The Effects of Chronic Treatment with *Morus bombycis* KOIDZUMI in Spontaneously Hypertensive Rats

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The present study was performed to evaluate the antihypertensive effects of *Morus bombycis* KOIDZUMI (MK) in spontaneously hypertensive rats (SHRs). In addition, the effects on vascular responses and cardiac functions were also investigated. In isolated rat aortic preparations, the 100% ethanol extract of MK exhibited a potent vascular relaxant effect with IC₅₀ value of 3.9 μg/ml, and this vasorelaxant effect was completely abolished by pretreatment of the aortic tissues with N⁵-nitro-L-arginine methyl ester or the denudation of endothelial layer. In isolated rat hearts, the MK extract significantly reduced cardiac functions such as left ventricular developed pressure and heart rate. In an antihypertensive study in SHRs, long-term administration with MK extracts (10, 30, 100 mg/kg) for 42 d dose-dependently decreased systolic blood pressure (approximately 20 mmHg). In SHRs, MK extract enhanced the aortic relaxation to acetylcholine and sodium nitroprusside after 42 d of treatment. In addition, lipid peroxidation and DNA damage in liver of SHRs were also attenuated by long-term treatment with MK extract. These results suggest that chronic treatment with MK extract exerts an antihypertensive effect in SHRs, and its direct vasorelaxant, negative inotropic actions, and anti-oxidant properties may contribute to reduce the elevated blood pressure.

Key words  *Morus bombycis*; antihypertension; vasorelaxation; spontaneously hypertensive rat; chronic treatment

Hypertension is considered as a major risk factor for development and progression of cerebro- and cardiovascular disorders. The pathogenesis of hypertension is accompanied with the increased oxygen free radical production, the decreased nitric oxide bioavailability in the vasculature and reactive oxygen species-mediated cardiovascular remodeling. Hypertensive patients show increased levels of plasma superoxide, hydrogen peroxide and lipid peroxide. In various rodent models of experimental hypertension, including spontaneously hypertensive rats (SHRs) and renal hypertensive rats, enhanced endothelial superoxide anion production leading to impairment of endothelium-dependent relaxation has been demonstrated.

The phytomedicine have been extensively used to prevent and heal of various cardiovascular problems for over a millennium in oriental countries. Over the last decade, interest about the effects of MK extract on pharmacological actions in cardiovascular disease associated with hypertension, particularly, on animal models of systemic hypertension. In the present study, we evaluated the antihypertensive effects of long-term administration with MK extract in SHRs. In addition, we also examined the effects on the vascular responses of isolated rat aorta and the cardiac functions of isolated rat heart.

**MATERIALS AND METHODS**

**Animals** All the experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health. Sprague-Dawley (S.D.) rats (Orient Company, Seoul, Korea) of weighing 380–420 g, were used for vascular response and cardiac function study. For *in vivo* antihypertensive study, male spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats were purchased from the Charles River Laboratories (Wilmington, MA, U.S.A.) and these rats housed four to a cage with free access to standard rat chow and water. Twelve weeks old SHRs and WKY rats were used for the present study.

**Plant Material and Preparation of Crude Extracts** The root bark of MK was collected from plants growing wild in the district of Kangwon in Korea. The samples were washed thoroughly in tap water, shade-dried, and powdered. Samples of 100 g each were extracted with 21 water, 80% ethanol, 100% ethanol, 80% methanol or 100% methanol for 3 d each time. The extracts were filtered with Whatman No. 2 filter paper, and the supernatant was lyophilized to produce a powder, which was then kept at 4°C.

