Long-Term Treatment With San’o-Shashin-To, a Kampo Medicine, Markedly Ameliorates Cardiac Ischemia-Reperfusion Injury in Ovariectomized Rats via the Redox-Dependent Mechanism

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Background: Hormone replacement therapy has failed to reduce ischemic cardiovascular events in climacteric women. To explore alternative therapy, we examined whether san’o-shashin-to (TJ-113), a kampo medicine, ameliorates cardiac ischemia-reperfusion (IR) injury in a climacteric rat model.

Methods and Results: Cardiac function and infarct size after IR were significantly exacerbated in ovariectomized rats as compared with sham-operated rats, whereas long-term treatment with a clinical dosage of TJ-113 for 4 weeks markedly improved these functional and morphological changes. Myocardial inducible nitric oxide synthase (iNOS) expression and peroxynitrite levels were significantly higher in ovariectomized rats compared with sham-operated rats, and long-term TJ-113 treatment significantly reduced these oxidative changes. Furthermore, myocardial manganese superoxide dismutase (Mn-SOD) activity was significantly lower in ovariectomized than in sham-operated rats, and long-term TJ-113 treatment significantly restored antioxidant activity. Importantly, those beneficial actions of TJ-113 were significantly inhibited by the estrogen receptor antagonist, fulvestrant, and the phytoestrogen, emodin, a TJ-113 ingredient, mimicked the actions of TJ-113, suggesting involvement of emodin in the effects of TJ-113.

Conclusions: These results provide the first evidence that long-term treatment with a clinical dosage of TJ-113 markedly ameliorates cardiac IR injury in ovariectomized rats via inhibition of iNOS expression, suppression of peroxynitrite formation, and restoration of Mn-SOD activity. TJ-113 may be a novel therapeutic option in the treatment of ischemic heart disease in climacteric women. (Circ J 2013; 77: 1827–1837)

Key Words: Ischemia-reperfusion injury; Kampo medicine; Ovariectomy; Oxidative stress; Rats

Ischemic heart disease (IHD) is the most common cause of death in many countries around the world. The prevalence of IHD is lower in pre-climacteric women than in men of similar age, but significantly increases in climacteric women because of the loss of the ovarian hormone, estrogen, which possesses a variety of cardiovascular protective actions, including mitigation of myocardial ischemia-reperfusion (IR) injury. Approximately 20–30% of climacteric women are diagnosed with a climacteric disorder, which manifests autonomic imbalance-like symptoms and/or psychiatric symptoms. Climacteric disorder is treated by hormone replacement therapy (HRT) with estrogen or with estrogen plus progesterone.
Observational studies have found a lower rate of IHD in climacteric women who take estrogen than in women who do not. Based on these results, the effect of HRT on the prevalence of HRT in climacteric women has been examined, but large randomized trials have revealed that, unfortunately, HRT does not reduce, but rather increases the rate of IHD events in climacteric women. Furthermore, it has also been reported that HRT possibly increases the risks of breast and uterine cancers, stroke, venous thromboembolism, and dementia in climacteric women. In consideration of these circumstances, the development of a safer and more effective alternative therapy for IHD in climacteric women is eagerly expected.

Kampo medicines are traditional Japanese pharmaceutical agents made from several distinct herbal preparations in fixed combinations, and are widely used not only in Japan but also in much of the world. San’o-shashin-to (code name: TJ-113) is a kampo medicine composed of 3 medicinal herbs (Scutellariae radix, Coptidis rhizoma and Rhei rhizoma; volume ratio = 1:1:1) and is used to treat climacteric disorder. To explore alternative therapy for IHD in climacteric women, in the present study we tested our hypothesis that long-term treatment with TJ-113 would ameliorate cardiac IR injury in a climacteric rat model.

