



Original article

## Waon therapy mobilizes CD34+ cells and improves peripheral arterial disease

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### KEYWORDS

Peripheral arterial disease;  
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Waon therapy;  
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### Summary

**Background:** We previously reported that Waon therapy upregulates endothelial nitric oxide synthase protein, and augments ischemia-induced angiogenesis in mice with hindlimb ischemia, and it improves limb ischemia in patients with peripheral arterial disease (PAD). The aim of this study was to investigate the underlying mechanism of Waon therapy for the treatment of patients with PAD, and to determine whether Waon therapy can mobilize blood-derived progenitor cells.

**Methods:** 21 consecutive PAD patients received standard medications, and were randomly divided into control ( $n = 10$ ) and Waon therapy groups ( $n = 11$ ). The Waon therapy group received Waon therapy daily for 6 weeks. The control group continued conventional therapy for 6 weeks. Leg pain was scored using a visual analogue scale. The ankle-brachial pressure index (ABPI) and the 6-min walking distance were measured at baseline and 6 weeks after therapy. Frequency of circulating CD34+ progenitor cell numbers was measured by quantitative real-time polymerase chain reaction, and the serum nitrate and nitrite levels were also measured at baseline and 6 weeks after therapy.

**Results:** The leg pain score, ABPI and the 6-min walking distance improved significantly after 6 weeks in the Waon therapy group, but not in the control group. Frequency of circulating CD34+ cells increased after 6 weeks of Waon therapy [ $2.0 \pm 1.2 (\times 10^{-4})$  at baseline to  $3.9 \pm 1.9 (\times 10^{-4})$ ,  $p = 0.015$ ], while it remained unchanged in the control group [ $1.8 \pm 1.8 (\times 10^{-4})$  at

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baseline to  $1.2 \pm 0.9 (\times 10^{-4})$ . Serum nitrate and nitrite levels increased significantly after Waon therapy ( $29.6 \pm 17.6$  to  $36.0 \pm 17.7 \mu\text{mol/ml}$ ,  $p < 0.05$ ), but not in the control group ( $34.4 \pm 9.4$  to  $38.3 \pm 8.8 \mu\text{mol/ml}$ ).

**Conclusion:** Waon therapy mobilized circulating endothelial progenitor cells and improved limb ischemia in patients with PAD. Waon therapy is a highly promising therapy for patients with PAD.

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## Introduction

Peripheral arterial disease (PAD) is associated with decreased functional capacity and quality of life. Furthermore, patients with PAD have a higher rate of limb amputation and an increased risk of death compared with the general population. Patients with chronic critical limb ischemia have a 20% mortality rate per year with many patients being poor candidates for revascularization procedures [1]. Therapeutic angiogenesis and vasculogenesis may provide a treatment option for those patients with critical limb ischemia who are not suited for conventional revascularization therapy [2]. Some attempts at therapeutic angiogenesis have induced gene transfer using naked plasmid DNA encoding vascular endothelial growth factor (VEGF), which has demonstrated some promise in improving symptoms of critical limb ischemia [3], although not all studies have shown a benefit [4,5]. In addition, research has examined endothelial progenitor cells, which originate from bone marrow and circulate in the peripheral blood, and are known to participate in postnatal neovascularization [6,7]. There is evidence that autologous implantation of bone marrow mononuclear cells could be effective for achieving therapeutic angiogenesis [8,9]. These therapeutic angiogenesis trials have shown promising early efficacy, but further trials are warranted.

We have developed a form of thermal therapy, termed Waon therapy, which uses a dry sauna maintained at a temperature of  $60^\circ\text{C}$  and differs from a traditional sauna. We previously reported that repeated Waon therapy improves hemodynamics and ameliorates symptoms and ventricular arrhythmias in patients with chronic heart failure (CHF) [10–12]. Furthermore, we reported that repeated Waon therapy improves vascular endothelial dysfunction in patients with CHF, and in patients with coronary risk factors such as hypertension, hyperlipidemia, diabetes mellitus, and smoking [13,14]. We also demonstrated that the molecular mechanism by which repeated Waon therapy improves vascular flow and endothelial function is through increased endothelial nitric oxide synthase (eNOS) expression [15,16].