**Measurement of Vasorelaxation in Isolated Rat Aorta** Thoracic aorta isolated from S.D. rats was cut into rings of 2—3 mm width with extreme care to preserve endothelium intact as previously reported. The aortic preparations were suspended between wire hooks in an organ bath containing 20 ml of Krebs’ bicarbonate buffer (mm: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; NaHCO₃, 25; MgSO₄, 1.2; KH₂PO₄, 1.2; and glucose, 11.0) bubbled with mixture gas (95% O₂, 5% CO₂) and maintained at 37°C. The aortic preparations were al...
lium-intact aortic preparations were pretreated with a force displacement transducer (Grass FT03, Grass Ins., Quincy, MA, U.S.A.) and displayed on a chart recorder (Multitorder MC 6625, Hugo Sachs Electronic, March, Germany). The aortic preparations were precontracted submaximally with phenylephrine (3 × 10⁻⁶ M). After the contraction was stabilized, acetylcholine (1 × 10⁻⁶ M) was added to confirm the presence of the endothelium. Then, the aortic preparations were washed out 3 times for 45 min, and rechallenged with phenylephrine. MK extracts with water, 80% ethanol, 100% ethanol, 80% methanol or 100% methanol were cumulatively added to the tissue bath after phenylephrine response reached the plateau. In separate experiments, the endothelium-intact arteries from SHRs were analyzed in arteries contracted by phenylephrine (3 × 10⁻⁷ M) and finally a cumulative concentration–response to acetylcholine or sodium nitroprusside was obtained.

Measurement of Blood Ions and Hepatic Malondialdehyde Levels At the end of 42 d experimental period, the blood sample was taken from the inferior vena cava of SHRs for determination of the blood ions (Rapidpoint 400, Bayer AG, Germany). The liver from SHRs were rapidly isolated and frozen in liquid nitrogen for the determination of hepatic malondialdehyde as an index of lipid peroxidation and DNA oxidative damage. The frozen liver was transferred into 1 : 2 w : v in Tris buffer (20 mM, pH 7.2) and homogenized using an Ultra-Turrax (Ika® Works, Model T25 Basic, Japan). After homogenates were centrifuged at 4000 × g for 10 min at 4 °C, the supernatant was taken for the assays of malondialdehyde contents. The malondialdehyde level was determined using a commercial immunoassay kit (Bioxytech MDA-586, Oxis-Research, Portland, Oregon, U.S.A.) according to the manufacturer. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard.

Single Cell Gel Electrophoresis Assay (Comet Assay) Analysis Comet assays were performed by using a modification of the method of Rojas et al. Briefly, liver tissue slices, which were cut in 20-μm-thick serial section with cryomicrotome (AS620, Life Science international LTD, Astomoor Cheshire, England), were incubated with collagenase A (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) for 30 min at 37 °C to separate the hepatocytes from the liver tissue before analysis. The isolated hepatocytes were embedded in a bed of 0.5% low-melting-point agarose with a cell density of about 200—300 cells per sample. Aliquots were placed on microscope slides that had been previously coated with 1% normal-melting point agarose. After the gel slides immersed for 3 h in ice-chilled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10.0, 10% DMSO, 1% Triton X-100), electrophoresis was conducted for 5 min at 30 V, 250 mA. The slides were washed with 1 M ammonium acetate in 70% ethanol and 1 mg/ml spermine in 70% ethanol before staining with SYBR Green (Sigma-Aldrich Co., St. Louis, MO, U.S.A.), a DNA intercalating fluorescent dye. Fluorescent stained nucleotides were obtained using the imaging system of Zeiss, Germany. Fluorescent stained nucleotides were quantified using image analysis CAPS program downloaded from http://www.casp.of.pl. The data shown represent the image analysis from three independent experiments.

Statistical Analysis All values are expressed as the mean±S.E.M. Significant difference between responses were analyzed by one-way analysis of variance (ANOVA) followed
by Dunnett’s test for multiple comparisons (Sigma Stat, Jandel, San Rafael, CA, U.S.A.). In all comparisons, the difference was considered to be statistically significant at \( p<0.05 \).

RESULTS

Vasorelaxant Effects on Isolated Rat Aortas  
To evaluate the peripheral vasorelaxant activities of MK extract, their effects on phenylephrine-induced aortic constriction were measured. The all kinds of extract of MK, except water extract of MK, produced concentration-dependent relaxations of the endothelium-intact aortic preparations (Fig. 1A). The 100% ethanol extract of MK (IC\(_{50}\) value: 3.9±0.68 \(\mu\)g/ml) exhibited more potent vascular relaxant effect than the MK extracts with 80% ethanol, 80% methanol and 100% methanol (IC\(_{50}\) values: 5.0±1.02, 6.3±0.73 or 8.4±1.03 \(\mu\)g/ml, respectively). The relaxant effect of 100% ethanol extract of MK in aortic preparations was completely abolished by denudation of the endothelial layer (Fig. 1B). Furthermore, pretreatment of aortic tissue with L-NAME (10\(^{-3}\) m) completely inhibited the MK extract-induced relaxation.

