Short Communication

**Comparison of the effects of keishibukuryogan and atorvastatin on balloon injury-induced intimal thickening in rat carotid artery**

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**Abstract**

HMG-CoA reductase inhibitors (statins) are frequently used to prevent and/or treat atherosclerosis because of their ability to lower blood cholesterol levels, the most crucial factor affecting atherosclerosis. Recently, keishibukuryogan (KBG), a Kampo medicine, has been reported to prevent atherosclerosis in high cholesterol diet (HCD)-fed rabbits without affecting the blood cholesterol level. Here, we compared the effects of KBG and atorvastatin (ATS) on balloon-induced intimal thickening of the carotid artery in rats fed a normal diet (ND) or an HCD. The inside of each rat carotid artery was denuded using a balloon catheter. ATS or KBG was administered orally to rats, and intimal thickening was assessed histologically two weeks after denudation. Under the ND-fed condition, the intimal thickness of the ATS-treated group was equal to that of the control group, but that of the KBG-treated group was significantly less than that of the control group. On the other hand, both ATS and KBG significantly attenuated intimal thickening under the HCD-fed condition. The serum cholesterol levels of the ATS- and KBG-treated groups were equal to that of the control group under the ND-fed condition. The serum cholesterol level under the HCD-fed condition was not affected by KBG but was slightly lowered by ATS. The malondialdehyde (MDA) content, an index of lipid peroxidation, was significantly increased in the balloon injured-vascular tissue, compared with in non-injured tissue. ATS and KBG did not affect the balloon injury-induced increase in MDA. These results suggest that ATS prevents intimal thickening by lowering the blood cholesterol level in part under hypercholesterolemic condition, whereas KBG prevents intimal thickening regardless of the blood cholesterol level. Oxidative stress is presumed to be at least partly involved in balloon injury-induced intimal thickening. However, neither ATS nor KBG prevented intimal thickening by suppressing oxidative stress.

**Key words** keishibukuryogan, atorvastatin, balloon injury, intimal thickening, cholesterol.

**Abbreviations** ACE, angiotensin-converting enzyme; AT1, angiotensin II type 1; ATS, atorvastatin; CMC-Na, carboxymethyl cellulose sodium; HCD, high cholesterol diet; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; I/M, intimal/medial; KBG, keishibukuryogan; LDL, low-density lipoprotein; MDA, malondialdehyde; ND, normal diet; PTCA, percutaneous transluminal coronary angioplasty.

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Introduction

Metabolic syndrome, which is comprised of a combination of medical disorders such as hypertension, dyslipidemia and diabetes related to visceral fat accumulation and insulin resistance, has recently become an important social problem. Insufficient attention to metabolic syndrome increases the risk of atherosclerosis, the most dangerous factor causing coronary heart disease and/or cerebral infarction. Therefore, countermeasures against atherosclerosis are very important for preventing these fatal diseases.

Percutaneous transluminal coronary angioplasty (PTCA) is often used to treat ischemic heart diseases associated with coronary artery stenosis. However, restenosis of the coronary artery occurs in 20 - 50% of all patients treated with PTCA, creating an obstacle for the long-term success of PTCA treatment.1) Although the mechanisms of restenosis have not yet been clarified, the exposure of the denuded intima to blood may trigger the development of platelet adhesion, in turn leading to restenosis.2) In addition, the proliferation of vascular smooth muscle cells and their migration to the intima play a key role in the development of atherosclerosis and/or post-PTCA restenosis.3,4)

Cholesterol, particularly low-density lipoprotein (LDL) cholesterol, is a crucial risk factor in the development of atherosclerosis. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme, is responsible for cholesterol biosynthesis, and inhibitors of HMG-CoA reductase (statins) are clinically used to decrease the total and LDL cholesterol levels in the blood. Thus, statins have been used as the treatment of first choice in the prevention and treatment of atherosclerosis.

Keishikuryogan (KBG) is a Kampo medicine that is often used for the treatment of various gynecological disorders such as hie-sho (chills) and menstrual and menopausal disorders. At present, apart from improving oketsu (blood stasis syndrome),5) KBG has been reported to inhibit the formation of atheromatous plaques in the thoracic aorta without affecting the blood cholesterol level in rabbits treated with a high cholesterol diet (HCD).6,7) Thus, KBG may be useful for the improvement and/or treatment of atherosclerosis.

In the present study, we examined the effect of KBG on balloon injury-induced intimal thickening in rat carotid artery, a model of atherosclerosis or post-PTCA arterial restenosis,8) and compared the effects with those of atorvastatin (ATS).9) Furthermore, the effects of both KBG and ATS on blood cholesterol levels were examined in ND- or HCD-fed rats, since hypercholesterolemia is a risk factor for atherosclerosis and post-PTCA restenosis.8,10) After balloon injury, angiotensin II is involved in the proliferation of vascular smooth muscle cells, leading to intimal thickening.11,12) Moreover, angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 (AT1) receptor antagonists reportedly attenuate the process of intima thickening.13,14) Consequently, the AT1 receptor antagonist losartan was used, as a positive control in the present study.