**Methods**

**Animals**

This study was approved by the Ethics Committee of Animal Care and Experimentation, University of the Ryukyus, Japan, and was carried out according to the Institutional Guidelines for Animal Experimentation and to the Law (No. 105) and Notification (No. 6) of the Japanese Government. The following 6 groups were studied: sham-operated female rats treated with tap water (vehicle); sham-operated female rats treated with TJ-113; ovariectomized female rats treated with tap water; ovariectomized female rats treated with TJ-113; ovariectomized female rats treated with TJ-113 plus fulvestrant; and ovariectomized female rats treated with emodin. In a separate experiment, male rats were used. Sham-operations and bilateral ovariectomies were performed in 9-week-old female Wister rats (Kyudo, Kumamoto, Japan) under general anesthesia with an intraperitoneal injection of 50 mg/kg sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA). Administration of TJ-113 (150 mg/day, Tsumura, Tokyo, Japan), fulvestrant (10 mg/kg), AdooQ BioScience, Irvine, CA, USA), emodin (1.7 mg/day, AdooQ BioScience), or tap water was started 10 days after the surgery, and continued for 4 weeks. TJ-113 was dissolved in warm water, and cooled to room temperature. Emodin was dissolved in 10% dimethyl sulfoxide (DMSO). Both these agents were given orally with a feeding needle to the rats once daily. Fulvestrant was dissolved in 10% DMSO, and given subcutaneously to the rats once weekly. When the effect of emodin or fulvestrant was examined, 10% DMSO was administered in the other groups in the same manner. The clinical dosage of TJ-113 was calculated by the authorized translation formula of drug doses from animal to human studies on the basis of the human adult’s clinical dosage (7.5 g/day).11

**Perfused Heart Experiment**

The rats were anesthetized with sodium pentobarbital (50 mg/kg, IP). Blood sampling was performed after intravenous injection of 1,000 IU/kg sodium heparin (Ajinomoto Pharmaceuticals Co, Ltd, Tokyo, Japan). The heart was then rapidly excised, cannulated retrogradely via the aorta, and set on a Langendorff apparatus, as reported previously. Perfusion of the isolated heart was carried out at a constant pressure of 100 cm H2O with 37°C Krebs-Henseleit solution of the following composition (mmol/L): NaCl 120, KCl 4.80, CaCl2 1.25, MgSO4 1.20, KH2PO4 1.20, NaHCO3 25.0 and glucose 11.0. The perfusate was oxygenated with 95% O2 and 5% CO2.

A saline-filled latex balloon was inserted into the left ventricle (LV), and the pressure was measured with a pressure transducer (TP-400T, Nihon Kohden, Tokyo, Japan). The latex balloon was loaded with 10 mmHg of initial LV end-diastolic pressure (LVEDP), and the balloon volume was kept constant throughout the experiments. The first derivative of LV pressure (dP/dt) was derived from differentiating the signal of the LV pressure electronically (ED-601G, Nihon Kohden). The LV developed pressure was obtained by subtracting LVEDP from the aortic pressure. Mean coronary perfusion flow was measured with a flow probe (FF-030T, Nihon Kohden) attached to the aortic cannula, which was connected to an electromagnetic flow meter (MFV-3200, Nihon Kohden).

After equilibration for 40 min, atrial pacing at 110% of own heart rate was started and maintained throughout the experiments. Global ischemia of the heart was induced by cessation of coronary perfusion for 30 min, and then the heart was reperfused with oxygenated Krebs-Henseleit solution for 60 min. Coronary effluent was collected every 30 min after reperfusion, and stored at 4°C until measurement. At the end of the experiments, some heart samples were quickly frozen by liquid nitrogen. The coronary effluent was centrifuged to remove blood cells, and the supernatant was used for measurement of the catecholamines.

### Table. Effects of Long-Term Treatment With Vehicle or TJ-113 on Baseline Values of In Vivo Hemodynamic Parameters in Sham or OVX