Effects on Cardiac Function in Isolated Rat Hearts

To measure the direct effects on the hearts, the cardiac functions of MK extract in hearts isolated from rats were measured. For the present study, the 100% EtOH extract of MK was used, because the vasorelaxant effect of MK extract was most potent on rat aortic preparations. The MK extract caused a concentration-dependent decrease in LVEDP, HR, and CFR (\( p<0.05 \) at 10, 30 \(\mu\)g/ml; Table 1), while LVEDP was significantly increased with the 100% EtOH extract of MK (\( p<0.05 \) at 10, 30 \(\mu\)g/ml).

Antihypertensive Effects in SHRs Chronically Treated with MK Extract

The effects of repeated oral administration with 100% EtOH extract of MK (10, 30, 100 mg/kg) for 42 consecutive days on SBP and HR in SHRs are shown in Fig. 2. The basal values of SBP and HR in WKY rats were 142±3.6 mmHg and 352±8.7 beats/min (\( n=12 \)), respectively. In contrast, the predose values of SBP and HR in SHRs were 192±2.3 mmHg and 418±4.6 beats/min (\( n=38 \)), respectively. In vehicle-treated SHRs, SBP was slowly increased with time-dependent manner (approximately 20 mmHg; Fig. 2A). The MK extract (30, 100 mg/kg) dose-dependently decreased SBP (approximately 20 mmHg; Fig. 2A). The MK extract (30, 100 mg/kg) dose-dependently decreased SBP (approximately 20 mmHg) decrease compared with vehicle-treated group, although the difference was not statistically significant. In all groups, HR was not different compared with vehicle-treated group (Fig. 2B). In addition, the gains of body weights were not different between groups (unpublished data).

Vasorelaxant Effects on Aortas from SHRs Chronically Treated with MK Extract

The effects of acetylcholine and sodium nitroprusside on the endothelium-intact aortic preparations from all groups of animals. Acetylcholine-induced relaxant response in aorta from vehicle-treated SHRs (\( E_{\text{max}} \): 43.3±3.4\% ) was significantly decreased compared with that of WKY rats (\( E_{\text{max}} \): 77.7±3.0\% ; Fig. 3A). This diminished relaxation in

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**Table 1. Effects of the Extract of *Morus bombycis* KOIDZUMI on Cardiac Function Parameters in Isolated Rat Hearts**

<table>
<thead>
<tr>
<th></th>
<th>Basal values</th>
<th>1 (\mu)g/ml</th>
<th>3 (\mu)g/ml</th>
<th>10 (\mu)g/ml</th>
<th>30 (\mu)g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left ventricular developed pressure (mmHg)</td>
<td>116±9.5</td>
<td>116±7.1</td>
<td>114±3.6</td>
<td>30±8.7*</td>
<td>6±0.6*</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>283±10.9</td>
<td>278±13.9</td>
<td>276±14.1</td>
<td>251±4.4</td>
<td>207±12.1*</td>
</tr>
<tr>
<td>Left ventricular end-diastolic pressure (mmHg)</td>
<td>11.0±2.96</td>
<td>6.8±1.8</td>
<td>5.0±1.9</td>
<td>27.0±6.5*</td>
<td>40.3±3.1*</td>
</tr>
<tr>
<td>Coronary flow rate (ml/min)</td>
<td>20.3±1.0</td>
<td>24.5±0.9</td>
<td>20.5±0.2</td>
<td>13.0±2.2*</td>
<td>4.5±0.9*</td>
</tr>
</tbody>
</table>

Values are expressed as the mean±S.E.M. (\( n=4-6 \)). *\( p<0.05 \), significantly different from basal values.
response to acetylcholine ($E_{\text{max}}$: 55.0±4.0%) was improved by treatment with MK extract, although it was not significant. Moreover, the decrease of vasorelaxation induced by sodium nitroprusside in SHRs ($E_{\text{max}}$: 77.1±2.4%) as compared with that of WKY rats ($E_{\text{max}}$: 97.1±0.9%) was significantly recovered by treatment with MK extract ($E_{\text{max}}$: 95.8±3.0%; Fig. 3B).