Nitric oxide (NO) is constitutively produced by eNOS, and is a mediator of angiogenesis. Waon therapy increases eNOS protein expression, blood flow, and capillary density in a mouse model of hindlimb ischemia [17]. Furthermore, Waon therapy does not increase blood flow and capillary density in eNOS-deficient mice, which demonstrates that eNOS is a critical regulator of angiogenesis during Waon therapy. Recently, we reported that Waon therapy is safe and improves leg pain, status, and blood flow in patients with PAD [18,19].

This study was performed to confirm the beneficial effects of Waon therapy using the infrared-ray dry sauna, and to investigate the underlying mechanism of why Waon

therapy may be beneficial in the treatment patients with PAD. In particular, we wanted to determine whether Waon therapy could mobilize blood-derived progenitor cells for local vascular regeneration.

## Methods

### Patients

The present study included patients with PAD with intermittent claudication for a minimum of 4 weeks without evidence of improvement despite conventional therapies. Inclusion criteria were (1) a resting ankle-brachial pressure index (ABPI)  $< 0.75$  in the affected limb on 2 consecutive examinations performed at least 1 week apart, (2) lower limb artery lesions were detected with computed tomographic angiography (CTA), magnetic resonance angiography (MRA) or color duplex ultrasound. 21 consecutive PAD patients were enrolled, and were randomly divided into control ( $n = 10$ ) and Waon therapy groups ( $n = 11$ ). They were allowed to continue taking antiplatelet drugs, provided that these therapies had been used for a minimum of 6 months before the enrollment. In the Waon therapy group, they had Waon therapy daily for 6 weeks. In the control group, they had only conventional PAD therapy for 6 weeks. All patients gave their written informed consent. This study design was unanimously approved by the Ethics Committee of Kagoshima University.

### Waon therapy

Waon therapy, a form of thermal therapy that uses a far infrared-ray dry sauna at  $60^\circ\text{C}$  and differs from the traditional sauna in that it has no hydration pressure, was performed as previously reported [18]. The patients were placed in a  $60^\circ\text{C}$  dry sauna system for 15 min; after leaving the sauna, they underwent bed rest with a blanket to keep them warm for an additional 30 min. All patients were weighed before and after the therapy, and oral hydration with water was used to compensate for weight lost due to perspiration.

Waon therapy was performed once a day, 5 days a week, for a total of 6 weeks in the Waon therapy group. To rule out an acute effect of Waon therapy, all measurements were performed before the first treatment and on the next day after the last treatment in the Waon therapy group.

### Measurements

Leg pain was scored using a visual analogue scale, with a marked 10-cm line extending from “no pain: 0” to “severest pain: 10”. Resting ABPI was calculated by the

quotient of the absolute ankle pressure to the right brachial pressure using an oscillometric method by Form/ABPI (Colin, Komaki, Japan) as reported previously [20]. Six-minute (6-min) walking distance was measured to evaluate exercise capacity. The serum nitrate (NO<sub>3</sub>), nitrite (NO<sub>2</sub>), and VEGF levels were measured. The pain score, ABPI, and 6-min walking distance were measured at baseline and after 6 weeks.

**Analysis of CD34 mRNA for detection of circulating CD34+ cells in the periphery**

To detect the shift of circulating CD34+ progenitor cells in the periphery with higher sensitivity, quantitative real-time polymerase chain reaction (PCR) for CD34 messenger RNA (mRNA) was performed with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control. Patients’ peripheral blood mononuclear cells were harvested at baseline and after 6 weeks. Peripheral blood mononuclear cells (PBMC) (approximately 10<sup>6</sup>/tube) were stored at -80 °C and later used for RNA extraction, cDNA synthesis, and quantitative real-time PCR.