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sham/vehicle</th>
<th>Sham/TJ-113</th>
<th>OVX/vehicle</th>
<th>OVX/TJ-113</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV developed pressure (mmHg)</td>
<td>116±8</td>
<td>107±10</td>
<td>120±6</td>
<td>114±1</td>
</tr>
<tr>
<td>dP/dtmax (mmHg/s)</td>
<td>8,500±1,600</td>
<td>7,200±1,300</td>
<td>7,900±500</td>
<td>7,800±800</td>
</tr>
<tr>
<td>dP/dtmin (–mmHg/s)</td>
<td>8,200±1,000</td>
<td>8,000±1,100</td>
<td>8,300±1,100</td>
<td>8,600±400</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>3±1</td>
<td>2±1</td>
<td>3±1</td>
<td>2±1</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>415±9</td>
<td>386±30</td>
<td>410±13</td>
<td>410±16</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>118±15</td>
<td>105±11</td>
<td>102±18</td>
<td>116±13</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>73±14</td>
<td>70±14</td>
<td>76±13</td>
<td>70±7</td>
</tr>
</tbody>
</table>

There were no significant differences in the baseline values of hemodynamic parameters among the 4 groups (n=7 for each group).

LV, left ventriclular; LVEDP, LV end-diastolic pressure; OVX, ovariectomized rats; Sham, sham-operated rats; TJ-113, san’o-shashin-to.
pressure control unit (FP891B, Scisense) and a pressure transducer (TP-400T, Nihon Kohden).

Myocardial Infarct Size
After the IR experiment, the heart was freshly isolated and cut into 2-mm slices using a heart slicer (RATH-A1-C, Muromachi Kikai, Tokyo, Japan). The heart slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37°C for

In Vivo LV Functional Analysis
The rats were anesthetized with sodium pentobarbital (60mg/kg, IP) and a 1.6Fr pressure catheter (FTS-1611B-0018, Scisense, Ontario, Canada) was inserted into the right carotid artery and advanced into the LV. The baseline values of LV pressure and systemic blood pressure were respectively measured with a nitrogen and stored at –80°C until measurement.

Figure 1. Effects of long-term treatment with vehicle or TJ-113 on cardiac performance in response to ischemia-reperfusion in sham-operated and ovariectomized female rats (n=7 for each group). (A) Representative tracing of left ventricular (LV) developed pressure and LVdP/dt. (B) LV developed pressure. *P<0.05 between sham-operated rats treated with vehicle and ovariectomized rats treated with vehicle, **P<0.01 between ovariectomized rats treated with vehicle and with TJ-113. (C) dP/dt%max. *P<0.05 between sham-operated rats treated with vehicle and ovariectomized rats treated with vehicle, **P<0.01 between ovariectomized rats treated with vehicle and with TJ-113. (D) dP/dtmin. **P<0.01 between sham-operated rats treated with vehicle and ovariectomized rats treated with vehicle, ***P<0.001 between ovariectomized rats treated with vehicle and with TJ-113. (E) Left ventricular end-diastolic pressure (LVEDP). *P<0.05 between sham-operated rats treated with vehicle and ovariectomized rats treated with vehicle. *P<0.05 between ovariectomized rats treated with vehicle and with TJ-113. (F) Coronary perfusion flow. Green triangle, sham-operated rats treated with vehicle; orange triangle, sham-operated rats treated with TJ-113; blue circle, ovariectomized rats treated with vehicle; red circle, ovariectomized rats treated with TJ-113.
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Griess method using an Eicom NOx analyzer (ENO-20, Eicom, Kyoto, Japan).

Western Blot Analysis
Western blot analysis was performed as reported previously.\textsuperscript{16} The membrane was immunoblotted with anti-endothelial nitric oxide synthase (eNOS; BD Transduction Laboratories, Franklin Lakes, NJ, USA), anti-neuronal NOS (nNOS, BD Transduction Laboratories), anti-inducible NOS (iNOS, BD Transduction Laboratories), anti-nitrotyrosine (Merck Millipore, Billerica, MA, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Sigma-Aldrich). After incubating with horseradish peroxidase-conjugated anti-mouse (BD Transduction Laboratories) or anti-rabbit IgG antibodies (Cell Signaling Technology, Danvers, MA, USA), blots were visualized by an enhanced chemiluminescence system (ECL Prime Western Blotting Detection Reagent, GE Healthcare, Little Chalfont, UK), scanned with a lumino image analyzer (LAS-4000 mini, Fuji Film), and analyzed with multi-gauge software (Fuji Film).

