The Effects of Cilostazol on Exercise-induced Ischaemia—reperfusion Injury in Patients with Peripheral Arterial Disease☆

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Abstracts *Objectives:* Cilostazol improves walking distance in peripheral arterial disease (PAD) patients. The study objectives were to assess the effects of cilostazol on walking distance, followed by the additional assessment of cilostazol on exercise-induced ischaemia—reperfusion injury in such patients.
Methods: PAD patients were prospectively recruited to a double-blinded, placebo-controlled trial. Patients were randomised to receive either cilostazol 100 mg or placebo twice a day. The primary end-point was an improvement in walking distance. Secondary end-points included the assessment of oxygen-derived free-radical generation, antioxidant consumption and other markers of the inflammatory cascade. Initial and absolute claudication distances (ICDs and ACDs, respectively) were measured on a treadmill. Inflammatory response was assessed before and 30 min post-exercise by measuring lipid hydroperoxide, ascorbate, α-tocopherol, β-carotene, P-selectin, intracellular and vascular cell-adhesion molecules (I-CAM and V-CAM), thromboxane B_2 (TXB_2), interleukin-6, interleukin-10, high-sensitive C-reactive protein (hsCRP), albumin—creatinine ratio (ACR) and urinary levels of p75TNF receptor. All tests were performed at baseline and 6 and 24 weeks.
Results: One hundred and six PAD patients (of whom 73 were males) were recruited and successfully randomised from December 2004 to January 2006. Patients who received cilostazol demonstrated a more significant improvement in the mean percentage change from baseline in ACD (77.2% vs. 26.6% at 6 weeks, $p = 0.026$ and 161.7% vs. 79.0% at 24 weeks, $p = 0.048$) as compared to the placebo. Cilostazol reduced lipid hydroperoxide levels compared to a placebo-related increase before and after exercise (6 weeks: pre-exercise: −11.8% vs.

+5.8%, $p = 0.003$ and post-exercise: −12.3% vs. +13.9%, $p = 0.007$ and 24 weeks: pre-exercise −15.5% vs. +12.0%, $p = 0.025$ and post-exercise: −9.2% vs. +1.9%, $p = 0.028$). β-Carotene levels were significantly increased in the cilostazol group, compared to placebo, before exercise at 6 and 24 weeks (6 weeks: 34.5% vs. −7.4%, $p = 0.028$; 24 weeks: 34.3% vs. 17.7%, $p = 0.048$). Cilostazol also significantly reduced P-selectin, I-CAM and V-CAM levels at 24 weeks as compared to baseline ($p < 0.05$). There was no difference between treatment groups for ascorbate, α-tocopherol, interleukin-6 and -10, hsCRP and p75TNF receptor levels.
Conclusions: Cilostazol significantly improves ACD, in addition to attenuating exercise-induced ischaemia—reperfusion injury, in PAD patients.

Intermittent claudication has been likened to ischaemia followed by reperfusion, which usually occurs during lower limb-revascularisation procedures, leading to an ischaemia—reperfusion injury.[1,2] This concept was first reported, in 1960, when two patients with acutely ischaemic lower limbs developed massive ischaemic myopathy and myoglobinuria after re-vascularisation.[3] Following ischaemia, the muscles may be salvaged by reperfusion. However, the re-introduction of oxygen to hypoxic muscles can also lead to damage by oxygen-derived free radicals (ODFR). Patients with intermittent claudication have been reported to demonstrate a similar exercise-induced inflammatory response with elevation in lipid peroxide and plasma thromboxane (TX) levels as early as 15 min following exercise associated with a corresponding reduction in antioxidant levels.[1,2,4] Khaira *et al.*,[4] in 1995, proposed that the presence of ischaemia—reperfusion events in patients with intermittent claudication leads to a systemic increase in vascular permeability as a result of endothelial injury or dysfunction, combined with the localised production of inflammatory mediators, including TX, as well as neutrophil, activation. Although initially protective, these mechanisms can become excessive, resulting in damaged tissue, impaired organ function and an abnormal fibro-proliferative response.[5]

Cilostazol, a 2-oxo-quinolone derivative, is a reversible, selective inhibitor of phosphodiesterase-3A (PDE-3A) with antiplatelet, antithrombotic, vasodilatory, antimitogenic and cardiogenic properties.[6] Cilostazol appears to increase intracellular cyclic adenosine monophosphate (cAMP) by inhibiting PDE-3A whilst reducing adenosine uptake into cells.[6,7] The adenosine augments the cAMP-elevating effect of PDE-3 inhibition in platelets and smooth muscle cells.[6]

Cilostazol has been shown to have possible attenuating effects on the inflammatory cascade from review of *in vitro*, animal and case-controlled human studies. Hakaim *et al.* demonstrated that a cilostazol infusion 10 min before re-vascularising an ischaemic leg prevented anterior compartment pressure increases.[8] In addition, Otsuki *et al.* reported an inhibition of tumour necrosis factor-α (TNF-α)- and vascular cell-adhesion molecule-1 (V-CAM-1)-mediated products with cilostazol. This inhibition may be via cAMP elevation in human vascular endothelial cells, thus preventing atherosclerosis.[9] Cilostazol also exerts beneficial effects on platelet-derived growth factor, monocyte chemo-attractant protein-1 and hepatocyte growth factor.[10] In Type-2 diabetes patients, cilostazol significantly reduces platelet-derived microparticles, activated platelets and soluble adhesion molecules.[11] Lee *et al.* suggested that cilostazol may also reduce current and future cardiovascular events through improvements in lipid profiles accompanied by a reduction in interleukin-6 (IL-6) levels.[12] Therefore, it is postulated that all of these cilostazol-mediated effects on the inflammatory cascade could lead to improvements both in lower limb function and longer-term cardiovascular outcomes.

