Effect of Waon Therapy on Oxidative Stress in Chronic Heart Failure

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Background: A previous report by our team showed that Waon therapy, using a far infrared-ray dry sauna at 60°C, improves cardiac and vascular function in patients with chronic heart failure (CHF). The purpose of the present study was to clarify the effect of Waon therapy on oxidative stress in CHF patients and investigate its mechanism by animal experiments.

Methods and Results: Forty patients with CHF were divided into control (n=20) and Waon therapy (n=20) groups. All patients received standard optimal medications for CHF. Waon therapy group was treated with Waon therapy daily for 4 weeks. After 4 weeks of Waon therapy, concentrations of hydroperoxide and brain natriuretic peptide (BNP) decreased significantly (hydroperoxide, 422±116 to 327±88 U.CARR, P<0.001; BNP, 402±221 to 225±137 pg/ml, P<0.001), and the nitric oxide metabolites increased (71.2±35.4 to 92.0±40.5 mmol/L, P<0.05). In contrast, none of these variables changed over the 4-week interval in the control group. Furthermore, animal experiments were performed using TO-2 cardiomyopathic hamsters. On immunohistochemistry, cardiac expression of 4-hydroxy-2-nonenal, a marker of oxidative stress, was decreased in the 4-week Waon therapy compared to untreated hamsters. On Western blotting, cardiac expressions of heat shock protein (HSP) 27, manganese superoxide dismutase and HSP32, which reduce oxidative stress, were significantly upregulated in the 4-week Waon therapy compared to untreated hamsters.


Key Words: Heart failure; Heat shock protein; Oxidative stress; Waon therapy
the severity of heart failure.16–18 Administration of angiotensin II receptor blockers (ARB), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), vitamin C, β-blockers, allopurinol and growth hormone-releasing peptide reduces oxidative stress and improves cardiac and vascular function in CHF.19–23 Furthermore, exercise training decreases oxidative stress in CHF.24

We have reported that 2 weeks of Waon therapy decreased urinary concentrations of 8-epi-PGF2α, a marker of oxidative stress, in patients with at least one atherosclerotic risk factor.25 However, the effect of Waon therapy on oxidative stress in CHF has not been elucidated. The purpose of the present study was to clarify the effect of Waon therapy on oxidative stress in CHF patients and address its mechanism using cardiomyopathic hamsters with heart failure.

**Methods**

**Clinical Study**

**Participants and Study Design** The study participants included 40 CHF patients who were admitted to Kagoshima University Hospital or Kagoshima City Medical Association Hospital between 2006 and 2009.

The inclusion criteria were the presence of symptomatic CHF, left ventricular ejection fraction (LVEF) <50% on echocardiography, and New York Heart Association (NYHA) functional classes II or III. Exclusion criteria were the presence of severe aortic stenosis, severe obstruction with hypertrophic obstructive cardiomyopathy, and NYHA functional class IV. Also excluded were patients who changed medications, such as angiotensin-converting enzyme inhibitors, ARB, β-blockers, statins, and allopurinol, because changes in medications might affect the oxidative stress.

All patients were treated with standard optimal therapy for at least 1 week after admission, and then were randomized to the Waon therapy group (n=20) or the control group (n=20). All patients continued optimal treatment for heart failure for an additional 4 weeks. The patients in the Waon therapy group received Waon therapy once a day, 5 days per week, for 4 weeks. The patients in the control group continued conventional treatment for 4 weeks.

All examinations were performed at baseline and on the next day after the last treatment of 4 weeks.

Informed consent was obtained from all patients prior to participation, and the protocol was approved by the Ethics Committee of the Faculty of Medicine, Kagoshima University.

**Waon Therapy** Waon therapy uses a far infrared-ray dry sauna, which is evenly maintained at 60°C and differs from the traditional sauna. Waon therapy has no hydration requirements, and is evenly maintained at 60°C and differs from the traditional sauna. Waon therapy was performed using an experimental far infrared-ray dry sauna system at 39°C for 15 min followed by 30°C for 20 min as reported previously.14 In this setting, their core temperatures were increased by 1°C and remained elevated for 20 min as shown in the clinical setting.2

**Measurements of Cardiac Function** In order to estimate the effect of Waon therapy on cardiac function in TO-2 hamsters, left ventricular (LV) +dP/dt and % fractional shortening (%FS) were measured at the age of 34 weeks as described previously.14,26 Millar catheter pressure transducers (Millar Instruments, Houston, TX, USA), which were cannulated into the right carotid artery, and echocardiography (Toshiba SSH-380A Power Vision, Toshiba Medical System, Tokyo, Japan) were used for the measurements.