Myocardial Malondialdehyde (MDA) Level
The extent of oxidative stress in the myocardial tissues was evaluated by the thiobarbituric acid method with some modifications. Accumulation of MDA was determined by image analysis of stained heart slices with a digital camera (EF-S 18-55 IS, Canon, Tokyo, Japan) and isometric and total areas were assessed with Image J software (NIH, Bethesda, MD, USA). Myocardial infarct size (%) was expressed as the ratio of ischemic area to total area.

Cardiac Enzyme Activity
Lactate dehydrogenase (LDH) activity in the coronary effluent was assayed with a LDH Kainos kit (Kainos Laboratories, Tokyo, Japan). Creatine kinase-MB (CK-MB) activity in the coronary effluent was determined by a colorimetric method using a Fuji Dry-Chem autoanalyzer (Fuji Film, Tokyo, Japan).

Myocardial ATP Level
Some myocardial tissues were lyophilized and homogenized with 0.6 mol/L perchloric acid. The homogenate was centrifuged at 16,000 g and 2°C for 15 min. The ATP level in the supernatant was determined by the firefly luminescence method with an ATP monitoring agent (LL-100-2, Toyo Ink, Tokyo, Japan) and a lumiphotometer (Minilumat LB9506, Berthold, Calmabacher, Germany).

Nitrite Plus Nitrate (NOx) Level
The NOx level in the coronary effluent was measured by the Griess method using an Eicom NOx analyzer (ENO-20, Eicom, Kyoto, Japan).

Figure 2. Effects of long-term treatment with vehicle or TJ-113 on myocardial infarct size and energy metabolism after ischemia-reperfusion in sham-operated and ovariectomized female rats. (A) Lactate dehydrogenase (LDH) activity in coronary effluent collected after 30 min of reperfusion (n=6 for each group). (B) Creatine kinase-MB (CK-MB) activity in coronary effluent collected after 60 min of reperfusion (n=6 for each group). (C) Myocardial infarct size evaluated by 2,3,5-triphenyltetrazolium chloride (TTC) staining. White color indicates myocardial infarction. Infarct size was calculated by dividing the ischemic area by the total area (n=6 for each group). (D) Myocardial ATP levels after ischemia-reperfusion (n=7 for each group). Sham, sham-operated rats; OVX, ovariectomized rats. *P<0.05, **P<0.01, ***P<0.001.
San’o-Shashin-To Ameliorates IR Injury

Results

Effects of Long-Term Treatment With TJ-113 on Cardiac Dysfunction Induced by IR in Ovariectomized Rats

We first examined the effects of long-term oral treatment with a clinical dosage of TJ-113 for 4 weeks on IR-induced cardiac dysfunction in ovariectomized rats. Before the Langendorff heart experiment, in the direct LV pressure measurements, there were no significant differences in the baseline values of in vivo hemodynamic parameters, including LV developed pressure (an index of general LV function), dP/dtmax (an index of systolic LV function), dP/dtmin (an index of diastolic LV function), or LVEDP (an index of general LV function), blood pressure, or heart rate among the 4 groups studied (sham-operated or ovariectomized rats that received long-term treatment with vehicle or TJ-113) (Table). In the Langendorff heart experiment, before exposure to myocardial ischemia, there were no significant differences in the baseline hemodynamic parameters, including LV developed pressure, dP/dtmax, dP/dtmin, or LVEDP among the 4 groups (data not shown). However, after exposure to myocardial ischemia for 30 min, in the ovariectomized rats as compared with the sham-operated rats, LV developed pressure, dP/dtmax, and dP/dtmin were significantly lower, and LVEDP was significantly higher, indicating exacerbation of cardiac dysfunction induced by IR in the ovariec-
Figure 4. Effects of long-term treatment with vehicle or TJ-113 on redox state after ischemia-reperfusion in sham-operated and ovariectomized female rats. (A) Myocardial superoxide production (n=7 for each group). (B) Western blotting for nitrotyrosine. (C) Myocardial nitrotyrosine levels of a 25-kDa protein (n=6 for each group). (D) Myocardial nitrotyrosine levels of a 65-kDa protein (n=6 for each group). (E) Myocardial nitrotyrosine levels of an 80-kDa protein (n=6 for each group). (F) Myocardial malondialdehyde (MDA) levels (n=7 for each group). (G) Myocardial manganese superoxide dismutase (Mn-SOD) activity (n=7 for each group). Sham, sham-operated rats; OVX, ovariectomized rats. *P<0.05, **P<0.01, ***P<0.001.
were upregulated in the heart of the ovariectomized rats. Myocardial iNOS protein expression, but not myocardial nNOS or eNOS protein expression, was significantly higher in the ovariectomized rats compared with the sham-operated rats (Figures 3B–D). Long-term treatment with TJ-113 significantly decreased the NOx levels in the coronary effluent and the myocardial iNOS expression in the ovariectomized rats (Figures 3A, D).