We conducted a prospective double-blinded, randomised, placebo-controlled clinical trial to assess the hypothesis that cilostazol improves walking distances as well as attenuates exercise-induced ischaemia—reperfusion injury in patients with intermittent claudication.

Methods

Patient recruitment and randomisation

Male and female (non-pregnant) patients between the ages of 30 and 90 years were eligible for recruitment to this single-centre clinical trial between December 2004 and January 2006. The study was approved by the local Research Ethics (Application No.147/04) and Hospital Clinical Governance Committees (BCH04059MO/L). The clinical trial was also registered with the European Clinical Trials Database (EUDRACT 2004-004846-42).

Patients were suitable for inclusion if they had intermittent claudication, defined as reproducible muscle discomfort in the lower limb produced by exercise and relieved by rest, with an ankle—brachial index less than 0.9, which had been stable on optimal medical therapy that included antiplatelet and lipid-lowering medication, cardiovascular risk assessment and treatment (e.g., hypertension) and smoking-cessation therapy combined with the provision of exercise advice for a period of 3 months.[13] Although exercise advice was provided during vascular consultations, there was no provision for accurate monitoring of exercise compliance in the community.[13] Patients were excluded if they had current or previous acute or critical limb ischaemia,[13] severe claudication that prohibited the use of treadmill testing as determined during pre-recruitment vascular assessments, an endovascular or surgical procedure within the preceding 6 months or a non-atherosclerotic co-morbidity that had limited their walking before the onset of claudication pain. Exclusion criteria also included a predisposition to bleeding, a history of uncontrolled cardiac, respiratory, renal or liver disease

or the use of omeprazole and diltiazem, as per cilostazol safety guidelines.

A power calculation for patient numbers based on a marker of inflammatory response as the primary outcome measure was desired. However, during protocol design, a suitable reference study assessing the exercise-induced inflammatory response in patients with intermittent claudication could not be identified. The power calculation for the study was, therefore, calculated from walking-distance indices derived from Dawson et al.[14] for two separate patient populations (normoglycaemic and diabetic).

Therefore, 30 patients per treatment group completing the trial would have a 90% power to detect a statistically significant ($p < 0.05$; two-tailed) difference in the change in maximal walking distance, between groups, of a magnitude of 45 m. This assumes a mean baseline maximal walking distance of 150 m and standard deviation of 54 m and an improvement of 30% in one group and no change in the other.[14] It was assumed that approximately 20% of patients would withdraw from the study due to an adverse reaction to the study medication (headache, diarrhoea or palpitations). A further 12 patients would be recruited to a total of 72 patients for each of the normoglycaemic and diabetic populations to account for drop-outs. Therefore, a total of 144 patients were required. Our study was performed on an intention-to-treat principle.

The primary end-points were, therefore, improved in initial and absolute walking distances. Secondary end-points included the assessment of ODFR generation, antioxidant consumption and other markers of the inflammatory cascade, including selectins, cell-adhesion molecules, TX, ILs and high-sensitive C-reactive protein (hsCRP).

Two baseline assessments, 4 weeks apart, facilitated stabilisation before treatment allocation. The mean of these two values was calculated as the baseline reading. Following written informed consent, patients were randomised into groups of four to receive cilostazol (100 mg) or matched placebo twice a day orally. Patient-treatment randomisation and allocation was performed independently by the Department of Research Pharmacology in the Belfast City Hospital. Both, the patient and the primary investigator, were blinded to study-drug allocation, which was completed using the sealed-envelope method. Follow-up clinical assessments were performed at 6 and 24 weeks. Study-drug un-blinding was performed at the end of the study, following the completion of all clinical assessments and laboratory analyses for all patients.

Walking assessment

Each patient rested for 30 min before commencement of testing using the same calibrated treadmill (Mortara Xscribe Treadmill, Mortara Instrument, Inc., US) at a constant speed of 2 mph (3.2 kph) and a 10% gradient with the primary investigator (MOD).[13] The distance when the onset of claudication symptoms was recorded as the initial claudication distance (ICD). The patient was advised to continue walking until claudication pain precluded continuation, which was defined as the absolute claudication distance (ACD).

Assessment of inflammatory response

All samples were collected after 30 min of rest before exercise and at 30 min following exercise. Plasma samples for ascorbate were centrifuged immediately. Urine was collected for analysis of p75TNF receptors. Serum was collected for all other assays. Serum was allowed to clot for 15 min and was then centrifuged. All samples were transferred to 2-ml tubes (Sarstedt, Ireland) and stored at $-80\,^{\circ}C$. Intra- and inter-assay coefficients of variation were within satisfactory limits according to the manufacturers' guidelines.

