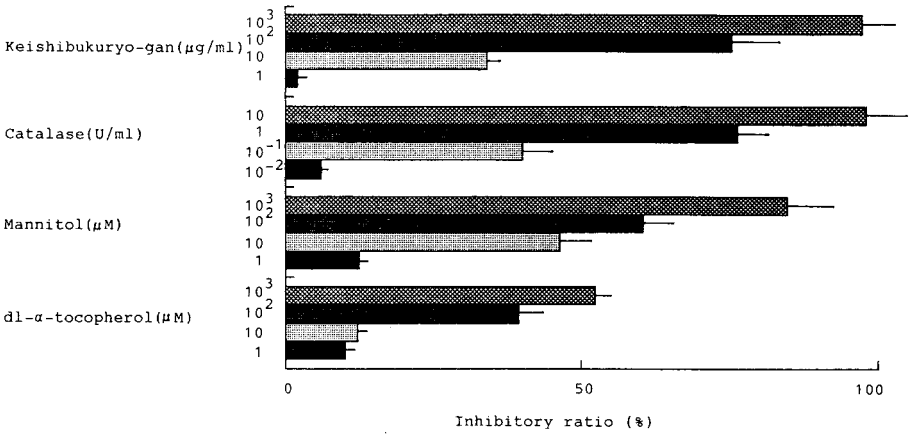

Fig. 1 Inhibitory effects of Keishi-bukuryo-gan on peroxidation of erythrocytes ghost by Fe^{2+} -H_2O_2 system.
Each value represents the mean±S.E. of 4 experiments.

system

Fig. 1 showed the inhibitory ratios of test materials on peroxidation of erythrocyte ghost by Fe^{2+} -H_2O_2 system. MDA produced in the absence of test material was 1.40±0.07 nmol/ml. Inhibitory ratios in the presence of 10^3 μg/ml Keishi-bukuryo-gan, 10 U/ml catalase, 10^3 μM mannitol and 10^3 μM dl-α-tocopherol were 97.60±5.3 %, 98.3±7.6 %, 84.7±6.5 % and 53.4±4.7 %, respectively. These inhibitory ratios increased in a concentration-dependent manner. (Fig. 1)

LPO of erythrocyte ghost catalyzed by xanthine-XOD system

Fig. 2 showed the inhibitory ratios of test

Table I Constitutional herbs in Keishi-bukuryo-gan.

Plant name	Composition (g)
Keishi (*Cinnamomum cassia* BLUME)	3.0
Shakuyaku (*Paeonia lactiflora* PALLAS)	3.0
Tonin (*Prunus persica* BATSCH)	3.0
Bukuryo (*Poria cocos* WOLF)	3.0
Botampi (*Paeonia suffruticosa* ANDREWS)	3.0

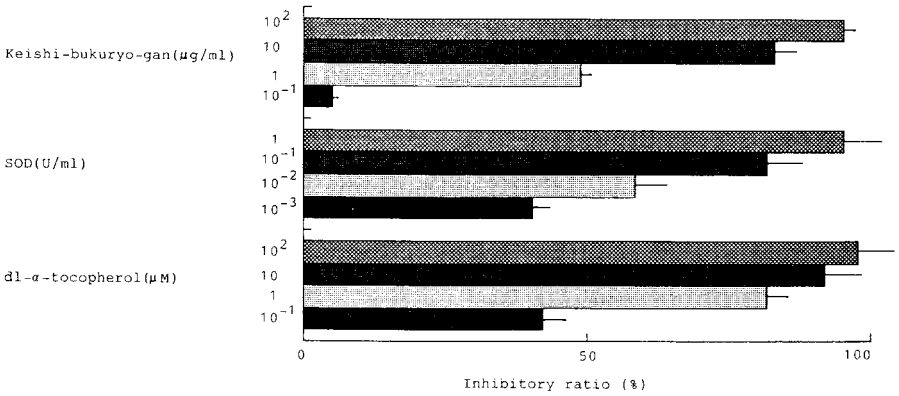

Fig. 2 Inhibitory effects of Keishi-bukuryo-gan on peroxidation of erythrocytes ghost by xanthine-XOD system.
Each value represents the mean±S.E. of 4 experiments.

Table II IC_{50} of Keishi-bukuryo-gan on peroxidation of erythrocyte ghost by Fe^{2+} -H_2O_2 and xanthine-XOD system.

Test material	IC_{50}	
	Fe^{2+} - H_2O_2 system	xanthine-XOD system
Keishi-bukuryo-gan	2.22×10 μg/ml	1.17 μg/ml
Catalase	1.94×10^{-1} U/ml	——
Mannitol	2.53×10 μM	——
SOD	——	303×10^{-3} U/ml
dl-α-tocopherol	8.06×10^{2} μM	5.95×10^{-1} μM

materials on peroxidation of eryhtrocyte ghost by xanthine - XOD system. MDA produced in the absence of test material was 0.132±0.010 nmol/ml. Inhibitory ratios in the presence of 10^2 μg/ml Keishi-bukuryo-gan, 10^2 μM dl-α-tocopherol and 1 U/ml SOD were 95.1±2.3 %, 97.7±2.3 % and 95.3 ±3.5 %, respectively. The inhibitory ratios increased in a concentration - dependent manner. (Fig. 2)

50 % inhibitory concentration (IC_{50}) on peroxidation of erythrocyte ghost by Fe^{2+}-H_2O_2 and xanthine-XOD system

IC_{50} shown in Table II was estimated from the results of Fig.s 1 and 2. It was impossible to compare with IC_{50} of test materials because test materials have different unites. (Table II)

Discussion

Cell membrane is mainly comprised of lipid (phospholipid, glycolipid, cholesterol, *etc.*) and protein. LPOs of cell membrane suggested peroxidations of its lipid and protein. We showed previously that Keishi-bukuryo-gan inhibits peroxidation of lecithin-liposome, a model of cell membrane, by active oxygen (hydroxyl radical or superoxide anion) derived from Fe^{2+}-H_2O_2 system and xanthine-XOD system.[4]

In the present study, we tried to reveal whether Keishi - bukuryo - gan has inhibitory effects on LPOs of native cell membrane by active oxygen derived from the same systems. We showed that the drug inhibited such LPOs in a similar manner as catalase, mannitol and dl-α-tocopherol. This result suggests that Keishi-bukuryo-gan has inhibitory effects on injury of cell membrane by oxidative stress derived from active oxygen.

Since it has been demonstrated that Keishi-bukuryo - gan has a scavenger - like effect on superoxide anion[8] and that Keishi, one of its constitutional herbs, inhibits the generation of hydroxyl radical,[9] the inhibitory effects of Keishi-bukuryo-gan on LPO of cell membrane seems to be responsible for its scavenger-like effect on superoxide anion and for inhibitory effects of Keishi on generation of hydroxyl radical.

MDA derived from LPO of erythrocyte membrane enhances the decrease in erythrocyte deformability.[10] Erythrocyte deformability is one of the essential factors of blood viscosity.[11] Terasawa *et al.* demonstrated that blood stagnation (Oketsu) is a microcircular injury on blood capillaries and is enhanced by the increase of blood viscosity.[12] Accordingly, it is suggested that Keishi-bukuryo-gan has a preventive effect on the decrease in blood viscosity. It could be concluded that the present study shows one anti-blood stagnation effect of Keishi-bukuryo-gan.

和文抄録

桂枝茯苓丸には，過酸化水素 -Fe^{2+} またはキサンチン -XOD 系で生成される活性酸素による赤血球ゴースト過酸化に対して抑制作用のあることが認められた。

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