**Effects of Long-Term Treatment With TJ-113 on Myocardial Oxidative Stress in Ovariectomized Rats**

We next studied the mechanism(s) by which long-term treatment with TJ-113 improved the IR-induced cardiac dysfunction in the ovariectomized rats. NOx levels in the coronary effluent, a marker of myocardial NO production, were significantly and markedly augmented in the ovariectomized rats as compared with the sham-operated rats (Figure 3A). Based on this evidence, we further examined which NOS isoforms were upregulated in the heart of the ovariectomized rats. Myocardial iNOS protein expression, but not myocardial nNOS or eNOS protein expression, was significantly higher in the ovariectomized rats compared with the sham-operated rats (Figures 3B–D). Long-term treatment with TJ-113 significantly decreased the NOx levels in the coronary effluent and the myocardial iNOS expression in the ovariectomized rats (Figures 3A, D).
Effects of Long-Term Treatment With TJ-113 on Mn-SOD Activity in Ovariectomized Rats

Because Mn-SOD (a 24-kDa protein) was considered to be tyrosine-nitrated in the heart of the ovariectomized rats, we further studied myocardial Mn-SOD activity in the ovariectomized rats. No significant differences in myocardial Cu,Zn-SOD plus EC-SOD activities were seen among the 4 groups (167±19 unit/mg wet weight in sham-operated rats treated with vehicle, 216±31 in the sham-operated rats treated with TJ-113, 121±23 in the ovariectomized rats treated with vehicle, and 169±26 in the ovariectomized rats treated with TJ-113). In contrast, myocardial Mn-SOD activity was significantly lower in the ovariectomized than in the sham-operated rats (Figure 4G). Long-term treatment with TJ-113 significantly restored the myocardial Mn-SOD activity in the ovariectomized rats (Figure 4G).

Effects of Long-Term Treatment With TJ-113 on Plasma Estradiol Levels in Ovariectomized Rats

Ovariectomy resulted in a significant reduction in plasma estradiol levels (28.3±5.0 pg/mL in the sham-operated rats treated with vehicle vs. 11.5±0.8 in the ovariectomized rats treated with vehicle; n=7 for each group, P<0.05). Long-term treatment with TJ-113 had no effect on the plasma estradiol levels in the sham-operated or ovariectomized rats (29.0±2.2 or 12.8±0.7, respectively) (n=7 for each group).

Effects of Long-Term Treatment With TJ-113 on Cardiac Dysfunction Induced by IR in Male Rats

Long-term treatment with TJ-113 significantly improved car-
Dosage of TJ-113 Used

The clinical dosage of TJ-113 in humans must be appropriately translated to the animal dosage. Although a simple conversion based on body weight was used in the past, it has recently been shown that a body surface area-based dose calculation is the most appropriate method. 

Body surface area correlates well across several mammalian species with several parameters of biology, including oxygen utilization, caloric expenditure, basal metabolism, blood volume, and renal function. In the present study, by using the body surface area-based conversion method, we calculated the dosage in rats from the clinical dosage of TJ-113, and therefore used a clinical therapeutic dosage of TJ-113. Although the body surface area-based conversion method does not take into account species differences in drug metabolism, a previous study reported that drug metabolism in rats and humans is similar.