Plasma lipid hydroperoxides
These were measured spectrophotometrically using the ferrous oxidation–xylenol orange version 1 assay (FOX 1).

Ascorbate
Concentrations were determined by the enzymatic oxidation of ascorbic acid and subsequent quinoxaline formation. The fluorescent derivative was measured on the Cobas Fara centrifugal analyser with fluorescent attachment.

Lipid-soluble antioxidants – α-tocopherol and β-carotene
These levels were measured using a high-performance liquid chromatography technique (HPLC).

P-selectin
Human serum P-selectin levels were determined using a quantitative sandwich immunoassay technique (R&D Systems Europe, Abingdon).

Intracellular and vascular cell-adhesion molecule and interleukins-6 and -10
These were measured using enzyme-linked immunosorbent assay (ELISA) kits from Eli-pair (Diaclone, Besançon, France) that were commercially available.

11-dehydro thromboxane B_2 (TXB$_2$)
TXB$_2$ was measured using the ACE® Competitive Enzyme Immunoassay (EIA) and an estimate of TXA$_2$ levels (Cayman Chemical, MI, USA) was provided.

Urinary p75TNF receptor
This receptor was measured by using a commercially available EASIA kit from Biosource (Nivelles, Belgium).

High-sensitive C-reactive protein (hsCRP)
This was measured using the commercially available quantex CRP Ultra Sensitive Biokit Assay (Biokit, Barcelona, Spain).

Statistical analysis

Descriptive statistics for patient demographics, medical co-morbidities and examination parameters were calculated as mean and standard error of the mean (SEM) or median and interquartile ranges (IQR). Quantitative end-points were analysed at the four assessment time points (-4, 0, 6 and 24 weeks). Mann–Whitney U (MWU) test was used to compare differences between the cilostazol and placebo

groups. Wilcoxon signed-rank test (WSR) was used to compare differences within one treatment group to the baseline. All statistics were two sided and a p-value of <0.05 was considered significant. Statistical analysis was performed using the SPSS statistical package (Version 12, SPSS® Inc., Chicago, USA).

Results

Recruitment

A total of 561 normoglycaemic and 113 diabetic medically fit patients, with previous optimisation of medical therapy, were assessed for potential trial recruitment. Of these participants, 92 normoglycaemic and 34 diabetic patients decided not to enrol citing personal reasons, while 389 and 53 patients, respectively, did not attend the research clinic despite the availability of multiple clinic appointments. Therefore, 106 peripheral arterial disease (PAD) patients (73 male) were recruited from December 2004 to January 2006 (median age: 66.5 years, range: 37–86 years). Twenty-six patients were diabetic (Table 1). The cilostazol and placebo treatment groups were not significantly different with regard to baseline characteristics (Table 2).

Walking assessment

Patients who received cilostazol demonstrated a more significant improvement in the mean percentage change from the baseline in ACD (77.2% vs. 26.6% at 6 weeks, $p = 0.026$ and 161.7% vs. 79.0% at 24 weeks, $p = 0.048$) as compared to the placebo. A mean percentage improvement from the baseline in ICD was also demonstrated in both the treatment groups (38.9% vs. 22.7% at 6 weeks, $p = 0.06$ and 67.0% vs. 51.6%, $p = 0.63$ at 24 weeks). However, this was not significant (Fig. 1).

Inflammatory response

Lipid hydroperoxides
The baseline pre- and post-exercise levels were similar in both groups. There was a significant reduction in the cilostazol group before and after exercise at 6 and 24 weeks (Table 3). A significant reduction was identified in the mean percentage change from the baseline for the cilostazol group as compared to an increase in the placebo group (6 weeks: pre-exercise: -11.8% vs. $+5.8\%$, $p = 0.003$ and post-exercise: -12.3% vs. $+13.9\%$, $p = 0.007$; 24 weeks: pre-exercise -15.5% vs. $+12.0\%$, $p = 0.025$ and post-exercise: -9.2% vs. $+1.9\%$, $p = 0.028$).

Ascorbate, corrected α-tocopherol and β-carotene
There was no difference between the cilostazol and placebo groups at any time point (Table 3). A trend for a higher residual ascorbate level appeared in the cilostazol group at 24 weeks (pre-exercise: 30.0% vs. 10.0%, $p = 0.24$ and post-exercise: 23.2% vs. 2.4%, $p = 0.55$). β-Carotene levels were significantly increased in the cilostazol group, as compared to the placebo group, before exercise at 6 and 24 weeks (6 weeks: 34.5% vs. -7.4%, $p = 0.028$; 24 weeks: 34.3% vs. 17.7%, $p = 0.048$).

P-Selectin, intracellular cell-adhesion molecule (I-CAM), V-CAM and 11-dehydro TXB$_2$
There were no significant differences in P-selectin, I-CAM, V-CAM and 11-dehydro TXB$_2$ levels between the groups at any time point (Table 4).