**Immunohistochemistry** The labeled streptavidin biotin method was performed using a Histfine kit (Nichirei, Tokyo, Japan) for immunohistochemistry as previously described.27 Briefly, cross-sections of hearts were incubated overnight with mouse monoclonal antibodies specific for 4-hydroxy-2-nonenal (4-HNE; Oxis, Foster City, CA, USA), which is a marker of oxidative stress.29,30,31 The specimens were then incubated with biotinylated anti-mouse IgG. They were developed with...
diaminobenzidine and counterstained with hematoxylin.

**ELISA** We assayed the concentration of cardiac 4-HNE in TO-2 hamsters using OxiSelect HNE-His Adduct ELISA Kit (Cell Biolabs, Inc, San Diego, CA, USA) according to the protocol supplied with the kit. In brief, 100 μl of the 10 μg/ml protein samples from whole hearts of TO-2 hamsters were probed with an anti-4-HNE antibody and absorbed on a microplate reader using 450 nm as the primary wavelength.

**Western Blotting** Western blotting was performed as described previously. In brief, 10 μg of protein samples from whole hearts of TO-2 hamsters were detected with the NuPAGE Electrophoresis System (NOVEX, San Diego, CA, USA) using rabbit polyclonal heat shock protein (HSP) 27, HSP32, manganese superoxide dismutase (Mn-SOD), copper/zinc-SOD (Cu/Zn-SOD) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p38 mitogen-activated protein kinase (p38MAPK), and phosphorylated p38MAPK (p-p38MAPK) antibodies (Cell signaling technology, Danvers, MA, USA). HSP27 and HSP32 are induced by several stimuli, including heat stimulation, and reduce oxidative stress.

Band density was determined by densitometry using NIH image software. Results were expressed as the ratio of the density of specific bands to the corresponding β-actin.

### Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Waon therapy (n=20)</th>
<th>Control (n=20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, years</td>
<td>64±14</td>
<td>65±13</td>
<td>0.77</td>
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<tr>
<td>Male, %</td>
<td>85</td>
<td>80</td>
<td>0.68</td>
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<tr>
<td>NYHA functional class (II/III)</td>
<td>17/3</td>
<td>18/2</td>
<td>0.63</td>
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<tr>
<td>Diagnosis, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>50</td>
<td>45</td>
<td>0.75</td>
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<tr>
<td>Ischemic cardiomyopathy</td>
<td>30</td>
<td>35</td>
<td>0.74</td>
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<tr>
<td>Valvular heart disease</td>
<td>10</td>
<td>5</td>
<td>0.55</td>
</tr>
<tr>
<td>Others</td>
<td>10</td>
<td>15</td>
<td>0.63</td>
</tr>
<tr>
<td>AF, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic AF</td>
<td>15</td>
<td>20</td>
<td>0.68</td>
</tr>
<tr>
<td>Paroxymal AF</td>
<td>30</td>
<td>10</td>
<td>0.11</td>
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<tr>
<td>Medication, %</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ACE inhibitor/ARB</td>
<td>100</td>
<td>95</td>
<td>0.31</td>
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<tr>
<td>β-blocker</td>
<td>90</td>
<td>95</td>
<td>0.55</td>
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<tr>
<td>Statin</td>
<td>20</td>
<td>30</td>
<td>0.47</td>
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<tr>
<td>Allopurinol</td>
<td>25</td>
<td>20</td>
<td>0.70</td>
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<tr>
<td>Calcium channel blocker</td>
<td>15</td>
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NYHA, New York Heart Association; AF, atrial fibrillation; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

