Original article

Walking training improves systemic and local pathophysiological processes in intermittent claudication

Running head: WT on pathophysiological processes in IC

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ABSTRACT

Objective: This study examined the impact of submaximal walking training (WT) on local and systemic NO bioavailability, inflammation and oxidative stress in patients with intermittent claudication (IC). **Design:** The study employed a randomized, controlled, parallel-group design and was performed in a single center. **Materials and Methods:** Thirty-two men with IC were randomly allocated to two groups: WT (n=16, 2 sessions/week, 15 cycles of 2-min walking at an intensity corresponding to the heart rate obtained at the pain threshold interspersed by 2-min of upright rest) and control (CO, n=16, 2 sessions/week, 30 min of stretching). NO bioavailability (blood NO and muscle nitric oxide synthase – eNOS); redox homeostasis (catalase – CAT, superoxide dismutase – SOD, lipid peroxidation – LPO measured in blood and muscle); and inflammation (interleukin-6 – IL-6, C-reactive protein – CRP, tumour necrosis factor α – TNF-α, intercellular adhesion molecules – ICAM, vascular adhesion molecules – VCAM measured in blood and muscle) were assessed at the baseline and after 12 weeks. **Results:** WT significantly increased blood NO, muscle eNOS, blood SOD and CAT, and muscle SOD and abolished the increase in circulating and muscle LPO observed in the CO group. WT decreased blood CRP, ICAM and VCAM and muscle IL-6 and CRP and eliminated the increase in blood TNF-α and muscle TNF-α, ICAM and VCAM observed in the CO group. **Conclusions:** WT at an intensity of pain threshold improved NO bioavailability and decreased systemic and local oxidative stress and inflammation in IC patients. The proposed WT protocol provides physiological adaptations that may contribute to cardiovascular health in these patients.

Keywords: Physical exercise; intermittent claudication; cardiovascular risk; atherosclerosis

WHAT THIS PAPER ADDS

Walking training near-maximal intermittent claudication (IC) symptoms is considered the best treatment for increasing walking capacity in patients with peripheral artery disease (PAD) and IC. However, ischemia and reperfusion induced by maximal walking produce oxidative stress and inflammation during and after the effort, which may contribute to PAD progression. This study showed that a submaximal walking protocol at an intensity of pain threshold besides increasing walking capacity is effective in ameliorating not only systemic but also local pathophysiological processes associated to PAD, which provides support for the use of submaximal WT protocols in clinical practice as suggested by the recent guidelines from Society for Vascular Surgery/American College of Cardiology/American Heart Association.

INTRODUCTION

Peripheral artery disease (PAD) affects more than 200 million people worldwide[1]. Intermittent claudication (IC), the leading PAD symptom, limits patients' walking capacity[2]. Additionally, patients with PAD and IC are accompanied by many pathophysiological alterations, such as decreased nitric oxide (NO) bioavailability[3], enhanced oxidative stress[3, 4] and increased inflammation[5], which increases cardiovascular risk and morbimortality[6, 7].

Cardiovascular mortality is the leading cause of death in these patients[7] with therapeutic interventions encouraged to ameliorate the abovementioned pathophysiological processes. Walking training (WT) is considered the best treatment to improve walking capacity in symptomatic PAD[2, 8] with walking to near-maximal IC symptoms producing greatest improvements[9, 10]. However, studies investigating the effects of WT on NO bioavailability[11, 12], oxidative stress[13, 14] and inflammation[13-18] in IC have provided controversial results with many of them showing no benefits[13, 16, 17, 19], which may be explained, at least in part, by the training protocols involving maximal ischemia.

Walking until maximal ischemia increases oxidative stress and inflammation during and after the effort[20], which may contribute to the progression of the disease[21], blunting the chronic benefits of WT. On the other hand, recent guidelines from the American College of Cardiology, American Heart Association and Society for Vascular Surgery[8-10] point out that pain-free and low-intensity walking protocols that avoid moderate-to-maximum claudication ischemia and pain can improve walking ability and functional status, being recommended for these patients. Thus, some useful and reproducible submaximal protocols for patients with PAD and IC have been created and tested[22-25]. Among them, we have developed a WT protocol based on the heart rate (HR) of pain threshold that is a reproducible physiological marker[23]. This training protocol has been shown to evoke low pain and produce significant physiological stimulus above the anaerobic threshold during its execution[23]. Additionally, it has improved

cardiovascular function and autonomic regulation in patients with IC after a period of training[22]. However, despite these benefits, little is known about its effects on important factors for vascular health, such as NO bioavailability, oxidative stress and inflammation.

Previous studies examining the effects of WT on biomarkers of inflammation and oxidative stress in patients with IC[11, 15-18, 20] have showed the systemic impact of WT. However, such analyses do not reflect the direct effects of WT on the source of these processes, the disease-affected muscle[26]. Thus, simultaneous examination of the systemic and local effects of submaximal WT on inflammatory, oxidative stress and NO bioavailability biomarkers may provide a more comprehensive understanding of the effects of WT on patients with IC management. Therefore, this study sought to determine if a submaximal walking training could improve systemic and local pathophysiological processes associated with PAD in patients with IC.