However, P-selectin levels were reduced in the cilostazol group before and after exercise when the 24-week levels were compared to baseline levels, with no difference seen in the placebo group (cilostazol: pre-exercise; $p = 0.001$ and post-exercise $p < 0.001$) (placebo: pre-exercise $p = 0.16$ and post-exercise $p = 0.18$) (Fig. 2). I-CAM and V-CAM levels

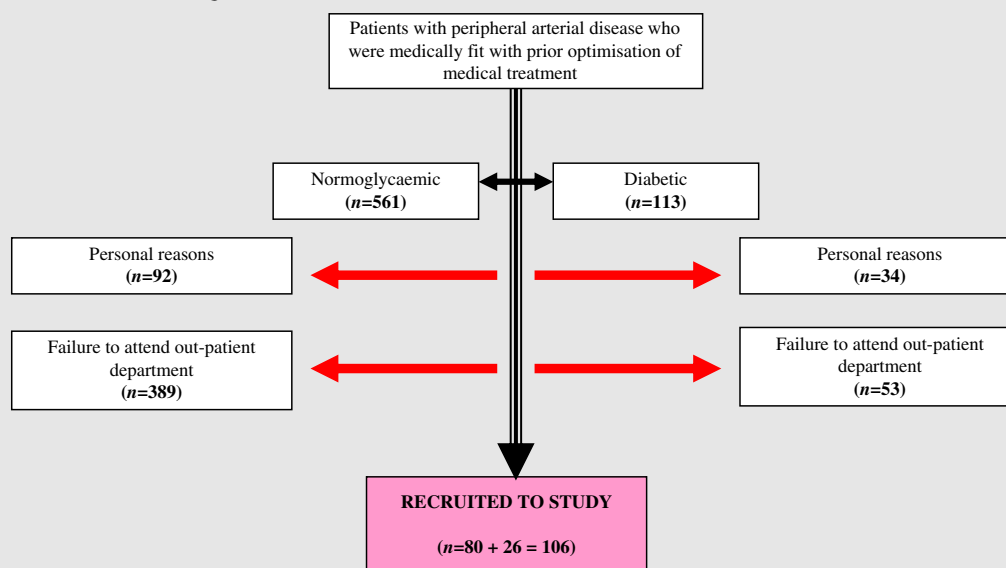Table 1   Patient recruitment algorithm

Table 2 Baseline patient demographics, co-morbidities and walking status (median, IQR unless stated as range) (Mann—Whitney *U* test)

Variable	Cilostazol	Placebo	*p*-Value
Number of patients	51	55	0.83
Sex	M = 34, F = 17	M = 39, F = 16	0.29
Median age in years (range)	64.2 (37–86)	66.1 (39–80)	0.29
Past medical history			
Hypertension	32 (62.7%)	37 (67.3%)	0.63
Hypercholesterolaemia	39 (76.5%)	42 (76.4%)	0.99
Diabetes	12 (23.5%)	14 (25.5%)	1.0
Angina	7 (13.7%)	3 (5.5%)	0.25
Myocardial infarction	9 (17.6%)	7 (12.7%)	0.46
Coronary artery bypass grafting	3 (5.9%)	5 (9.1%)	0.91
Cerebrovascular accident	3 (5.9%)	3 (5.5%)	0.78
Carotid endarterectomy	2 (3.9%)	3 (5.5%)	0.28
Abdominal aortic aneurysm	0 (0%)	1 (1.8%)	1.0
Previous vascular arterial bypass/endovascular intervention	4 (7.8%)	6 (10.9%)	0.74
Medical therapy			
Aspirin	37	44	0.94
Clopidogrel	12	8	0.65
Warfarin	2	3	0.97
Statin	48	52	0.93
ACE inhibitors	15	18	0.72
ACE II antagonists	8	4	0.18
B-blocker	9	14	0.34
Calcium antagonist	9	12	0.60
Diuretic	18	18	0.78
Walking capabilities			
Limb affected	L = 8, R = 8, Both = 35	L = 6, R = 7, Both = 42	0.72
Duration of symptoms in years	2.0 (1.0–5.5)	3.0 (1–5)	0.59
Initial claudication distance (metres)	69.7 (50.1–94.8)	63.9 (45.2–85.8)	0.67
Absolute claudication distance (metres)	144.4 (99.7–204.3)	138.6 (101.7–193.8)	0.44
Index limb ABI	0.77 (0.65–0.89)	0.74 (0.52–0.88)	0.10
Smoking history			
Smoking status			
Non-smoker	5	3	0.43
Ex-smoker	23	22	
Current smoker	23	30	
Packs cigarettes/day	1 (1–1.63)	1 (0.75–2.25)	0.49
Smoking duration (years)	40 (32.5–50)	40 (20–55)	0.11
Pack years	42 (33–63)	40 (20–50)	0.50

were reduced in the cilostazol group before exercise and in the I-CAM levels after exercise when the 24-week levels were compared to baseline levels, with no difference in the placebo group (cilostazol pre-exercise: I-CAM $p = 0.003$ and V-CAM $p = 0.016$ and post-exercise: I-CAM $p = 0.04$ and V-CAM $p = 0.16$). There was a reducing trend in the cilostazol group at 6 weeks when the mean percentage change in 11-dehydro TXB$_2$ levels was compared to the baseline.

Interleukin-6, interleukin-10, p75TNF receptor and hsCRP
There was no significant difference between the cilostazol and placebo groups at any time point (Table 5). However, a clear disparity in the IL levels between the treatment groups was demonstrated, due to a large number of equivocal or sub-threshold readings.