### Table 2. Changes in Clinical Variables at Baseline and After 4 Weeks

<table>
<thead>
<tr>
<th></th>
<th>Waon therapy (n=20)</th>
<th>Control (n=20)</th>
<th>Comparison at baseline P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, kg</td>
<td>58.2±16.5</td>
<td>57.6±16.1</td>
<td>&lt;0.05</td>
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<tr>
<td>HR, beats/min</td>
<td>78±12</td>
<td>71±11</td>
<td>0.058</td>
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<tr>
<td>SBP, mmHg</td>
<td>103±18</td>
<td>96±21</td>
<td>0.12</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>61±11</td>
<td>56±14</td>
<td>0.082</td>
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<tr>
<td>CTR, %</td>
<td>56.3±6.2</td>
<td>53.0±5.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>31.8±11.3</td>
<td>35.8±13.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BNP, pg/ml</td>
<td>402±221</td>
<td>225±137</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>UA, mg/dl</td>
<td>6.65±1.88</td>
<td>6.24±1.51</td>
<td>0.15</td>
</tr>
</tbody>
</table>

BW, body weight; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; CTR, cardiothoracic ratio; LVEF, left ventricular ejection fraction; BNP, brain natriuretic peptide; UA, uric acid.

**Figure 1.** Changes in oxidative stress after 4 weeks of Waon treatment. The plasma concentrations of hydroperoxide decreased significantly after 4 weeks of Waon therapy. *P<0.001 vs. baseline.
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**Figure 2.** Changes in nitric oxide metabolites after 4 weeks of treatment. The plasma concentration of nitric oxide metabolites increased significantly in the Waon therapy group, but did not change in the control group. *P<0.05 vs. baseline.

**Figure 3.** Effects of Waon therapy on the expression of 4-HNE in hearts. (A) Immunoreactivity of 4-hydroxy-2-nonenal (4-HNE) decreased in hearts of TO-2 hamsters treated by 4-week Waon therapy compared to the untreated hamsters. (B) ELISA revealed that 4-week Waon therapy decreased the concentration of cardiac 4-HNE of TO-2 hamsters significantly compared to untreated hamsters (Waon therapy group, 3.57±0.96 vs. untreated group, 5.53±1.17μg/ml, P<0.05, n=8 per group).
Statistical Analysis
Values are expressed as means±SD. Statistical analysis was performed using Stat View Version 5.0 software. Comparisons of baseline clinical characteristics between the 2 groups were performed using Pearson’s chi-square test or Student’s unpaired t-test. Within-group changes between before and 4 weeks after treatment were evaluated by paired t-tests. In the animal experiments, the results of the 2 groups were compared by Student’s unpaired t-test. Statistical significance was accepted when the P-value was <0.05.

Results
Clinical Examination
Patient Characteristics  The patients’ baseline characteristics are shown in Table 1. There were no significant differences in age, gender, NYHA functional class, causative heart diseases, atrial fibrillation and medication between the 2 groups at baseline. In addition, as shown in Table 2, there were no significant differences in body weight, HR, SBP, DBP, CTR, LVEF, BNP, and uric acid between the 2 groups at baseline.

Changes in Clinical Variables After 4 Weeks  During the study, none of the patients treated with Waon therapy had worsened clinical symptoms. The changes in clinical variables after 4 weeks are summarized in Table 2. Body weight, CTR, and BNP decreased significantly after 4 weeks of Waon therapy compared to baseline, but they did not change in the control group. In addition, echocardiography demonstrated that LVEF increased significantly after 4 weeks of Waon therapy, but did not change in the control group. There were no significant differences in HR, SBP, DBP, and uric acid after 4 weeks of Waon therapy. In the control group, there were no significant differences in these clinical variables after 4 weeks of treatment.

Plasma Concentrations of Hydroperoxide  The changes in plasma concentrations of hydroperoxide, which is an index of oxidative stress, are shown in Figure 1. The plasma concentration of hydroperoxide decreased significantly after 4 weeks of Waon therapy, whereas it did not change in the control group after 4 weeks of treatment (Waon therapy group, 422±116 to 327±88 U.CARR, P<0.001; control group, 422±71 to 431±85 U.CARR, P=0.59). There was no significant difference between the 2 groups at baseline (P=0.99).