MATERIALS AND METHODS

The study's protocol followed the Declaration of Helsinki, was registered at the Brazilian Clinical Trials database (http://www.ensaiosclinicos.gov.br, RBR-3pq58k), and was approved by an Ethic Committee of Human Research at the University of São Paulo (process 667.382). Written informed consent was obtained prior to participation.

Participants

Patients with PAD and IC symptoms enrolled in a tertiary centre specialized in vascular disease were invited to participate. Patients were included if they met the following criteria: (i) male; (ii) aged ≥ 50 years; (iii) diagnosed with Fontaine stage II and Rutherford stages 1–3 of PAD; (iv) ankle brachial index (ABI) at rest ≤ .90 in at least one lower limb; (v) absence of non-compressible arteries; (vi) resting systolic and diastolic blood pressure (SBP and DBP) lower than 160 and 105 mmHg, respectively; (vii) absence of revascularization surgery or angioplasty in the last year; (viii) not receiving β-blocker, non-dihydropyridine calcium channel antagonist, anticoagulant clopidogrel and insulin; (ix) ability to walk at least 2 min at 3.2 km/h on a treadmill; (x) ability to undertake an incremental treadmill test limited by symptoms of IC; (xi) absence of myocardial ischemia or complex arrhythmias during a treadmill test. Patients were excluded if they: (i) presented diabetes with clinical autonomic neuropathy; (ii) presented other medical comorbidities (such as cardiomyopathies, neurodegenerative conditions and others) that prevent exercise execution; and iii) changed medication during the study.

Study Design

This randomized, controlled, parallel-group study was performed in a single center. Patients who fulfilled the study criteria after preliminary evaluations underwent the

experimental protocol in which the study's outcomes were evaluated at baseline and after 12 weeks of intervention. Participants were randomly assigned, via a computer random number generator (https://www.randomizer.org), into 2 groups: control (CO) and walking training (WT).

Preliminary Procedures

Patients were interviewed to identify age, presence of cardiovascular disease and risk factors, comorbid conditions, and current medication. ABI was evaluated as previously described[2]. Body mass and height were assessed to calculate body mass index. Auscultatory BP was measured in triplicate after 5 min of seated rest in two visits and the mean value was calculated. Diabetic patients (n=9, 28,1%) underwent the American Diabetes Association's battery for detecting cardiovascular autonomic neuropathy[27].

Afterwards, the patients underwent a graded maximal walking test on a treadmill (Imbrasport, ATL, Porto Alegre, Brazil) with speed set at 3.2 km/h and grade increased by 2% every 2 min until maximal leg pain[28]. Patients were monitored by a 12-lead ECG (Welch Allyn, Inc., Cardio Perfect MD, New York, USA). Heart rate (HR) at the pain threshold was recorded when the patients first reported claudication pain. This index has been previously shown to have high reliability and good agreement (ICC = 0.92; SEM = 3.2 bpm; SDD = 8.8 bpm)[29]. Claudication onset distance (COD) and total walking distance (TWD) were also recorded.

Experimental protocol

For all evaluations, patients were instructed to maintain similar routines in the prior 24h and to avoid physical exercise for the previous 48h, alcoholic beverages for 24h, and smoking on the day of the sessions. Patients had to take their medication as normal and to attend to the laboratory in fasted state. Laboratory temperature was kept between 20–22°C.

Patients arrived at 7:00 a.m. and received a standardized meal (two cereal bars and 50 ml of juice). Then, a catheter was inserted into the antecubital vein and was kept patent by salinization. They rested in the supine position until 8:00 a.m., when assessments were initiated. HR and auscultatory blood pressure were measured in triplicate and the mean value was calculated. Afterwards, venous blood and muscle biopsy samples were collected.

Patients were informed about their allocated group after the initial evaluation, while researchers who assessed the main outcomes (biomarkers analyses) of the study were blinded to the group allocation.

Outcomes

The primary outcomes were blood and muscle inflammatory and oxidative stress biomarkers. Secondary outcomes were walking capacity and cardiovascular function. (For the full list of biomarkers evaluated and their definitions, please see the Measurements section.)

Measurements

Cardiovascular function

HR was determined from ECG (EMG System do Brazil, EMG 030110/00B, Brazil). Auscultatory SBP/DBP was measured using a mercury sphygmomanometer (Unitec, São Paulo, Brazil). Rate product pressure (RPP) was calculated as HR x SBP.

Blood analysis

Blood samples (16 ml) were collected into standard EDTA–treated vacutainer tubes, centrifuged and plasma aliquots stored at –80ºC. Enzyme-linked immunosorbent assays (ELISA) were used to evaluate plasma concentrations of C-reactive protein (CRP), tumour necrosis factor-α (TNF-α), vascular cell adhesion molecule (VCAM), intercellular adhesion

molecules (ICAM) and interleukin-6 (IL-6), according to the manufacturer's instructions (Cayman Chemical – USA and R&D Systems – USA). Plasma concentrations of superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) were analysed by specifics kits (Cayman Chemical – USA). Plasma NO was analysed by chemiluminescence method with a specific analyser (Sievers ® Nitric Oxide Analyzer NOA 280, USA).