Total RNA was extracted from patients’ PBMC using the guanidine isothiocyanate method (RNeasy Mini Kit, Qiagen, Hilden, Germany), and was then transcribed to cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. Real-time quantitative PCR analysis was performed using a LightCycler system and LC FastStart DNA Master Hybridization Probes (Roche Diagnostics), and 1 µl of cDNA in a final reaction volume of 20 µl. Primers for human CD34 (GenBank accession no. M81104) were designed as follows: (forward) 5’-CTCAGTGCTACTGCTGGTCT-3’ and (reverse) 5’-GGAATAGCTCTGGTGGCTT-3’. The taqman probe sequence was 5’-FAM-CAACTTATGAAAAGCACCAATCTGACCTG-TAMRA-3’. Thermal cycler parameters included 10 min at 95 °C, 40 cycles of 95 °C for 10s, and 62 °C for 20s. The size of the amplicon of the CD34 gene was 143 bp. The expression level of the GAPDH (GenBank accession no. M33197) gene was measured as an internal reference with a standard curve for determining the integrity of template RNA for all specimens.

All primers were purchased from Nihon Gene Research Laboratories (Sendai, Japan). The frequency of CD34+ cells in the periphery was determined as the ratio of the mRNA level of these genes as follows: absolute copy number of CD34 gene in 1 µl of cDNA/absolute copy number of GAPDH gene in 1 µl of cDNA.

**Statistical analysis**

All data are expressed as mean values ± SD. Data at baseline and after 6 weeks were compared using the paired *t*-test. Differences in patients’ characteristics were evaluated using the chi-squared test and the unpaired *t*-test. A value of *p* < 0.05 was considered statistically significant.

**Results**

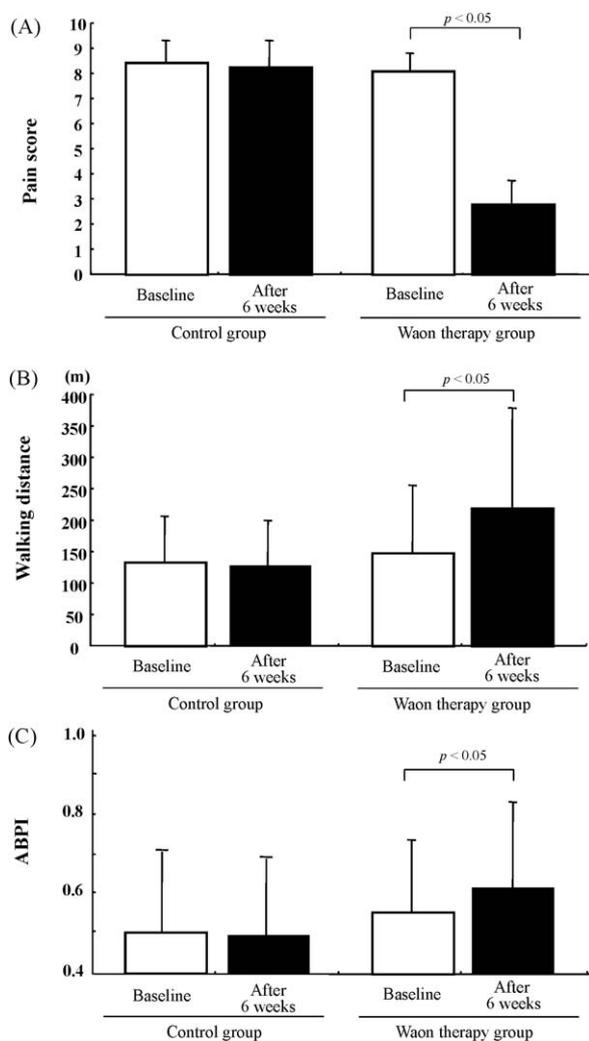
**Clinical characteristics**

The patients’ clinical characteristics are summarized in Table 1. There were no significant differences in age, body mass index, ABPI, hyperlipidemia, diabetes mellitus, hypertension, and smoking between the control and the Waon therapy groups. In addition, there were no significant differences in the use of medications for PAD, such as antiplatelets, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, or statins between the two groups. There was a significant difference in gender between the two groups.