Figure 4. Effects of Waon therapy on the cardiac expressions of HSP27 and HSP32 assessed by Western blotting. (A) The representative Western blotting bands of HSP27 and HSP32 expressions in the whole hearts of TO-2 hamsters are shown. (B) Cardiac expressions of HSP27 and HSP32 in TO-2 hamsters increased significantly after 4 weeks of Waon therapy compared to those in the untreated group. *P<0.01 vs. untreated hamsters, n=8 per group.
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Plasma Concentrations of Nitrate and Nitrite  The nitric oxide metabolites, nitrate and nitrite, were measured at baseline and 4 weeks after treatment (Figure 2). The plasma concentration of nitric oxide metabolites increased significantly in the Waon therapy group, but did not change in the control group (Waon therapy group, 71.2±35.4 to 92.0±40.5 μmol/L, P<0.05; control group, 96.2±90.2 to 77.4±43.5 μmol/L, P=0.23). There was no significant difference between the 2 groups at baseline (P=0.26).

Animal Experiments

Effect of Waon Therapy on Cardiac Function  In order to examine the effect of Waon therapy on oxidative stress in CHF, animal experiments using TO-2 cardiomyopathic hamsters were performed. First of all, the effect of Waon therapy on cardiac function in TO-2 hamsters was confirmed. Waon therapy significantly increased the LV +dP/dt of TO-2 hamsters compared to untreated hamsters (LV +dP/dt: Waon therapy group, 5,880±1,640 vs. untreated group, 4,180±660 mmHg/s, P<0.01, n=11 per group, %FS: Waon therapy group, 23.3±4.3 vs. untreated group, 16.5±4.2%, P<0.01, n=11 per group).

Effect of Waon Therapy on Oxidative Stress  Immuno-histochemistry using 4-HNE antibody, which is a marker of oxidative stress, was performed to analyze the effect of Waon therapy on oxidative stress in the failing heart. Cardiac 4-HNE immunoreactivities were lower in TO-2 hamsters following 4-week Waon therapy than in untreated hamsters (Figure 3A), which indicates that Waon therapy decreases oxidative stress in the failing heart.

ELISA of 4-HNE also revealed that 4-week Waon therapy decreased the concentration of cardiac 4-HNE of TO-2 hamsters significantly compared to untreated hamsters (Waon therapy group, 3.57±0.96 vs. untreated group, 5.53±1.17 μg/ml, P<0.05, n=8 per group, Figure 3B).

Effect of Waon Therapy on HSP27 and HSP32 Expressions  The representative Western blotting bands of HSP27 and HSP32 expression in the whole hearts of TO-2 hamsters are shown in Figure 4. Cardiac expressions of HSP27 and 32 were significantly upregulated in hamsters treated with 4-week
Waon therapy compared to untreated hamsters (HSP27, Waon therapy group, 1.25±0.30 vs. untreated group, 0.27±0.16 arbitrary units, n=8 per group, P<0.01; HSP32, Waon therapy group, 0.83±0.18 vs. untreated group, 0.54±0.12 arbitrary units, n=8 per group, P<0.01).

**Effect of Waon Therapy on SOD Expression**
Cardiac expression of Mn-SOD was significantly upregulated in hamsters treated with 4-week Waon therapy compared to untreated hamsters (Waon therapy group, 0.83±0.20 vs. untreated group, 0.52±0.16 arbitrary units, n=8 per group, P<0.01; Figure 5). However, cardiac expression of Cu/Zn-SOD was not changed by Waon therapy (Cu/Zn-SOD, Waon therapy group, 1.14±0.08 vs. untreated group, 1.21±0.16 arbitrary units, n=8 per group, P=0.29, Figure 5).

**Effect of Waon Therapy on the Activation of p38MAPK**
Waon therapy did not change cardiac expression of p38MAPK. However, cardiac expression of p-p38MAPK was upregulated in hamsters treated with 4-week Waon therapy compared to untreated hamsters. The ratio of p-p38MAPK to p38MAPK in TO-2 hamsters was significantly higher than that in the untreated group. (Waon therapy group, 0.84±0.18 vs. untreated group, 0.53±0.14 arbitrary units, n=8 per group, P<0.01; Figure 6.)

**Discussion**
The present clinical study demonstrated that 4 weeks of Waon therapy improved cardiac function, decreased plasma hydroperoxide concentrations and increased plasma nitrite and nitrate concentrations. In addition, 4 weeks of Waon therapy improved cardiac function and decreased oxidative stress in failing hearts of TO-2 hamsters. Furthermore, it appears that Waon therapy reduces the oxidative stress through HSP27, HSP32 and Mn-SOD.