Muscle biopsy

Tissue samples (~70 to 100 mg) from the gastrocnemius muscle of the leg with the lowest ABI were removed by biopsy following Bergstrom's technique[30]. The samples were cleaned (blood and excessive connective tissue removed), immediately frozen in liquid nitrogen, and stored at -80ºC.

The mRNA levels of inflammatory biomarkers (IL-6, CRP, TNF-α, VCAM, ICAM) and endothelial nitric oxide synthase (eNOS) were assessed by real time-PCR (SYBRGreen PCR Master Mix and ABI PRISM 7500 Sequence Detection System, Applied Biosystems). Cyclophilin mRNA levels were used as a reference gene expression level. Relative quantities of target gene expressions were compared after normalization to the reference gene value (ΔCT). Changes in mRNA expression were calculated using the differences in ΔCT between the target gene expressions and cyclophilin (ΔΔCT) and the equation $2^{-\Delta\Delta CT}$. Results are expressed as a percentage of the value from the baseline assessment of the CO group.

For SOD, CAT and LPO analyses, muscle biopsies were homogenized (1:4 wt/vol) in potassium phosphate-buffered saline (50 mM, pH 7.8), centrifuged and stored at 4ºC. SOD maximal activity was analysed based on the inhibition of xanthine/xanthine oxidase-driven cytochrome C and expressed as U/mg. For CAT activity analysis, the rate of hydrogen peroxide (H_2O_2) decomposition was assessed. LPO was evaluated as an index of skeletal muscle oxidative injury by the ferrous oxidation-xylenol orange technique (FOX2).

Interventions

WT group followed a protocol already used in this population[22, 23]. Patients from this group executed two supervised training sessions per week. Each session consisted of 15 bouts of 2-min walking on a treadmill interspersed by 2-min of upright rest, resulting in a 60 min total session with 30 min of active walking. During walking bouts, intensity was adjusted to maintain HR at the HR obtained at the pain threshold[23] evaluated in the graded maximal test executed in the preliminary procedures. A range of 4 bpm above or below the HR obtained at this threshold was used to determine the upper and lower limits for training. Thus, during training sessions, treadmill speed was set at 3.2 km/h and slope was adjusted when necessary to keep HR (measured by a thorax monitor Polar A3, Helsinki, Finland) within the desired range (i.e. in each 2-min walking bout, if HR during walking was below the lower HR limit, the slope was increased in the next bout, and if HR was above the upper limit, slope was decreased in the next bout).

In this population, the inclusion of a CO group that had to move for supervised sessions was essential to assure that improvements obtained by the WT group could not be attributed to the active commuting necessary to get to the training sessions. Stretching was chosen as the control intervention because it is an alternative mode of physical activity that has been shown not to modify walking capacity nor cardiovascular function and regulation in patients with IC[22, 23]. Thus, CO group performed stretching classes for 30 min, twice a week. In each class, approximately 20 stretching exercises were performed for all body segments. Each exercise was executed 2-3 times with passive technique and maximal stretch maintained for 20 s.

Statistical analyses

The required sample sizes were calculated based on a pilot study that showed differences between the groups of: CRP = 500 pg/ml; TNF-α = 0.29 pg/ml; ICAM = 25 ng/ml;

VCAM = 85 ng/ml; LPO = 6.40 µM; NO = 1.78 µM; and CAT = 4.91 $nmol.min^{-1}.ml^{-1}$. Considering a power of 80% and an alpha error of 5%, the minimum sample size required was calculated to be 30 subjects.

Normality of data distribution and homogeneity of variance were evaluated, respectively, by Shapiro–Wilks and Levene tests. Baseline groups' characteristics were compared by *t* or chi-square tests. The effects of training were assessed via mixed 2-way (group vs. phase) ANOVAs (Statsoft, Statistic for Windows 4.3, Oklahoma, USA) and Newman–Keuls post-hoc tests were used when necessary. $P \leq .05$ was considered as significant and data were presented as mean ± SE.

RESULTS

The study flowchart is presented in Figure 1. Seventy-eight patients were contacted, 54 provided informed consent, 35 undertook the baseline assessments, and 32 completed the study (16 in each group), which represents a loss of 40.7% of the sample that had signed the informed consent. The baseline characteristics were similar between the groups (Table 1).

*** INSERT FIGURE 1***

*** INSERT TABLE 1***

WT increased COD (212 ± 15 vs 372 ± 48 m, P=.004) and TWD (572 ± 35 vs 947 ± 61 m, P<.001) while no change was observed in CO (COD = 231 ± 16 vs 191 ± 47 m, and TWD = 593 ± 73 vs 633 ± 75 m; P=.54 and P=.48, respectively).

Significant interactions were identified for SBP, HR and RPP (Table 2). These variables were similar between the groups at baseline and decreased significantly only after WT.

INSERT TABLE 2

Systemic biomarkers are shown in Figure 2. Significant interactions were identified for plasma NO (p=.001), SOD (p=.009), CAT (p=.004), LPO (p=.030), CRP (p=.007), TNF-α (p=.005), ICAM (p=.008) and VCAM (p<.001), showing that WT had a significant effect differently from CO on these variables. Baseline levels of these variables were similar between the groups. WT significantly increased NO, SOD and CAT, and prevented the increase of LPO observed in the CO group. Additionally, WT significantly decreased CRP, ICAM and VCAM, and prevented the increase in TNF-α observed in the CO group.