Materials and Methods

Animals: Male rats (Sprague-Dawley, 340 - 370 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan) and used in the experiments after one week of acclimation. The animals were housed in an air-conditioned room (23 ± 2 °C; humidity: 55 ± 10%) under a 12-h dark/12-h light cycle (lights on at 8:00 - 20:00) with free access to water and food. This study was approved by the Animal Experiment Committee of Products Evaluation and Quality Analysis Research Laboratories, Kracie Pharma, Ltd.

Balloon injury of rat carotid artery: Balloon injury of the rat carotid artery was performed according to the method of Kim et al.15) Briefly, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Nembutal; Dainippon-Sumitomo Pharma, Osaka, Japan). A balloon catheter (2F Fogarty catheter; Edwards Life Science, Irvine, CA, USA) was inserted into the blood vessel from the left femoral artery and the tip was advanced into the left common carotid artery. Subsequently, the tip of the balloon catheter was inflated with saline (0.02 mL) and pulled back slowly by about 10 mm to denude the endothelium. Thereafter, the tip was shrunk and advanced again to the initial position. This endothelial denudation was performed three times.

In the animals fed a normal diet (ND), normal pellet
diet (CE-2; Clea Japan, Tokyo, Japan) was freely given to rats during the experimental period starting three days prior to balloon denudation. In the animals fed an HCD, pellets containing 1% cholesterol and 1% cholic acid were freely given in the same manner.

**Histological evaluation of intimal thickening:** At two weeks after balloon denudation, the rats were anesthetized with urethane (240 mg/kg, i.p.; Wako Pure Chemicals, Osaka, Japan) and blood was collected from the abdominal aorta. The carotid artery was fixed by infusing it with 10% phosphate-buffered neutral formalin from the left ventricle. Subsequently, the fixed carotid artery was removed and embedded in paraffin. The paraffin block was sliced into 4-μm-thick sections (5 sections for each artery) and stained with elastica van Gieson’s solution to produce cross-sectional histological specimens of the artery. An intact artery specimen was made using the non-denuded opposite carotid artery of the control rat in the same manner. These arterial specimens were observed microscopically and the intimal and medial areas were measured using an image analyzer (Micro Analyzer; Nihon Poladigital, KK, Tokyo, Japan). Subsequently, the intimal/medial area ratio (I/M ratio) was calculated as an index of the intimal thickening of the artery.

**Measurement of serum cholesterol level:** Blood was centrifuged (3000 rpm for 10 minutes at room temperature) to obtain the serum and the total and LDL cholesterol levels in the sera were measured using the Cholesterol E-Test Wako and the L-Type Wako LDL-CM (Wako Pure Chemicals, Osaka, Japan), respectively.

**Measurement of lipid peroxides in vascular tissue:** In the HCD-fed animals, the malondialdehyde (MDA) content in the vascular tissue after balloon denudation was determined as an index of lipid peroxidation or oxidative stress. The MDA content in the vascular tissues was measured according to the method of Muscoli et al., with slight modifications. Namely, the carotid artery was removed after infusing it with cold saline from the left ventricle under urethane anesthesia 7 days after balloon denudation. About 0.01 g of tissue was cut off and homogenized with 0.2 mL of chloroform:methanol (2:1). Then, 2 mL of chloroform:methanol (2:1) and 0.8 mL of distilled water were added to the homogenate and centrifuged. After centrifugation, the chloroform layer was collected and the chloroform was volatilized using nitrogen gas. The residues were suspended in 0.1 mL of saline, and the samples were stored at -80 °C until MDA measurement.

To measure the MDA content, 0.02 mL of 8.1% sodium dodecyl sulfate, 0.15 mL of 20% acetic acid (pH 3.5), 0.15 mL of 0.8% TBA reagent (2-thiobarbituric acid dissolved in 20% acetic acid) and 0.4 mL of distilled water were added to the stored samples. The mixture was stirred vigorously and then heated in a boiling water bath for 60 minutes. After cooling the mixture in ice, 1.6 mL of n-butanol:pyridine (15:1) was added and the mixture was centrifuged (3000 rpm for 10 minutes at room temperature) to separate the butanol layer (upper layer). Subsequently, the absorbance of the butanol layer was measured at 532 nm and the concentration was calculated using a standard curve.