Langendorff Heart Model

There are at least 2 ways to study cardiac IR injury: a temporary coronary artery ligation model, and the Langendorff heart model. The temporary coronary artery ligation model can access regional cardiac ischemia and reperfusion in vivo, but cardiac performance is influenced by hemodynamic changes, baroreceptor reflex, and humoral factors. The Langendorff heart model, on the other hand, can evaluate global cardiac IR in vitro, and is useful for analyzing the effect of agents on the heart. In order to evaluate the cardiac effects of TJ-113, we used the Langendorff heart model in the present study.

Discussion

The major novel findings of the present study are that long-term oral treatment with the clinical dosage of TJ-113 markedly improved cardiac dysfunction and infarct size following IR in ovariectomized rats through its inhibition of iNOS expression, suppression of peroxynitrite formation, and restoration of Mn-SOD activity. These results provide the first evidence that long-term treatment with TJ-113 protects the heart against IR injury in a climacteric rat model.
Cardioprotective Effect of Long-Term Treatment With TJ-113 on IR Injury in Ovariectomized Rats

Ovariectomy reportedly reduces cardiac function and increases myocardial infarct size in response to IR and the present study confirmed those findings. Long-term treatment with TJ-113 amended IR-induced cardiac dysfunction in ovariectomized rats. In addition, long-term treatment with TJ-113 prevented the release of cardiac enzymes and enlargement of infarct, and increased the myocardial ATP levels, a marker of myocardial energy metabolism, in the ovariectomized rats. Because long-term treatment with TJ-113 had no effect on coronary perfusion flow in the ovariectomized rats, it is likely that the beneficial effects of TJ-113 were not caused by alterations in coronary flow. It is thus evident that long-term treatment with TJ-113 exerted a cardioprotective effect against cardiac dysfunction and infarct size after IR in ovariectomized rats.

Effect of Long-Term Treatment With TJ-113 on Myocardial iNOS Expression in Ovariectomized Rats

We next examined the mechanism(s) for the beneficial effects of TJ-113 on IR injury in the ovariectomized rats. We analyzed the kinetics of NO because it is a key determinant of cardiac pathophysiology. Ovariectomy resulted in increased NOx levels in the coronary effluent, and in selective upregulation of myocardial iNOS expression, both of which were blocked by long-term treatment with TJ-113. Although iNOS has dual roles in cardiac pathophysiology, myocardial iNOS expression in response to IR has been shown to exert a deleterious effect. Thus, it is possible that the suppression of myocardial iNOS expression was involved in the beneficial effects of TJ-113 on IR injury in the ovariectomized rats.

Effect of Long-Term Treatment With TJ-113 on Myocardial Oxidative Stress in Ovariectomized Rats

Peroxynitrite is a potent oxidant generated by the reaction of NO with superoxide. In the present study, ovariectomy led to increased myocardial levels of superoxide and nitrotyrosine (a marker of peroxynitrite formation), indicating the presence of myocardial oxidative stress in the ovariectomized rats. Consistent with our findings, it has been revealed that cardiomyocyte overexpression of iNOS in mice results in oxidative stress and peroxynitrite formation. Peroxynitrite generated at reperfusion has been indicated as inducing peroxidation of membrane lipids of cardiomyocytes and cardiac dysfunction in reperfused rabbit hearts. In agreement with that evidence, increased myocardial nitrotyrosine levels were associated with enhanced myocardial MDA levels, a marker of lipid peroxidation, and cardiac dysfunction in the present ovariectomized rats. Long-term treatment with TJ-113 normalized the increases in myocardial levels of superoxide, nitrotyrosine, and MDA in the ovariectomized rats. It is thus likely that attenuation of myocardial oxidative stress was involved in the beneficial effects of TJ-113 on IR injury in the ovariectomized rats.

NO is a potent vasodilator. Although NOx levels in the coronary effluent were increased in the ovariectomized rats after IR, coronary perfusion flow did not change. Because myocardial superoxide levels were concomitantly enhanced in the ovariectomized rats after IR, inactivation of NO by superoxide might result in the lack of change in coronary perfusion flow.