**Effects on Oxidative Status in SHRs Chronically Treated with MK Extract**

The changes of blood ions from SHRs which were daily treated with 100% EtOH extract of MK (10, 30, 100 mg/kg) for 42 consecutive days were measured (Table 2). The concentration of Na$^+$ ions in blood was slightly increased in vehicle-treated SHRs compared with WKY rats, while the concentrations of K$^+$ and Ca$^{2+}$ ions were tended to be decreased, as previous report. 15) MK extract has shown to recover the changes of blood ions of SHRs although it was not significant. Hepatic malondialdehyde was significantly increased in vehicle-treated SHRs (48.4±3.2 nM/g) compared with that of WKY rats (29.2±1.1 nM/g; Fig. 4A). This increase in liver lipid peroxidation was significantly reduced by MK extract (34.2±2.9, 30.4±3.1, 35.8±2.8 nM/g at 10, 30, 100 mg/kg, respectively, $p<0.05$). DNA damage in liver cells as expressed the comet tail moment was also significantly increased in vehicle-treated SHRs (48.0±2.7%) compared with that of WKY rats (22.8±1.6%; Fig. 4B). This increase in oxidative base damage to liver DNA was significantly reduced by MK extract (38.6±2.4, 32.3±1.1% at 10, 30 mg/kg, respectively, $p<0.05$).

**DISCUSSION**

We demonstrated that chronic treatment with MK extract for 42 d exerts the antihypertensive effects in SHRs. In addition, we showed that MK extract causes the vasorelaxant effects in isolated rat aortic preparations and the negative in-

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**Fig. 2.** Effects of Long-Term Administration with the Extract of *Morus bombycis Koidzumi* (MK; p.o., Once a Day for 42 d) on Systolic Blood Pressure (SBP; A) and Heart Rate (HR; B) in Spontaneously Hypertensive Rats

Results were expressed as changes from baseline SBP and HR measured on day 1 before treatment of MK extract. Vehicle (open circles); captopril 30 mg/kg (closed circles); *Morus bombycis Koidzumi*, 10 (open triangles), 30 (closed triangles) and 100 mg/kg (open squares); Wistar-Kyoto rats (closed squares). Values are expressed as the mean±S.E.M (n=8). *p<0.05, significantly different from the vehicle-treated group.

**Fig. 3.** Acetylcholine- (A) and Sodium Nitroprusside (B)-Induced Relaxation in Thoracic Aorta from Spontaneously Hypertensive Rats with Long-Term Administration of the Extract of *Morus bombycis Koidzumi* (p.o., Once a Day for 42 d)

Vehicle (open circles), captopril 30 mg/kg (closed triangles), *Morus bombycis Koidzumi* 100 mg/kg (open inverted triangles), Wistar-Kyoto rats (closed squares). Values are expressed as the mean±S.E.M (n=8). *p<0.05, significantly different from the vehicle-treated group.

**Table 2.** Effects of Long-Term Administration with the Extract of *Morus bombycis Koidzumi* (p.o., Once a Day for 42 d) on Ions in Blood from Spontaneously Hypertensive Rats

<table>
<thead>
<tr>
<th>Ion conc. in blood</th>
<th>Vehicle (—)</th>
<th><em>Morus bombycis Koidzumi</em> (mg/kg)</th>
<th>Captopril (mg/kg)</th>
<th>WKY (—)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>30</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>Na$^+$ (mmol/l)</td>
<td>147.0±1.1</td>
<td>145.7±0.2</td>
<td>145.1±0.1</td>
<td>145.9±0.8</td>
</tr>
<tr>
<td>K$^+$ (mmol/l)</td>
<td>3.69±0.03</td>
<td>3.60±0.04</td>
<td>3.60±0.06</td>
<td>3.84±0.04</td>
</tr>
<tr>
<td>Ca$^{2+}$ (mmol/l)</td>
<td>1.25±0.01</td>
<td>1.23±0.01</td>
<td>1.25±0.01</td>
<td>1.27±0.01</td>
</tr>
<tr>
<td>Cl$^-$ (mmol/l)</td>
<td>103.4±0.3</td>
<td>104.3±0.3</td>
<td>102.9±0.1</td>
<td>103.4±0.2</td>
</tr>
</tbody>
</table>

Values are expressed as the mean±S.E.M (n=8). WKY, Wistar-Kyoto rats.
ototropic effects in isolated rat hearts. The 100% EtOH extract among several MK extracts exhibited the most potent vasorelaxant effect on phenylephrine-induced contraction of aortic preparations (IC_{50} value; 3.9 µg/ml). This MK extract-induced relaxation in rat aortic preparations was completely inhibited by removing of functional endothelium or pretreatment of N^{G}-nitro-L-arginine methyl ester, a well known non-selective nitric oxide synthase inhibitor. These results suggest that the vasorelaxation caused by MK extract may be mediated endothelium-dependent nitric oxide signaling pathway.