Albumin—creatinine ratio (ACR)
The baseline pre- and post-exercise ratios were similar between the study groups. There was a significant reduction in the ACR before and after exercise at 6 weeks (0.70 μg mg^{-1} vs. 1.35 μg mg^{-1}, $p = 0.039$ and 1.30 μg mg^{-1} vs. 2.70 μg mg^{-1}, $p = 0.043$) and before exercise at 24 weeks (0.60 μg mg^{-1} vs. 1.50 μg mg^{-1}, $p = 0.017$) when the cilostazol group was compared to the placebo group (Table 5).

Study completion

A total of 91 patients completed the clinical trial. This included 15 patients who failed to complete the study, which corresponded to a 14.2% drop-out rate (cilostazol: 8
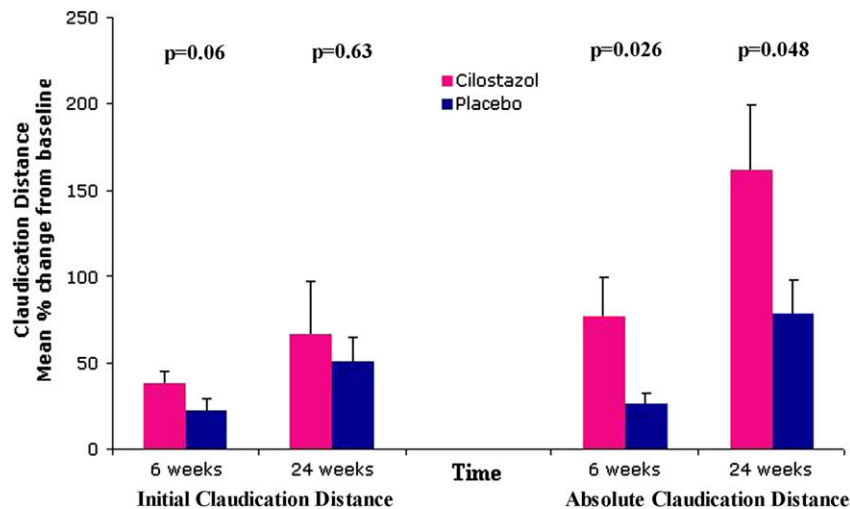
Figure 1 Mean percentage change from baseline in initial and absolute walking distances at 6 and 24 weeks (Mean, SEM) (Mann—Whitney U test).

out of 51; 15.7% and placebo: seven out of 55; 12.7%). In the cilostazol group, six patients withdrew because of side effects, one patient was non-compliant with treatment after the 6th-week assessment point and one patient had a serious adverse event unrelated to the cilostazol treatment due to a gastrointestinal bleed secondary to an increased consumption of alcohol whilst on holiday after 23 weeks in the study. In the placebo group, two patients withdrew due to side effects, two patients were withdrawn after the 6th-week assessment by the primary investigator due to the onset of critical ischaemia, two patients were non-compliant with treatment after the 6th-week assessment point and one patient had a serious adverse event secondary to the addition of two additional antihypertensive agents, which resulted in acute renal failure that settled with conservative management. Following completion of the clinical trial, patients were continued on cilostazol therapy, if appropriate, or commenced on cilostazol if they had been prescribed the placebo treatment. All patients were subsequently reviewed in the routine vascular outpatient clinic thereafter as ethical approval only facilitated inclusion of study assessments at the 6- and 24-week time points.

Discussion

Ischaemia—reperfusion injury can occur in patients with lower limb ischaemia during walking and resting and after arterial bypass surgery.[1] Elevations in lipid peroxide and plasma TX levels have been shown to occur 15 min following exercise that is associated with a corresponding reduction in antioxidant levels.[1,4] Khaira et al.[4] demonstrated that this ischaemia—reperfusion injury leads to a systemic increase in vascular permeability due to endothelial injury combined with localised production of pro-inflammatory mediators.

This study demonstrated lower lipid hydroperoxide levels in the cilostazol group, compared to an actual mean percentage increase from the baseline in the placebo group. This reduction was identified at 6 weeks and

continued to be present until the 24th-week assessment, similar to other reports.[15] In contrast to this study, all other studies were performed on animal models or in in vitro laboratory-based human cell lines. These lipid hydroperoxide changes may be related to a cilostazol-mediated improvement in lipid homeostasis, since modified low-density lipoprotein (LDL) is fundamental to the process of lipid peroxidation.[12] Although it is unclear why the placebo group demonstrated an increase in lipid hydroperoxide levels, possible reasons include an increase in oxidative stress secondary to an increase in walking distance (26.6% at 6 weeks and 79% at 24 weeks). These effects in the placebo group also demonstrate that exercise does lead to activation of the inflammatory cascade via free-radical generation. However, the longer-term consequences of an exercise-induced inflammatory response in claudicating patients require further study, but our data demonstrate that cilostazol may have an important therapeutic role.

The oxidative damage associated with ODFR may also lead to the consumption of endogenous antioxidants, such as ascorbate and α-tocopherol, which act as ODFR scavengers to prevent oxidation of proteins and lipids.[16] PAD patients have lower baseline vitamin C concentrations, especially those with a low ACD. A low concentration of vitamin E has also been shown to correlate with maximal lipid peroxidation after lower limb arterial bypass.[16] The oral administration of vitamins C and E reduces exercise-induced oxidative injury and interferes with the expression of other inflammatory mediators such as selectins and I-CAM.[17] The lack of significance in ascorbate concentrations in this study may be due to these antioxidants requiring a longer duration of therapy before any noticeable change occurs. This could explain the minimal percentage change detected at 6 weeks, while an increase in the cilostazol group was detected at 24 weeks.