Effect of Long-Term Treatment With TJ-113 on Myocardial Mn-SOD Activity in Ovariectomized Rats

Peroxynitrite causes tyrosine nitration and malfunction of proteins. In the present study, the significant tyrosine nitration of a 25-kDa protein was noted in the ovariectomized rats after IR. Judging from its molecular weight, this protein was considered to be Mn-SOD. Mn-SOD is a major cardiovascular antioxidant enzyme localized in mitochondria, and catalyzes the elimination of superoxide anions in cells. Thus, we further examined myocardial Mn-SOD activity in the ovariectomized rats. There are three distinct SOD isoforms: Cu,Zn-SOD, EC-SOD, and Mn-SOD. Potassium cyanide selectively inhibits Cu,Zn-SOD and EC-SOD activities, but not Mn-SOD activity. On the basis of this principle, Mn-SOD activity was assayed in the presence of 1 mmol/L potassium cyanide, and Cu,Zn- plus EC-SOD activities were estimated by subtracting the Mn-SOD activity from the total SOD activities. In the present study, we provide novel evidence that ovariectomy selectively reduces myocardial Mn-SOD activity, but not myocardial Cu,Zn-SOD or EC-SOD activities, after IR in ovariectomized rats. Notably, reduced myocardial Mn-SOD activity was restored by long-term treatment with TJ-113. Mn-SOD has been reported to exert an inhibitory effect on tyrosine nitration induced by peroxynitrite in a rat renal transplantation model. It is thus conceivable that restoration of myocardial Mn-SOD activity was also involved in the beneficial effects of TJ-113 on IR injury in the ovariectomized rats.

No Involvement of Estrogen in the Beneficial Effects of TJ-113 on IR Injury in Ovariectomized Rats

It has been elucidated in rats that females have greater resistance to cardiac IR injury than males through the effect of estrogen, and that treatment with estrogen mitigates cardiac IR injury in ovariectomized rats. In the present study, long-term treatment with TJ-113 did not affect plasma estrogen levels in the ovariectomized rats, suggesting that the beneficial effects of TJ-113 on the IR injury in the ovariectomized rats were not related to changes in plasma estrogen levels.

Involvement of the Estrogen Receptor Pathway in the Beneficial Effects of TJ-113 on Cardiac IR Injury in Ovariectomized Rats

The cardioprotective effects of TJ-113 were noted under low plasma estrogen levels in both ovariectomized female rats and male rats, whereas no such effect was seen under high plasma estrogen levels in sham-operated female rats. Thus, we hypothesized that estrogenic activity may have mediated the cardioprotective effects of TJ-113 on IR injury in the ovariectomized rats. Co-treatment with the estrogen receptor antagonist, fulvestrant, significantly inhibited the beneficial effects of TJ-113 on cardiac function and infarct size after IR in the ovariectomized rats, suggesting that TJ-113 exerted its cardioprotective action in the ovariectomized rats through the estrogen receptor pathway.

Cardioprotective Effect of Long-Term Treatment With Emodin on IR Injury in Ovariectomized Rats

TJ-113’s ingredient, emodin, has been reported to be a phytosterogen with an affinity for the estrogen receptor. Thus, we further examined whether emodin mimics the cardioprotective effects of TJ-113 in ovariectomized rats. Long-term treatment with emodin significantly improved cardiac IR injury in the ovariectomized rats, as in the case with TJ-113. It is thus conceivable that emodin was in part involved in the cardioprotective actions of TJ-113 in the ovariectomized rats.

Conclusions

In summary, we were able to demonstrate that long-term oral treatment with a clinical therapeutic dosage of TJ-113 mark-
edly ameliorated cardiac dysfunction and infarct size following IR in ovariectomized rats via a redox-dependent mechanism. Our findings provide the first evidence of cardioprotective actions of TJ-113 against IR injury in a climacteric rat model. Because kampo medicines are made from natural products, such as the roots of plants, they are considered to have no serious side effects. Indeed, we did not observe any overt side effects in the rats that were subjected to long-term treatment with TJ-113. Thus we conclude that TJ-113 may be a safe and effective novel therapeutic option in the treatment of IHD in climacteric women.

Acknowledgments

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Disclosures

Conflict of Interest: None.

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