**Drug administration:** Dried extract powder of keishi-bukuryogan (KBG, Lot No. 06091211; Kracie Pharma, Ltd., Tokyo, Japan) was used in this study. It is composed of equal part of five kinds of crude drugs including Cinnamomi Cortex, Poria, Moutan Cortex, Persicae semen and Paeoniae Radix. Drugs were suspended in 0.5% carboxymethyl cellulose sodium (CMC-Na) aqueous solution and administered orally to the rats once a day for three days before the balloon denudation and for the next two weeks. Control rats were similarly treated with 0.5% CMC-Na solution alone. The dose of each drug was as follows: atorvastatin (ATS; Zhejiang Neodankong Pharmaceutical, Zhejiang, China), 15 mg/kg; KBG, 1600 mg/kg; and losartan potassium (LKT Laboratories, Inc., St. Paul, MN, USA), 20 mg/kg. The ratio of the doses of ATS and KBG was equalized to that of the clinical doses of both drugs (i.e., ATS:KBG = 20 mg/day:2300 mg/day = 15 mg/kg:1600 mg/kg).

**Statistical analysis:** The results were expressed as the mean ± S.E.M. The statistical difference between each group was assessed using a Student t-test and was judged significant when the P value was under 0.05 (P<0.05).
Results

Examination of ND-fed animals

Effects on body weight: No significant differences in the body weight (g) were observed between the control and drug-treated groups two weeks after balloon denudation (control: 429 ± 5, n=24; ATS: 424 ± 3, n=24; KBG: 424 ± 3, n=21; losartan: 429 ± 4, n=24).

Effects on balloon-induced intimal thickening: The I/M ratio of each group two weeks after balloon denudation is shown in Fig. 1. The I/M ratio of the ATS-treated group was similar to that of the control group. On the other hand, the I/M ratios of the KBG- and losartan-treated groups were significantly lower than that of the control group, although the values were similar between the two groups.

Effects on serum cholesterol level: No significant differences in the serum total cholesterol level (mg/dL) were observed between the control and drug-treated groups two weeks after balloon denudation (control: 59.0 ± 1.8, n=24; ATS: 60.7 ± 1.9, n=24; KBG: 59.5 ± 1.6, n=21; losartan: 58.9 ± 1.5, n=24).

Examination of HCD-fed animals

Effects on body weight: No significant differences in the body weight (g) were observed between the ND-fed control, HCD-fed control and drug-treated groups two weeks after balloon denudation (ND-fed control: 436 ± 4, n=19; HCD-fed control: 428 ± 4, n=19; ATS: 419 ± 5, n=19; KBG: 422 ± 6, n=16; losartan: 420 ± 5, n=19).

Effects on balloon-induced intimal thickening: The I/M ratio of each group two weeks after balloon denudation is shown in Fig. 2. The I/M ratio of the HCD-fed control group tended to be larger than that of the ND-fed control group. In contrast to the ND-fed animals, however, the I/M ratio of the ATS-treated group, as well as those of the KBG- and losartan-treated groups, was significantly lower than that of the control group. However, no differences in the values were observed among the three drug-treated groups. Typical appearances of the cross-sections of the carotid arteries are shown in Fig. 3.

Effects on serum cholesterol level: The serum total and LDL cholesterol levels of each group two weeks

Fig. 1 Effects on balloon injury-induced intimal thickening of the carotid artery in normal diet-fed rats.
- I/M ratio = intimal area/medial area.
- C: Control (n=24), A: Atorvastatin 15 mg/kg (n=24), K: Keishibukuryogan 1600 mg/kg (n=21), L: Losartan 20 mg/kg (n=24).
- Each column and bar represents the mean ± S.E.M.
- *p<0.05, **p<0.01, compared with Control (t-test).
after balloon denudation are shown in Fig. 4. The serum total and LDL cholesterol levels were significantly higher in the HCD-fed control group than in the ND-fed control group. Both cholesterol levels of the ATS-treated group were slightly lower than those of the HCD-fed control group, although the cholesterol levels of the KBG- and losartan-treated groups were not different from those of the control group.

**Effects on lipid peroxidation in vascular tissue:** The MDA content as an index of lipid peroxidation in the vascular tissues of each group 7 days after balloon denudation is shown in Fig. 5. The MDA content was significantly higher in the balloon-denuded vascular tissue than in non-denuded tissue. Neither ATS nor KBG affected the balloon denudation-induced increase in the MDA content.

![Image of graph showing I/M ratio](image-url)

**Fig. 2** Effects on balloon injury-induced intimal thickening of the carotid artery in high cholesterol diet (HCD)-fed rats.
N: Normal diet-control (n=19), C: Control (n=19), A: Atorvastatin 15 mg/kg (n=19), K: Keishibukuryogan 1600 mg/kg (n=16), L: Losartan 20 mg/kg (n=19).
Each column and bar represents the mean ± S.E.M.
*p<0.05, **p<0.01, compared with HCD-Control (t-test).