Other researchers have reported that the resistance of LDLs to oxidation may not actually correlate with vitamin E content and that other lipid-soluble antioxidants, such as ubiquinol-10, may be a more important first-line antioxidant.[18] The changes in all of these antioxidants may not be

Table 3 Lipid hydroperoxides and antioxidant levels before and after exercise at baseline, 6 and 24 weeks (median, IQR) (Mann—Whitney - U test)

Parameter	Assessment	Pre-exercise			Post-exercise		
		Cilostazol	Placebo	p-Value	Cilostazol	Placebo	p-Value
Lipid hydroperoxide (nmol/l)	Baseline	0.55 (0.36—0.81)	0.63 (0.43—0.95)	0.26	0.61 (0.34—0.79)	0.64 (0.46—0.89)	0.27
	6 Weeks	0.46 (0.33—0.61)	0.64 (0.47—0.85)	0.001	0.49 (0.37—0.60)	0.68 (0.53—0.86)	<0.001
	24 Weeks	0.45 (0.33—0.61)	0.61 (0.46—0.82)	0.001	0.46 (0.32—0.61)	0.62 (0.51—0.88)	0.005
Ascorbate (μmol/l)	Baseline	20.5 (10.2—42.0)	28.6 (11.4—42.3)	0.63	22.7 (9.5—42.8)	30.2 (14.3-47.4)	0.54
	6 Weeks	14.2 (3.2—40.9)	23.0 (5.5—35.8)	0.54	16.1 (5.3—47.7)	26.1 (12.4—41.3)	0.37
	24 Weeks	24.8 (3.2—42.1)	24.2 (5.6—45.4)	0.85	27.4 (4.5—43.7)	27.2 (6.5—49.1)	0.63
Corrected α-tocopherol (μmol/l)	Baseline	6.77 (5.55—8.13)	6.43 (5.76—7.25)	0.35	6.46 (5.38—7.91)	6.43 (5.72—7.34)	0.69
	6 Weeks	6.11 (5.50—7.56)	6.41 (5.84—7.28)	0.89	6.06 (5.30—7.46)	6.29 (5.70—6.98)	0.66
	24 Weeks	6.35 (5.52—7.83)	6.92 (6.05—7.89)	0.28	6.79 (5.54—7.99)	6.99 (6.33—8.05)	0.36
β-Carotene (μmol/l)	Baseline	0.20 (0.13—0.29)	0.21 (0.13—0.32)	0.88	0.20 (0.12—0.28)	0.21 (0.12—0.33)	0.81
	6 Weeks	0.21 (0.12—0.38)	0.19 (0.08—0.31)	0.26	0.21 (0.14—0.38)	0.19 (0.10—0.29)	0.25
	24 Weeks	0.25 (0.14—0.41)	0.21 (0.09—0.33)	0.35	0.28 (0.11—0.38)	0.19 (0.11—0.35)	0.26

Table 4 Selectins and cell-adhesion-molecule levels before and after exercise at baseline, 6 and 24 weeks (median, IQR) (Mann—Whitney - U test)