Oxidative stress is implicated in the pathogenesis of heart failure. Reactive oxygen species (ROS) are produced in the failing myocardium, and ROS causes progression of heart failure. Increased ROS in CHF impairs vascular endothelial function, which is represented by the endothelium-dependent vasodilatory response, through decreased NO bioavailability induced by decreased eNOS activity, including decreased eNOS expression and increased eNOS uncoupling. As vascular endothelial function is one of the most important factors affecting clinical symptoms in CHF, therapy that improves vascular endothelial dysfunction is considered to be an ideal treatment for CHF. Furthermore, increased oxidative stress induces apoptosis of cardiomyocytes, resulting in further impairment of the failing myocardium. Therefore, therapies that decrease oxidative stress are important to improve vascular endothelial function and cardiac function in CHF.

SOD is well known as a key anti-oxidant enzyme, and some kinds of HSP, such as HSP27 and HSP32, reduce oxidative stress. It has been reported that overexpression of HSP27 attenuated doxorubicin-induced cardiac dysfunction through the decreases of oxidative stress and apoptosis in hearts of HSP27 transgenic mice. We demonstrated that Waon therapy upregulated the cardiac expressions of HSP27 and Mn-SOD in TO-2 cardiomyopathic hamsters. In addition, Waon therapy was shown to increase cardiac expression of HSP32 in TO-2 hamsters. HSP32 is also known as Heme Oxygenase-1, and it plays a role in cellular protection against injury caused by ROS. HSP32 degrades the pro-oxidant heme and catalyzes it into biliverdin and bilirubin, which function as anti-oxidants. Hearts of heterozygous HSP32 knockout mice, subjected to ischemia/reperfusion injury, had increased oxidative stress and infarct size compared to wild type mice. In contrast, hearts of HSP32 transgenic mice had reduced oxidative stress and infarct size compared to wild type mice when they were subjected to ischemia/reperfusion injury. These results clarified the cardio-protective effect of HSP32. Given the results of the clinical study and the animal experiments presented in this paper, the increases of HSP27, Mn-SOD and HSP32 by Waon therapy appear to reduce oxidative stress and improve cardiac function in CHF.

It is reported that whole-body hyperthermia with 15 min
42°C hot water bathing increases cardiac Mn-SOD through the production of TNF-α and IL-1β of normal rats.47 TNF-α activates the translocation of NF-κB and increases Mn-SOD.48 However, Waon therapy did not modulate the cardiac expression of TNF-α and NF-κB signaling in TO-2 hamsters with heart failure (data not shown). We think that TNF-α and NF-κB have already increased in heart failure, therefore, the mechanisms by which Waon therapy increases Mn-SOD in failing hearts might not involve TNF-α/NF-κB pathway. It is reported that p38MAPK is activated by several stress factors, and is involved in the induction of HSP and Mn-SOD.49 In this study, we demonstrated that Waon therapy increased cardiac p-p38MAPK in TO-2 hamsters with heart failure. We believe that Waon therapy activates p38MAPK signaling, which leads to the induction of HSP and Mn-SOD.

Oxidative stress is also involved in the pathogenesis of arteriosclerosis and major cardiovascular diseases.50 We reported that Waon therapy for 2 weeks improved impaired vascular endothelial function in the setting of atherosclerotic risk factors, such as hypertension, hypercholesterolemia, diabetes mellitus, obesity, and smoking.12 In addition, we demonstrated that 2 weeks of Waon therapy significantly decreased urinary 8-epi-PGF2α levels in patients with at least 1 atherosclerotic risk factor, when compared to those of patients who did not undergo Waon therapy.11 Thus, Waon therapy reduces oxidative stress and improves vascular function in patients with atherosclerotic risk factors.

Although the plasma concentrations of nitrite and nitrate were significantly lower in patients with atrial fibrillation than in the control subjects,51 there is no significant difference in the incidence of atrial fibrillation between 2 groups in the present study. There are limitations in this study. It is difficult to get rid of the bias in data, because this study is not blind or a cross over test.

Conclusion
Waon therapy decreases oxidative stress and is an innovative non-pharmacological therapy for patients with CHF.

References