*** INSERT FIGURE 2***

Local muscle biomarkers are shown in Figure 3. Significant interactions were identified for muscle eNOS (p=.050), CAT (p=.036), LPO (p=.035), IL-6 (p=.043), CRP (p=.026), TNF-α (p=.017), ICAM (p=.008) and VCAM (p=.006), showing that WT had a significant effect different from CO on these variables. Baseline levels of these variables were similar between groups. WT significantly increased eNOS and CAT, and prevented the increase in LPO observed in the CO group. Additionally, WT significantly decreased IL-6 and CRP, and prevented the increase in TNF-α, ICAM and VCAM observed in the CO group.

INSERT FIGURE 3

DISCUSSION

The findings of this study were that, in IC patients, 12 weeks of submaximal WT: i) increased walking capacity (COD, TWD); ii) ameliorated cardiovascular function (decrease SBP, HR and RPP; iii) increased systemic and local NO bioavailability; iv) decreased systemic and local oxidative stress (blunted the increase in LPO observed in CO); and v) decreased systemic and local inflammation (decreased blood CRP, ICAM and VCAM; decreased muscle IL-6 and CRP; and blunted the increase in blood TNF- and muscle TNF-α, ICAM and VCAM observed in CO).

As expected, the employed submaximal WT protocol improved walking capacity and cardiovascular function[22]. COD and TWD improvements (75.5 and 65.5%, respectively) were similar to those obtained with supervised maximal WT protocols[9, 31]. Furthermore, the decrease of 10±2 mmHg in SBP obtained in this study may have clinical impact since a reduction of 5 mmHg is associated with decreased incidence of cardiovascular events[32, 33]. Similarly, studies using other walking protocols based on the individual pain threshold speed[24] also reported positive claudication, functional and hemodynamic adaptation[25, 34], corroborating the applicability of protocols that avoid maximum ischemia for managing patients with IC.

The novelty of the present investigation was the benefits of WT on NO bioavailability, oxidative stress and inflammation. The increases in plasma NO and muscle eNOS suggest a global effect of WT, enhancing whole body NO bioavailability. Systemic NO response is similar to previous studies[11, 12]. However, the present findings extended this knowledge to eNOS mRNA levels within the muscle affected by PAD. It is possible that increased local shear stress within active muscles during walking increased eNOS gene expression, leading to NO production and release to the blood[35, 36]. These responses may improve endothelial function, contributing to an improvement in walking capacity[22] and a better cardiovascular prognosis[37].

WT also increased systemic and local antioxidant defense as demonstrated by increases of blood SOD and CAT activities and muscle CAT activity. These increases may have prevented the increase in oxidative stress observed in CO (i.e. increase in LPO), reflecting a beneficial effect of WT on redox homeostasis. Previous studies reported controversial results regarding the effects of WT on oxidative stress biomarkers assessed in the blood[13, 14, 38]. Thus, the present results expand the knowledge by demonstrating that submaximal WT was able to increase antioxidant defense and prevent increased oxidative stress not only in blood but also inside the muscle. It is interesting to note that although CAT activity increased in both blood and muscle, muscle SOD activity did not increase. Increased SOD activity after training was observed mainly in muscles predominantly composed by oxidative fibers[39]. As patients with IC have reduced oxidative fibers within the affected muscles[40], this characteristic may explain, at least in part, the absence of increase in muscle SOD activity after WT. The mechanisms by which WT increased antioxidant response were not investigated, but it has been suggested that the oxidative stress generated during walking may activate pathways that lead to chronic adaptations[41].

A reduction in systemic inflammation with WT has also been previously reported[13-15, 18, 20], and the present study expands this finding to the muscle affected by the disease, the potential major source of inflammation in IC[26]. The mechanisms responsible for this anti-inflammatory adaptation were out of the scope of this study. However, a reduction in circulating immune cells related to inflammatory mediators may be involved, as aerobic training was reported to decrease the number of circulating monocytes and a decrease in TNF-α production[42, 43]. Potentially, WT may inhibit cytokine release, as muscle contractions increase IL-10 from regulatory T-cells[44] which inhibits the expression of pro-inflammatory cytokines[45]. Further, the improvement of walking capacity may promote fewer episodes of ischemia and

reperfusion throughout the day, resulting in less local inflammation[20]. Finally, the improvement in oxidative stress may also impact decreasing inflammation[46].

The effects of WT on NO bioavailability, oxidative stress and inflammation may have important clinical implications for IC patients. Increased NO bioavailability may improve endothelial function, blood flow and vasodilatory capacity[22], contributing to increase walking capacity, mobility and independent living. As endothelial dysfunction[47], oxidative stress[48] and inflammation[21] are associated with morbimortality and disease progression in IC, their improvement may reduce cardiovascular risk and ameliorate prognosis. Thus, the present study provided novel evidence (systemic and local) about the benefic effects of a submaximal WT on key factors associated with PAD progression and cardiovascular risk (a complete hypothetic model can be seen in graphical abstract).