All patients enrolled in the trial completed the study without any adverse events. During the trial, no patients developed significant laboratory test abnormalities, including those for blood cell counts, electrolyte concentrations, chemistry and lipid profiles, tumor markers, and inflammatory markers. No changes in diabetic retinopathy were observed on fundoscopic examination, and no latent neoplasms developed. Transient leg pain during Waon therapy was seen, but it disappeared after a few sessions of Waon therapy.

**Table 1** Characteristics of patients.

	Control (n = 10)	Waon therapy (n = 11)	p-Value
Gender (male/female)	10/0	7/4	<0.05
Age (years)	75 ± 5	75 ± 6	N.S.
BMI	24.0 ± 1.3	23.0 ± 1.9	N.S.
ABPI	0.50 ± 0.21	0.55 ± 0.22	N.S.
Hyperlipidemia, n (%)	5 (50%)	7 (63%)	N.S.
Diabetes mellitus, n (%)	6 (60%)	8 (72%)	N.S.
Hypertension, n (%)	9 (90%)	8 (72%)	N.S.
Smoking, n (%)	8 (80%)	8 (72%)	N.S.
<b>Medications</b>			
Antiplatelets, n (%)	10 (100%)	11 (100%)	N.S.
ACEI or ARB, n (%)	4 (40%)	5 (45%)	N.S.
Statin, n (%)	5 (50%)	6 (54%)	N.S.
PGI2 analogue, n (%)	3 (33%)	3 (27%)	N.S.



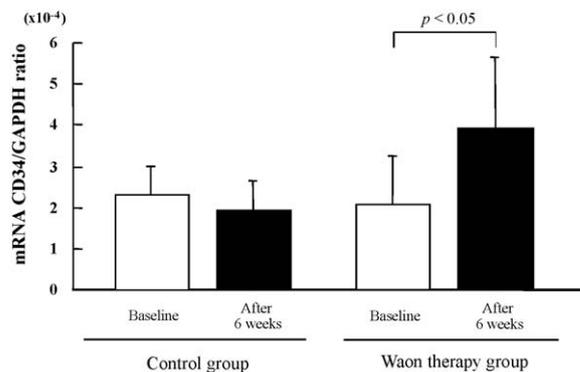
**Figure 1** Changes in leg pain, walking distance, and ankle-brachial pressure index (ABPI). (A) Pain score evaluated using a visual analogue scale (10: severe pain; 0: no pain). (B) 6-min walking distance. (C) ABPI open bars: at baseline; solid bars: after 6 weeks.

### Changes in leg pain, walking distance, and ABPI

Therapeutic benefit was demonstrated by regression of leg pain in all patients treated with Waon therapy. The pain score evaluated using a visual analogue scale was significantly decreased in the Waon therapy group after 6 weeks ( $8.1 \pm 0.7$  to  $2.8 \pm 1.1$ ,  $p < 0.05$ ), while it remained unchanged in the control group ( $8.4 \pm 0.8$  to  $8.2 \pm 1.1$ , Fig. 1A).

Exercise performance improved significantly in the Waon therapy group (Fig. 1B). After 6 weeks of Waon therapy, the 6-min walking distance increased from  $149 \pm 104$  m at baseline to  $230 \pm 154$  m at 6 weeks after Waon therapy ( $p < 0.01$ ), while it did not change in the control group ( $136 \pm 76$  to  $132 \pm 72$  m, Fig. 1B).

The mean ABPI increased significantly from  $0.55 \pm 0.20$  at baseline to  $0.60 \pm 0.22$  after 6 weeks in the Waon therapy group ( $p < 0.01$ , Fig. 1C), but it did not change in the control group ( $0.50 \pm 0.21$  to  $0.49 \pm 0.20$ ).