| Parameter	Assessment	Pre-exercise			Post-exercise		
		Cilostazol	Placebo	p-Value	Cilostazol	Placebo	p-Value
P-selectin (ng/ml)	Baseline	97.8 (74.4—119.8)	88.4 (73.1—109.5)	0.27	95.1 (67.7—124.5)	90.6 (70.9—110.5)	0.50
	6 Weeks	96.9 (79.7—113.8)	88.3 (75.4—105.0)	0.20	99.9 (73.7—123.3)	90.3 (73.7—109.5)	0.27
	24 Weeks	94.0 (69.8—116.5)	88.6 (70.8—102.0)	0.51	90.5 (69.8—113.0)	85.5 (67.8—104.0)	0.63
Intracellular cell-adhesion-molecule (ng/ml)	Baseline	841.0 (711.8—1040.8)	858.0 (674.5—970.5)	0.68	841.0 (722.5—1055.5)	853.0 (722.5—1011.5)	0.78
	6 Weeks	815.5 (703.5—1079.5)	866.0 (698.5—1008.3)	0.84	871.5 (712.0—1073.5)	893.0 (705.3—1052.5)	0.94
	24 Weeks	818.5 (649.5—961.8)	807.0 (681.5—957.0)	0.82	829.0 (681.5—1020.0)	837.0 (693.0-1019.0)	0.91
Vascular cell-adhesion-molecule (ng/ml)	Baseline	903.5 (788.0—1110.8)	1027.0 (782.5—1155.0)	0.58	935.5 (806.0—1153.0)	1020.0 (811.5—1142.5)	0.66
	6-weeks	921.5 (789.0—1079.5)	949.5 (810.8—1135.0)	0.47	877.0 (795.5—1106.8)	979.0 (831.5—1118.5)	0.24
	24-weeks	881.5 (762.0—1046.3)	989.0 (777.0—1097.0)	0.36	919.0 (783.5—1050.5)	1002.0 (811.5—1157.0)	0.18
Thromboxane B_2 (pg/ml)	Baseline	864.6 (342.6—1612.9)	744.9 (509.5—2288.1)	0.74	722.8 (408.1—1228.4)	953.9 (458.6—1959.6)	0.19
	6 Weeks	580.2 (344.5—1410.7)	886.1 (465.6—2026.6)	0.16	564.1 (332.5—1423.1)	1194.8 (493.2—2134.2)	0.065
	24 Weeks	637.0 (431.9—1578.6)	787.8 (377.8—1663.6)	0.63	801.0 (444.9—1697.4)	942.0 (393.6—1971.7)	0.60
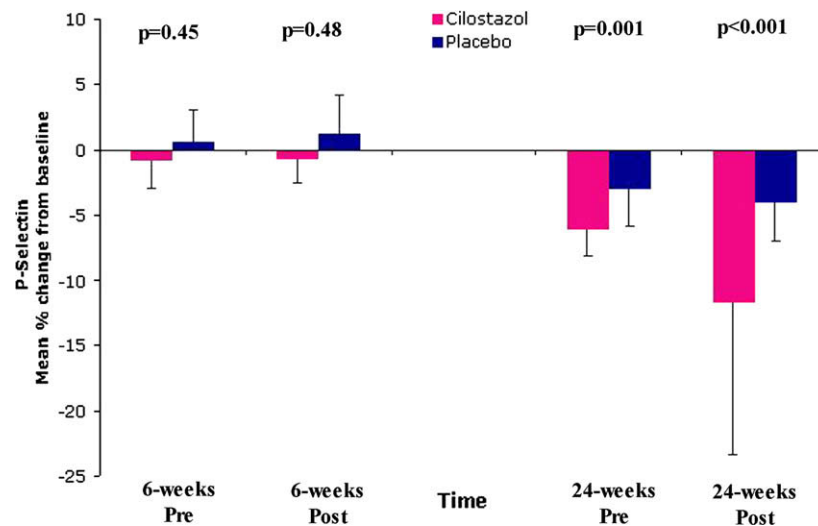
Figure 2   Mean percentage change from baseline in P-selectin levels (Mean, SEM) (Wilcoxon signed-rank test).

as profound in the claudicating patient as compared to more severely affected patients undergoing operative intervention.[16] Although cilostazol has no definite effect on other antioxidants in PAD patients, our results show an increased level of β-carotene, possibly from a relative sparing of this antioxidant, due to a reduction in lipid hydroperoxide levels (ODFR activity). Although the evidence is tenuous, it may explain the changes observed in the vitamin C concentrations.

In addition to direct cell-wall lipid peroxidation, ODFR can cause damage indirectly by activating platelets and leucocytes. These effects may be mediated via the selectins and cell-adhesion molecules, which affect both platelets and leucocytes, whilst propagating the inflammatory cell—endothelial interaction.[19,20] The reduction in P-selectin levels in the cilostazol group at 24 weeks as compared to the baseline is similar to the results of Jagroop et al. who reported reduced levels in PAD patients who were using combination antiplatelet (aspirin and clopidogrel) therapy.[21] A similar effect was reported in an in vitro model where cilostazol inhibited both platelet aggregation and P-selectin release.[22] Cilostazol also reduced cell-adhesion-molecule levels, which are important factors in atherosclerotic lesions as they facilitate leucocyte attachment to the endothelium, accounting for an increased risk of cardiovascular disease.[23]

Similarly, a longer treatment duration may be necessary to demonstrate the beneficial effects of cilostazol on the adhesion molecules. The TXA_2 is an important stimulant of platelet aggregation and vasoconstriction and acts as a powerful chemo-attractant for further neutrophil activation during ischaemia—reperfusion injury.[24] The TXA_2 effects on neutrophils and platelets is mediated through multiple factors, including I-CAM-1.[25] This study, however, found no difference between groups with regards to TXB_2, an index commonly used to estimate TXA_2 levels.

Elevated levels of IL-6 and TNF-α production have been reported following ischaemia—reperfusion injury.[26] Lee et al. have previously demonstrated that 8 weeks of cilostazol therapy in patients with intermittent claudication significantly reduced IL-6 levels with correlation to

improved triglyceride and high-density lipoprotein profiles.[12] However, this study had only 16 patients per treatment limb. Contrary to these findings, our study did not demonstrate such a response or difference between treatment groups for IL-6 levels. As the detection of circulating, free-TNF-α is unpredictable due to its short half-life and sporadic release, we measured soluble TNF receptors (p75TNF-R), which are more stable with a longer half-life. Edrees et al. reported increased urinary p55TNF-R in patients with intermittent claudication and critical limb ischaemia undergoing lower limb arterial bypass.[27] Like IL-6, we found no difference in p75TNF receptor levels between treatment groups.