The main practical implication of the present results is to support the use of submaximal WT protocols in patients with PAD and IC. Maximal WT (i.e. near-maximal claudication symptoms) has been consistently recommended[9, 10] for clinical practice due to walking capacity improvement obtained[9]. However, high levels of pain, ischemia and reperfusion induced by maximal effort increase oxidative stress and inflammation during and after walking[11, 20], which may favor atherosclerotic process, contributing to PAD progress. By showing that a submaximal WT protocol can increase walking capacity and improve oxidative stress and inflammation, the present results support its use in the clinical treatment of PAD and IC as suggested by the recent guidelines from Society for Vascular Surgery/American College of Cardiology/American Heart Association[8-10]. Future studies, however, should directly compare maximal and submaximal WT protocols effects on these outcomes.

This study has limitations that should be acknowledged. It only included men with specific disease characteristics, which limits the extrapolation of these results. There is evidence that women with PAD and IC present distinct walking capacity[49, 50], biomarkers[50],

and cardiovascular variables[51], and respond differently to training[52, 53] than men. Thus, future studies should examine the impact of the proposed WT protocol in women and compare it with men's responses. Additionally, patients at other stages of the disease may present less or more difficult to perform WT, which may produce different results that should be addressed in the future. Patients receiving medications that directly affected cardiac autonomic regulation were excluded due to the possibility that these drugs blunt adaptations to exercise[54, 55], which could minimize biomarkers responses to training. As many patients use these medications, future studies should broaden their examination to include such patients and check whether they present the similar adaptations to WT. Several muscle biomarkers were measured by mRNA expression that despite being widely used and represent gene transcription, may or may not be an indicative of functional protein. Finally, although the sample size was adequate for the whole groups' comparisons, it does not allow stratifications within the groups, which could strength the discussion. Futures studies are encouraged to use sample sizes that allow groups stratification.

In conclusion, a 12-week of WT at an intensity of pain threshold improves NO bioavailability and decreased systemic and local oxidative stress and inflammation in patients with PAD and IC.

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CONFLICTS OF INTEREST

There is no conflict of interest.

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TABLES

596 Table 1. Baseline characteristics of patients in the control (CO) and walking training (WT)
597 groups.

	CO (N = 16)	WT (N = 16)	P
Age (years)	69 ± 3	66 ± 2	.36
Body mass index (kg/m^2)	25.4 ± 0.9	25.7 ± 0.8	.79
Diagnosis of PAD			
ABI	0.62 ± 0.04	0.64 ± 0.03	.67
COD (m)	231 ± 22	212 ± 20	.51
TWD (m)	593 ± 88	572 ± 47	.83
Cardiovascular measures			
Systolic blood pressure (mmHg)	137 ± 5	136 ± 2	.78
Diastolic blood pressure (mmHg)	79 ± 2	83 ± 2	.21
Heart rate (bpm)	75 ± 3	70 ± 2	.15
Comorbidities			
Obesity – n (%)	1 (6.2)	1 (6.2)	.99
Hypertension – n (%)	12 (75.0)	11 (68.8)	.69
Diabetes Mellitus – n (%)	5 (31.2)	4 (25.0)	.69
Dyslipidemia – n (%)	14 (87.5)	16 (100.0)	.14
Current Smokers – n (%)	4 (25.0)	6 (37.5)	.45
Alcohol intake – n (%)	4 (25.0)	6 (37.5)	.43
Chronic obstructive pulmonary disease – n (%)	1 (6.2)	0 (0.0)	.31
Heart Disease/Stroke – n (%)	3 (18.7)	3 (18.7)	.99

Atrial Fibrilation – n (%)	0 (0.0)	0 (0.0)	---
Valve disorder – n (%)	0 (0.0)	0 (0.0)	---
Drug Therapy			
Aspirin – n (%)	15 (93.8)	15 (93.8)	.99
Statin – n (%)	14 (87.5)	16 (100.0)	.14
Oral hypoglycemic – n (%)	5 (31.2)	4 (25.0)	.69
Antihypertensive agent			
Angiotensin converting enzyme inhibitor – n (%)	6 (37.5)	9 (56.2)	.48
Diuretic – n (%)	6 (37.5)	2 (12.5)	.10
Dihydropyridine calcium channel antagonist – n (%)	1 (6.2)	5 (31.2)	.078
Angiotensin receptor antagonists – n (%)	3 (18.7)	0 (0.0)	.074

598 Data are mean ± SE or n and percentage (%). PAD – peripheral artery disease; ABI – ankle-
599 brachial index; COD – claudication onset distance; TWD – total walking distance. Obesity
600 defined as body mass index $\geq$ 30 kg/m^2. Diabetes, hypertension, dyslipidemia, chronic
601 obstructive pulmonary disease, heart disease and stroke defined by previous diagnosis.
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612 Table 2. Cardiovascular variables measured at baseline and after 12 weeks for the control (CO)
613 and walking training (WT) groups.