**Figure 2** Changes in frequency of mobilized circulating CD34+ cells. Open bars: at baseline; solid bars: after 6 weeks. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

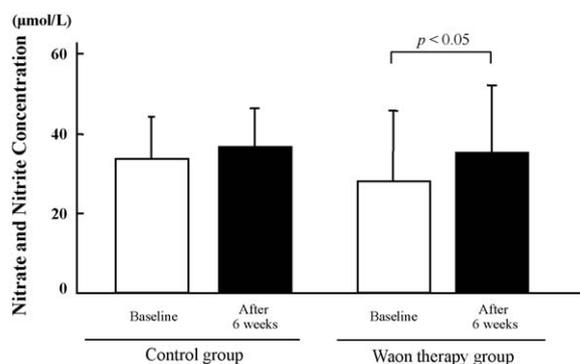
### Detection of circulating CD34+ cells by quantitative real-time PCR

Frequency of circulating CD34 positive cell numbers was measured at baseline and after 6 weeks by quantitative real-time PCR using the mRNA CD34/GAPDH ratio (Fig. 2). In the PAD group, the mRNA CD34/GAPDH ratio increased 2.0-fold, from  $2.0 \pm 1.2$  ( $\times 10^{-4}$ ) at baseline to  $3.9 \pm 1.9$  ( $\times 10^{-4}$ ) after 6 weeks of Waon therapy ( $p = 0.015$ ), while the mRNA CD34/GAPDH ratio in the control group was  $1.8 \pm 1.8$  ( $\times 10^{-4}$ ) at baseline and  $1.2 \pm 0.9$  ( $\times 10^{-4}$ ) after 6 weeks (no significant difference). Repeated Waon therapy appears to mobilize progenitor cells among PAD patients, and this might be helpful for the repair or regeneration of damaged vessels.

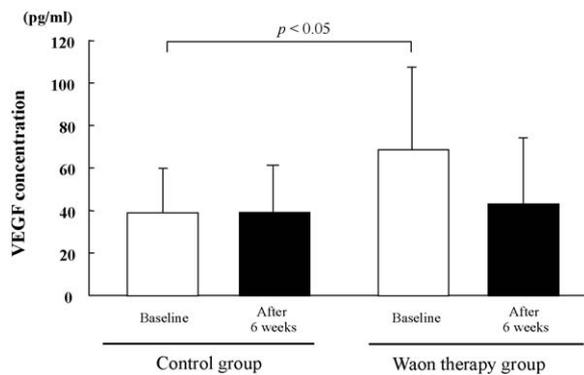
### NO production and VEGF levels

Serum nitrate ( $\text{NO}_3$ ), nitrite ( $\text{NO}_2$ ), and VEGF levels were measured in both groups. In the Waon therapy group, the serum nitrate and nitrite levels increased significantly after 6 weeks of Waon therapy ( $29.6 \pm 17.6$  to  $36.0 \pm 17.7$   $\mu\text{mol/ml}$ ,  $p < 0.05$ ), while it remained unchanged in the control group ( $34.4 \pm 9.4$  to  $38.3 \pm 8.8$   $\mu\text{mol/ml}$ , Fig. 3).

There were no significant differences in VEGF levels at baseline and after 6 weeks in both groups (control group:  $39.2 \pm 21.7$  to  $42.5 \pm 21.6$  pg/ml; Waon therapy group:  $70.2 \pm 46.3$  to  $40.2 \pm 40.5$  pg/ml, Fig. 4). VEGF level at base-



**Figure 3** Changes in serum nitrate and nitrite levels. Open bars: at baseline; solid bars: after 6 weeks.



**Figure 4** Changes in serum vascular endothelial growth factor (VEGF) levels. Open bars: at base line; solid bars: after 6 weeks.

line of Waon therapy group was significantly higher than that of control group ( $p = 0.041$ ).