In isolated rat hearts, the 100% EtOH extract of MK caused significant decrease in LVDP, HR and CFR, and increase in LVEDP, as an indicator of cardiac contraction, with concentration-dependent manner (p<0.05 at 10, 30 µg/ml). These results suggest that MK extract possesses negative inotropic and chronotropic effects in hearts isolated from rats. These phenomena have generally been shown in hearts treated with antihypertensive agents, such as calcium channel blockers and angiotensin converting enzyme inhibitors. It seems that the direct vasorelaxant and negative inotropic effects of MK extract may contribute to alleviate the development of hypertension.

In SHR study, repeated oral administration with 100% EtOH extract of MK (10, 30, 100 mg/kg) for 42 consecutive days dose-dependently decreased SBP compared with that of vehicle-treated SHRs. SBP in SHRs treated with MK extract (30, 100 mg/kg) was decreased by approximately 20 mmHg (ca. 10%) compared with that of vehicle-treated SHRs. Another objective of our study was to investigate whether long-term treatment with MK extract improves the endothelial function of conductance arteries. It is known that structural and functional alterations of the vascular endothelium occur in many pathologies, including arterial hypertension. These alterations are important in the mechanisms that determine blood pressure, the regression of which is generally regarded as an important target of antihypertensive therapy. We observed that the relaxant activity due to acetylcholine in rat aorta was significantly decreased in vehicle-treated SHRs than in WKY rats. It has been speculated that impaired endothelium-dependent relaxation induced experimentally by acetylcholine in aorta from SHRs may result from decreased nitric oxide synthesis, and perhaps from oxygen free radicals. In the present study, the diminished relaxation in response to acetylcholine in SHRs was improved by long-term treatment with MK extract. Moreover, it is worth mentioning that unlike other antihypertensive substances, long-term treatment with MK extract was able to improve the sensitivity to sodium nitroprusside in aortic preparations of SHRs. These results suggest that the chronic administration with MK extract may restore the impaired endothelial-dependent and -independent vascular dilation on aortic preparations from SHRs. To investigate that the anti-hypertensive effects of MK extract may be associated with a reduced oxidant status due to its anti-oxidant properties, lipid peroxidation (hepatic malondialdehyde) and oxidative base damage to liver DNA (% tail DNA) in SHRs was measured. Because the liver plays major roles in the metabolism and elimination of reactive oxygen species as well as in producing the anti oxidant enzymes protecting tissues from free radicals, the hepatic MDA and DNA damage value reflect not only the liver oxidative status but also probably more general indices of the oxidative status and lipid peroxidation. The long-term treatment with MK extract significantly reduced hepatic MDA and DNA damage value in SHRs.

In conclusion, the results from the present study suggest that long-term administration of Morus bombycis Koizumi (MK) extract causes the antihypertensive effects in SHRs, and its direct vasorelaxant effects, negative inotropic actions, and anti-oxidant properties may contribute to reduce the elevated blood pressure. In addition, the vasorelaxant effects of MK extract might be mediated through the increase in release of nitric oxide from endothelial cells. However, further studies are necessary to clearly elucidate the constituents responsible for these responses and the underlying mechanisms.

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Fig. 4. Effects of Long-Term Administration with the Extract of Morus bombycis Koizumi (p.o., Once a Day for 42 d) on Malondialdehyde Contents (A, n=8) and DNA Damage (B, n=3—4) in Liver from Spontaneously Hypertensive Rats

DNA damage was measured as percentage of tail DNA. Veh, Vehicle; Capto, Captopril; MK, extract of Morus bombycis Koizumi; WKY, Wistar-Kyoto rats. * p<0.05, significantly different from the vehicle-treated group.
724 (2003).