	CO (N = 16)		WT (N = 16)		
	Baseline	12 weeks	Baseline	12 weeks	P
Cardiovascular function					
Systolic BP (mmHg)	136 ± 4	141 ± 5	137 ± 3	126 ± 3*#	<.001
Diastolic BP (mmHg)	75 ± 2	76 ± 2	79 ± 2	77 ± 2	.082
HR (bpm)	69 ± 2	69 ± 3	66 ± 2	62 ± 2*#	.038
RPP (bpm.mmHg)	9293 ± 356	9684 ± 512	8998 ± 357	7798 ± 365*#	<.001

614 Data are mean ± SE. BP – blood pressure; HR – heart rate; RPP – rate pressure product. * =
615 different from baseline within the group (P≤.05); # = different from CO at the same study phase
616 (P≤.05).
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FIGURE LEGENDS

Graphical abstract. Hypothetic model for the local and systemic NO bioavailability, oxidative stress and inflammation responses after walking training in patients with IC. Oxygen – O2; Endothelial nitric oxide synthase – eNOS; ROS – reactive oxygen species; Nitric oxide – NO; Superoxide dismutase – SOD; Catalase – CAT; Lipid peroxidation – LPO; Baroreflex sensitivity – BRS; Sympathetic nervous system – SNS; Parasympathetic nervous system – PNS; Blood pressure – BP. → maintain; ↓ decrease; ↑ increase.

Figure 1. Study flowchart: Enrolment, Randomization, Interventions and Follow-up.

Figure 2. Blood biomarkers of nitric oxide bioavailability, oxidative stress and inflammation measured at baseline and after 12 weeks in the control (CO – N = 16) and walking training (WT – N = 16) groups. (A) nitric oxide – NO; (B) superoxide dismutase – SOD; (C) catalase – CAT; (D) lipid peroxidation – LPO; (E) interleukin-6 – IL-6; (F) C-reactive protein – CRP; (G) tumour necrosis factor-α – TNF-α; (H) intercellular adhesion molecule – ICAM; and (I) vascular cell adhesion protein – VCAM. * = different from baseline within the group (P≤.05); # = different from the CO at the same study phase (P≤.05).

Figure 3. Muscle biomarkers of nitric oxide bioavailability, oxidative stress and inflammation measured at baseline and after 12 weeks in the control (CO – N = 16) and walking training (WT – N = 16) groups. (A) endothelial nitric oxide synthase – eNOS; (B) superoxide dismutase – SOD; (C) catalase – CAT; (D) lipid peroxidation – LPO; (E) interleukin-6 – IL-6; (F) C-reactive protein – CRP; (G) tumour necrosis factor-α – TNF-α; (H) intercellular adhesion molecule – ICAM; (I) vascular cell adhesion protein – VCAM. * = different from baseline within the group (P≤.05); # = different from the CO at the same study phase (P≤.05).

