Combined Aspirin and Cilostazol Treatment is Associated with Reduced Platelet Aggregation and Prevention of Exercise-Induced Platelet Activation

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Exercise

Abstract  Background: Cilostazol has proven efficacy in increasing walking distance in claudicants, but it has not been demonstrated to be more effective than placebo in secondary cardiovascular prevention. The direct effect of exercise on platelet function remains less well defined. We have investigated the effect of combination treatment with aspirin and cilostazol on platelet activity in claudicants subjected to repeated treadmill exercise.

Methods: Nineteen claudicants completed a double-blind, randomised, controlled, cross-over trial. Each subject received a 2-week course of aspirin (75 mg) and placebo and aspirin and cilostazol (100 mg twice daily). Following each 2-week treatment period, patients participated in a standardised treadmill test (3.2 km h⁻¹, 10° incline) walking to maximal claudication distance. The exercise was repeated thrice in total, and blood was sampled before and after exercise. Platelet activation was measured using free platelet counting aggregation, flow cytometry for surface markers of platelet activation and soluble P-selectin assay.

Results: Compared to aspirin and placebo, combination treatment with aspirin and cilostazol was associated with reduced arachidonic-acid-induced platelet aggregation (p < 0.01, Wilcoxon signed-rank test). Aspirin and placebo treatment were associated with elevated P-selectin expression, platelet-monocyte aggregation and reduced CD42b expression (p < 0.05, Wilcoxon signed-rank test) post-exercise. No difference was seen in spontaneous platelet aggregation whilst soluble P-selectin was reduced post-exercise with combination treatment with aspirin and cilostazol (p < 0.05, Wilcoxon signed-rank test).

Conclusions: Combination treatment with aspirin and cilostazol results in suppression of platelet activation and reduces the effect of exercise on platelets. The benefit seen may be a result of cilostazol enhancing the inhibitory effect of aspirin on the cyclo-oxygenase pathway.

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Cilostazol is a type III phosphodiesterase inhibitor licensed for use in intermittent claudication. A meta-analysis of eight multicentre drug trials involving over 2700 claudicants has indicated that cilostazol is beneficial in improving both initial and absolute claudication distance. Comparable results have also been reported by a Cochrane review.

By inhibiting phosphodiesterases, cilostazol prevents the hydrolysis of cyclic adenosine monophosphate (cAMP). The consequential rise in intracellular cAMP is responsible for its antiplatelet and vasodilatory properties. In addition, cAMP is an inhibitor of the cyclo-oxygenase pathway, and hence reduces production of TxA2. Within vascular smooth muscle, cAMP inhibits the release of stored intracellular Ca²⁺, and hence suppresses the activity within contractile proteins. While the direct antiplatelet effects of cilostazol have been confirmed, any possible clinical benefits require further work. In the context of coronary artery disease, a beneficial effect has been seen in the prevention of restenosis after percutaneous coronary intervention. Compared to placebo, cilostazol has also been shown to be useful in the prevention of secondary stroke, although there was not a significant benefit over aspirin. Although the risk of intra- and post-operative bleeding requires further investigation, the results of a study by Wilhite et al. reassuringly suggested cilostazol does not significantly prolong bleeding time.

Although the effect of a combination therapy with aspirin remains undefined, any synergistic antiplatelet effect may support the use of cilostazol in patients with peripheral arterial disease (PAD). Further studies are needed to ascertain both the antiplatelet effect of combination therapy and the potential clinical benefits. This study was performed as a pilot study to assess whether combination therapy with cilostazol and aspirin had an antiplatelet effect that exceeded that of aspirin alone and attenuated the increased platelet activation seen in PAD patients following exercise.