## Discussion

We have developed thermal therapy using a 60°C dry sauna and showed that it improves hemodynamic parameters, endothelial function, clinical symptoms, and prognosis in many CHF patients [10–12, 21–23]. Generally, thermal therapy at very high temperature was originally used to treat localized cancer. However, the therapy we developed to treat cardiovascular diseases is quite different in that it consists of systemic soothing warmth that comfortably refreshes the mind and body. Therefore, we have changed the name from thermal to Waon therapy, since “Waon” in Japanese means soothing warmth [24].

In the present study, it was confirmed that Waon therapy is safe and potentially effective for patients with severe PAD, as evidenced by substantial decreases in the pain score and increases in ABPI and walking distance.

The goal in this study was to examine the underlying mechanism of Waon therapy for the treatment of PAD, and to see whether Waon therapy could mobilize blood-derived progenitor cells for local vascular regeneration. Recently, flow cytometry has been used to detect CD34+ progenitor cells in the periphery; however, the frequency of CD34+ cells in the periphery under normal, steady state conditions is less than 0.01%, which is just at the limit of the detectable range of flow cytometers. It is widely known that granulocyte-colony stimulating factor (G-CSF) can mobilize a large number of CD34+ hematopoietic progenitor cells from the bone marrow, which can be easily detected by flow cytometry. Nonetheless, we speculated that progenitor mobilization by our Waon therapy might be much weaker than that with G-CSF administration. To detect the change in circulating CD34+ cells with higher sensitivity, we developed a quantitative real-time PCR for CD34 mRNA using the GAPDH gene as an internal control, and found that circulating CD34+ cells were mobilized by repeated Waon therapy in patients with PAD.

We previously reported that angiogenesis was induced via eNOS using Waon therapy in mice with hindlimb ischemia [17]. NO is a mediator of angiogenesis and plays a key role in angiogenesis induced by Waon therapy. Repeated Waon

therapy increases cardiac output, shear stress of the vessel wall, and, ultimately, eNOS expression.

PAD patients are characterized by reduced systemic NO bioactivity. The impaired neovascularization in mice lacking eNOS is related to a defect in progenitor cell mobilization. eNOS expressed by bone marrow stromal cells influences recruitment of stem and progenitor cells [25]. In the present study, Waon therapy increased NO and circulating endothelial progenitor cells (EPC), which suggests that this therapy may mobilize EPC through an eNOS-dependent mechanism.

Some strategies to regulate the mobilization of progenitor cells through the eNOS pathway have been reported [25]. Statin therapy has improved EPC mobilization, myocardial neovascularization, left ventricular function, and survival after experimental myocardial infarction [26]. Estrogen therapy has augmented EPC incorporation into sites of myocardial neovascularization after myocardial infarction [27] and physical training also has increased EPCs and enhanced angiogenesis [28].

NO is an angiogenic mediator acting downstream of VEGF; impaired angiogenesis in eNOS-deficient mice was not improved by administration of VEGF [29], and transgenic mice overexpressing eNOS did not show a greater increase in VEGF expression in hindlimb ischemia [30]. Although Waon therapy did not increase expression of VEGF in the ischemic hindlimb, this therapy augmented angiogenesis by increasing eNOS expression in the course of ischemia-induced VEGF expression [17]. Therefore, we think CD34+ cells were recruited in PAD patients who had the ischemia-induced VEGF. Furthermore, in this study, the concentration of VEGF which was upregulated by ischemia decreased in the course of improvement of limb ischemia after Waon therapy.

## Study limitations

There are several limitations in this study. VEGF levels at baseline of the two groups were different. This difference may have an influence on results, although Waon therapy did not increase the VEGF levels. Further large study is required to resolve this grouping problem.

There was no significant correlation between CD34+ cells, NO, and VEGF in the present study. There may be a time lag for a rise of these factors. Further study that measures these factors in several points will clarify the correlation of these factors.

## Conclusions

Waon therapy mobilized circulating endothelial progenitor cells and improved limb ischemia in PAD patients. Waon therapy is a highly promising therapy for PAD patients.

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