FIGURE 1

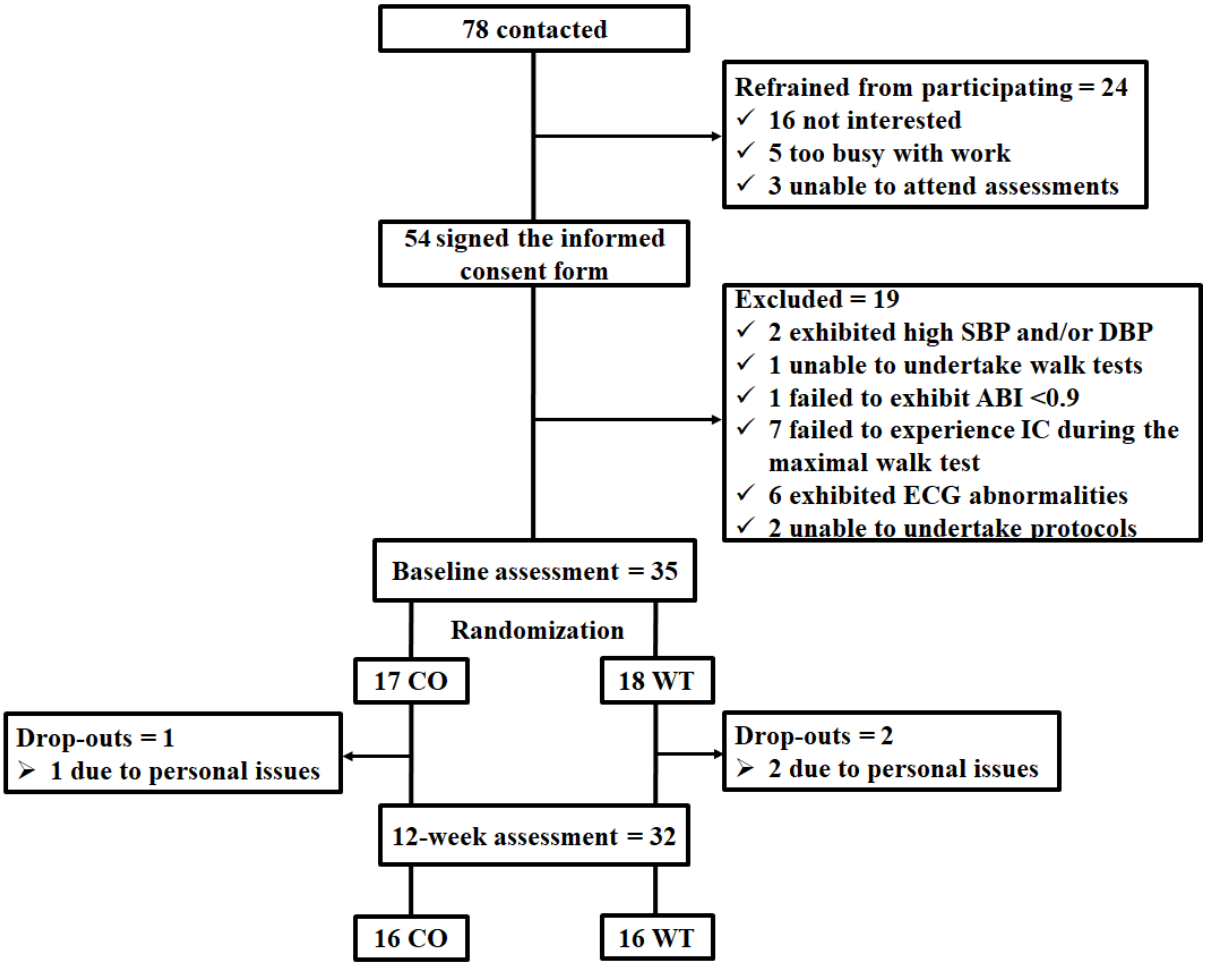
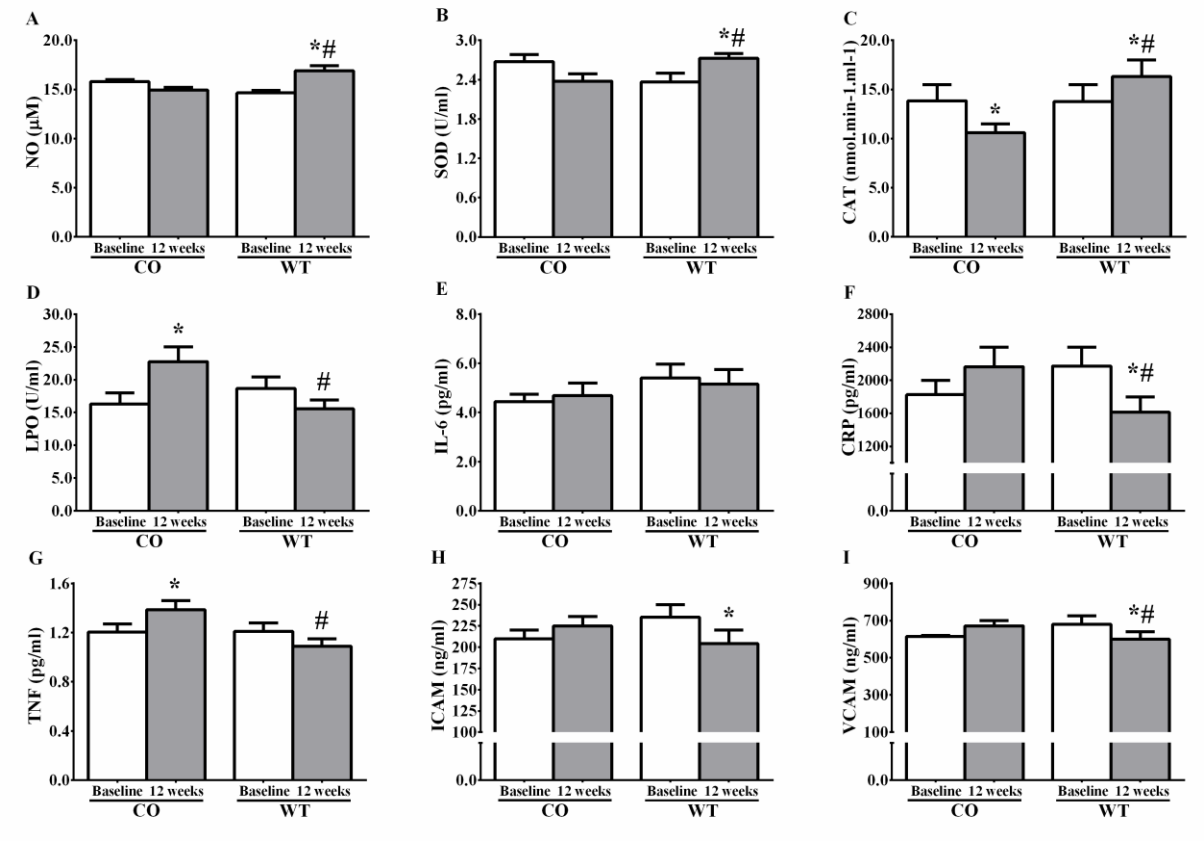
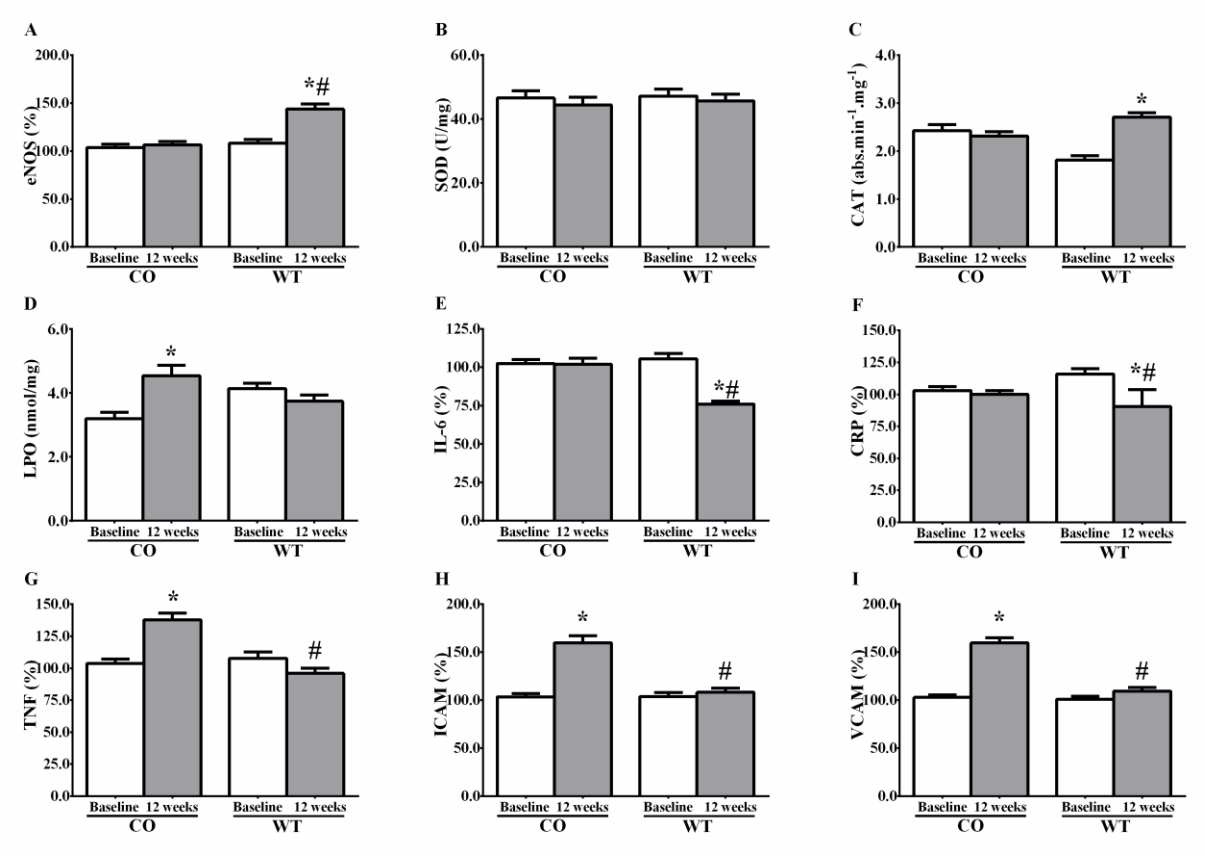