However, there were limitations regarding our IL analyses. During lower limb arterial bypass, IL-6 concentrations have been shown to peak between 12 and 24 h after declamping, with a subsequent rapid return to normal values. Therefore, our 30-min post-exercise sampling time frame may not detect this peak IL-6 response and may account for a large amount of our samples recording a $0\,pg\,ml^{-1}$ value. Previous perioperative aortic aneurysm and lower limb studies from our unit have used wider sampling windows ranging from the immediate post-operative period to 48 h following the primary surgery.[27] These assessment points were not possible in our study as patients were only assessed in the outpatient department. The detection limit of $6.25\,pg\,ml^{-1}$ for the assays used in this study may not have been sufficiently sensitive and, thus, any value below this threshold was recorded as $0\,pg\,ml^{-1}$. The use of a high-sensitivity IL-6 ELISA would be optimal, with detection limits of $0.2\,pg\,ml^{-1}$.[12] Blood sampling from a remote site, such as the antecubital fossa, may have missed the localised cytokine burst due to systemic circulatory dilution. Furthermore, the volume of tissue affected by ischaemia—reperfusion injury is small in claudication, affecting only the calf in most patients as compared to multiple organs being affected after aortic surgery.

Ischaemia—reperfusion injury can lead to local and remote organ damage, such as an increase in glomerular permeability and trans-capillary leak of plasma proteins, which subsequently results in an increase in renal albumin

Table 5 Albumin—creatinine ratio and cytokine and high-sensitive C-reactive protein levels before and after exercise at baseline, 6 and 24 weeks (median, IQR) (Mann—Whitney U test).

Parameter	Assessment	Pre-exercise			Post-exercise		
		Cilostazol	Placebo	p-Value	Cilostazol	Placebo	p-Value
Interleukin-6 (pg/ml)	Baseline	57.2 (35.7)	8.2 (6.6)	0.41	60.9 (39.9)	8.4 (6.9)	0.40
	6 Weeks	33.1 (26.7)	10.2 (9.3)	0.22	36.5 (29.4)	12.9 (11.3)	0.44
	24 Weeks	35.9 (28.8)	12.7 (8.8)	0.64	44.6 (33.3)	6.9 (3.8)	0.99
Interleukin-10 (pg/ml)	Baseline	254.0 (150.8)	2.54 (1.4)	0.11	186.3 (120.1)	1.88 (0.78)	0.16
	6 Weeks	195.9 (140.6)	0.31 (0.31)	0.05	208.0 (144.3)	27.9 (26.8)	0.37
	24 Weeks	172.5 (124.4)	17.3 (15.6)	0.31	112.0 (106.9)	1.78 (1.22)	0.32
p75 TNF receptor (ng/ml)	Baseline	5.24 (2.18–10.06)	5.64 (3.74–11.15)	0.35	6.48 (2.24–11.75)	7.79 (4.33–15.2)	0.16
	6 Weeks	5.68 (2.31–11.12)	5.50 (3.33–13.5)	0.15	7.74 (2.97–11.3)	9.08 (3.48–15.4)	0.19
	24-Weeks	4.48 (2.99–7.96)	6.08 (2.79–11.35)	0.31	5.16 (3.21–9.33)	5.48 (3.63–19.5)	0.29
Albumin—creatinine ratio (μg/mg)	Baseline	0.95 (0.7–1.81)	1.55 (0.6–3.15)	0.18	1.18 (0.59–3.3)	2.05 (0.8–4.9)	0.07
	6 Weeks	0.70 (0.4–2.03)	1.35 (0.53–4.18)	0.039	1.30 (0.4–4.5)	2.70 (0.95–6.1)	0.043
	24 Weeks	0.60 (0.3–2.3)	1.50 (0.5–6.0)	0.017	1.5 (0.4–4.0)	2.3 (0.8–6.58)	0.10
High-sensitive C-reactive protein (μg/dl)	Baseline	215.6 (137.8–472.4)	299.8 (169.4–544.4)	0.44	N/A		
	6 Weeks	197.5 (147.1–677.2)	247.8 (136.5–578.2)	0.95			
	24 Weeks	259.9 (139.8–526.3)	218.9 (122.7–533.8)	0.70			

excretion.[28] This increased urinary ACR occurs in patients with claudication following treadmill exercise.[1,28] Hickey et al.[28] suggested that such increases in urinary ACR relate to the degree of muscle ischaemia and that this increase is reduced following successful lower limb arterial bypass. Our results imply that cilostazol may exert this reno-protective role through the reduction in ODFR generation.

CRP is emerging as a predictor of future cardiovascular events and is associated with PAD.[29] Contrary to studies by Tisi et al., who reported a reduction in CRP in claudicants in a supervised exercise program, we did not demonstrate any difference between the cilostazol and placebo groups.[30] However, their study was conducted over a longer period of time, which suggests that a further period of time may be required to elicit this effect in patients with intermittent claudication. In addition, cilostazol may not ameliorate CRP levels in patients with mild-to-moderate PAD.

In conclusion, cilostazol significantly attenuates an exercise-induced host-inflammatory response in patients with PAD with significant improvements in levels of lipid hydroperoxides, β-carotene, P-selectin and cell-adhesion molecules. Although the mechanism of action for cilostazol remains unclear, free-radical generation and antioxidant consumption are key pathways for the modulation of host-inflammatory response in patients with intermittent claudication. The role of cytokines remains unresolved. The short post-exercise sampling from a remote venous site may have led to undetectable concentrations. The lack of significance in some of the parameters may also be due to the cilostazol group walking further and thus suffering the same degree of ischaemia—reperfusion injury as the placebo group.

Conflicts of Interest

Study Funding

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