![Image of histological sections](image-url)

**Fig. 3** Typical appearances of the cross-sections of the carotid arteries after balloon injury in high cholesterol diet (HCD)-fed rats.
X: Non-denuded, N: Normal diet-control, C: HCD-Control, A: HCD-Atorvastatin 15 mg/kg, K: HCD-Keishibukuryogan 1600 mg/kg, L: HCD-Losartan 20 mg/kg.
Original magnification: × 100, bar: 0.2 mm.
Discussion

In the present study, the effects of ATS and KBG on the intimal thickening of rat carotid arteries induced by balloon injury were investigated under elevated and normal blood cholesterol levels. In ND-fed animals with normal cholesterol levels, ATS did not have any effects on the serum cholesterol level and balloon-induced intimal thickening. KBG and losartan also did not affect the serum cholesterol level, but both drugs significantly reduced intimal thickening.

In the HCD-fed animals with high cholesterol levels, on the other hand, ATS slightly lowered the serum cholesterol level and significantly reduced intimal thickening, compared with the values of the corresponding control group. KBG and losartan, similar to the results in ND-fed animals, both significantly reduced intimal thickening caused by balloon denudation without affecting the serum cholesterol levels.

Blood cholesterol is thought to be a promoting factor in the intimal thickening caused by balloon injury.8,10 The present results also showed that intimal thickening tended to be accelerated in HCD-fed animals, in which the blood cholesterol levels were significantly higher than those in ND-fed animals. Therefore, ATS presumably reduces intimal thickening by lower-
ing the blood cholesterol level in part under hypercholesterolemic condition but does not reduce intimal thickening under normocholesterolemic condition, where ATS has no effect on the blood cholesterol level. However, only a small difference in the degree of intimal thickening was noted between the ND- and HCD-fed control groups, in contrast with the marked differences in their serum cholesterol levels. In addition, in HCD-fed animals, ATS almost completely counteracted the acceleration of intimal thickening, despite having only a slight inhibitory effect on the serum cholesterol level. These findings suggest that cholesterol is only partly involved in the promotion of intimal thickening after balloon injury, and that other important factors are also involved. In recent years, the effects of statins on the clinical prevention or amelioration of atherosclerosis have been attributed to other actions independent of their blood cholesterol lowering effect (i.e., pleiotropic effects). Therefore, ATS might also inhibit intimal thickening through such pleiotropic effects.

KBG and losartan prevented intimal thickening without affecting the blood cholesterol level not only in ND-fed animals, but also in HCD-fed animals. In the balloon injury model, angiotensin II is known to promote the proliferation of vascular smooth muscle cells; in contrast, ACE inhibitors or angiotensin II receptor antagonists inhibit balloon injury-induced intimal thickening. The results for losartan in the present study agreed with those of previous reports. As for KBG, Sekiya et al. reported that KBG inhibited the formation of atheromatous plaques in the thoracic aorta in HCD-fed rabbits without affecting the blood cholesterol level. They suggested that the improvement of endothelial dysfunction or the suppression of oxidative stress was involved in the mechanisms of KBG’s action. Oxidative stress is considered to play an important role in the intimal thickening induced by balloon injury, since the MDA level, an index of lipid peroxidation or oxidative stress, increases in vascular tissue following balloon denudation. Therefore, we examined whether KBG inhibited balloon-induced intimal thickening by suppressing oxidative stress. The MDA level was elevated in the vascular tissue after balloon injury, confirming the involvement of oxidative stress as mentioned above. However, the administration of KBG did not affect this increase in the MDA level. The reason why KBG did not show an antioxidative effect in the present study, in contrast to previous reports, remains unclear. Differences in animal species, the methods used to produce the experimental models, areas of MDA measurement and so on are all probable explanations. An increase in reactive oxygen species (ROS) levels through the activation of NAD(P)H oxidase reportedly occurs in the carotid artery after balloon injury. In addition, ATS has been suggested to prevent balloon-induced intimal thickening in ovariectomized rats by inhibiting NADPH oxidase-dependent ROS production. These results indicate that detailed studies are needed in the future to elucidate the effects of KBG on oxidative stress using indexes other than MDA.

As described above, statins such as ATS are clinically used to prevent atherosclerosis by lowering blood cholesterol levels. However, if high doses of statins are required to lower blood cholesterol levels sufficiently, the risk of serious adverse effects, such as rhabdomyolysis, increases. The results of the present study suggest that KBG may be useful for preventing atherosclerosis regardless of the blood cholesterol level. Therefore, KBG may be clinically effective against atherosclerosis not only when used alone, but also when used in combination with statins. Consequently, KBG might be useful for reducing the required dose of statins, thereby lowering the risk of adverse effects from such medicines.

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