78 contacted
Refrained from participating = 24
✓ 16 not interested
✓ 5 too busy with work
✓ 3 unable to attend assessments
54 signed the informed consent form
Excluded = 19
✓ 2 exhibited high SBP and/or DBP
✓ 1 unable to undertake walk tests
✓ 1 failed to exhibit ABI <0.9
✓ 7 failed to experience IC during the maximal walk test
✓ 6 exhibited ECG abnormalities
✓ 2 unable to undertake protocols
Baseline assessment = 35
Randomization
17 CO
18 WT
Drop-outs = 1
➢ 1 due to personal issues
Drop-outs = 2
➢ 2 due to personal issues
12-week assessment = 32
16 CO
16 WT

FIGURE 2


A
NO (µM)
20.0
15.0
10.0
5.0
0.0
*#
Baseline 12 weeks
CO
Baseline 12 weeks
WT
B
SOD (U/ml)
3.0
2.4
1.8
1.2
0.6
0.0
*#
Baseline 12 weeks
CO
Baseline 12 weeks
WT
C
CAT (nmol.min-1.ml-1)
20.0
15.0
10.0
5.0
0.0
*
*#
Baseline 12 weeks
CO
Baseline 12 weeks
WT
D
LPO (U/ml)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
#
Baseline 12 weeks
CO
Baseline 12 weeks
WT
E
IL-6 (pg/ml)
8.0
6.0
4.0
2.0
0.0
Baseline 12 weeks
CO
Baseline 12 weeks
WT
F
CRP (pg/ml)
2800
2400
2000
1600
1200
0.0
*#
Baseline 12 weeks
CO
Baseline 12 weeks
WT
G
TNF (pg/ml)
1.6
1.2
0.8
0.4
0.0
*
#
Baseline 12 weeks
CO
Baseline 12 weeks
WT
H
ICAM (ng/ml)
275
250
225
200
175
150
125
100
0.0
*
Baseline 12 weeks
CO
Baseline 12 weeks
WT
I
VCAM (ng/ml)
900
700
500
300
100
0.0
*#
Baseline 12 weeks
CO
Baseline 12 weeks
WT

677 **FIGURE 3**



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