Materials and Methods

Study design

A double-blind, randomised, placebo-controlled, cross-over trial design was used. A cross-over design was chosen to account for any potential confounding effects of smoking, co-morbidities and medication on platelet function. Using a table of block randomisation, patients were randomised to start either aspirin/placebo or aspirin/cilostazol and then treated with the alternative combination therapy. Both the patient and the principal investigator were blinded. Treatment packs were dispensed by the hospital pharmacy. The treatment regimens revealed only after the completion of the study by all participants and analysis of the assays. Each patient was subjected to a 14-day period of treatment with aspirin (75 mg) and placebo (twice daily) and aspirin and cilostazol (100 mg twice daily). A 10-day ‘washout’ period was allowed between treatments. On the final day of each treatment, the patients were brought to the hospital and performed a standardised treadmill test with blood sampling as previously described. The local ethical committee and the Research and Development committee approvals were obtained. The study was performed according to the declaration of Helsinki. PAD patients with symptoms of intermittent claudication were recruited from outpatients. All patients were treated with 75 mg aspirin daily, prior to recruitment, as part of their routine antiplatelet therapy.

Inclusion and exclusion criteria

Patients were included if they had PAD with intermittent claudication, ankle–brachial pressure index (ABI) < 0.9 and a radiologically (either angiographically or with duplex) proven lower limb disease. All patients were aged between 40 and 80 years, were able to complete a treadmill exercise test and were on 75 mg aspirin (prior to commencement of cilostazol or placebo) as part of their routine antiplatelet therapy. Patients were excluded if they had any other co-morbidities known to alter platelet activity. Specifically, chronic renal failure, diabetes mellitus, invasive malignancy, current infectious process (including gangrene), platelet count < 150 or > 450 and any concurrent platelet disorders were the exclusion criteria. Patients taking steroids, anticoagulants and antiplatelet agents (other than aspirin) were also excluded. Patients with co-morbidities preventing completion of a treadmill test, for example, arthritis, pulmonary disease and heart failure, were also excluded. Cilostazol is metabolised by hepatic cytochrome P-450 enzymes, hence patients taking any medication that induced or inhibited cytochrome P-450 activity were also excluded.

Treadmill testing

Patients were asked to remain on clear fluids only for 12 h prior to attending hospital for the treadmill test, aimed at minimising the effect of dietary agents on platelet activity. Transport to the hospital was provided, and, on arrival, patients were rested supine for 90 min prior to the treadmill test (3.2 km h⁻¹ at a 10° incline). All participants were asked to walk to their maximal claudication distance. This was repeated thrice in total with brief rest periods in between to allow resolution of calf pain. Blood samples were taken before commencement of exercise and after completion of all exercises (1 min and 40 min after completion of the third cycle of claudication, respectively).

Assessment of platelet activation

Platelet activation was assessed by a variety of methods. Flow cytometry was used to measure the expression of platelet P-selectin, glycoprotein (GP) Ib-IX-V and platelet–monocyte aggregates (PMAs). Whole blood free platelet aggregation was used to measure spontaneous platelet aggregation and arachidonic acid (AA)-induced aggregation. ELISA was used to quantify changes in soluble P-selectin.

Flow cytometry was performed using a whole-blood two-colour staining technique to quantify platelet P-selectin and GP Ib-IX-V. The method for P-selectin has been previously described. GP Ib-IX-V expression was measured with a similar technique but using fluorescein isothiocyanate (FITC)-conjugated CD61 antibodies (Dako, Cambridgeshire,
UK) to identify the platelet population and PE-conjugated
CD42b antibody (Dako, Cambridgeshire, UK) to quantify GP
Ib-IX-V complex. Then, 15 000 platelet events were
collected, and the results were expressed as percentage
cells positive for P-selectin and relative median fluorescence
(RMF) for GP Ib-IX-V. A whole-blood three-colour staining
technique was used for PMA estimation as previously
described. Following a collection of 30 000 events the
results were expressed as percentage cells positive for the
anti-CD61 antibody. For each marker, isotypic control
samples were run in parallel.

Whole blood free platelet aggregation was measured
using the method previously described by Robless et al. This
technique measures percentage reduction in platelets
following stirring of blood using a platelet aggregometer
(Chronolog Whole blood Aggregometer 590-2D, Chronolog,
Havertown, PA, USA). Platelet count was measured using
the whole blood platelet Coulter counter (Coulter T-890
counter, Coulter Electronics, Luton, UK). Spontaneous
platelet aggregation (SPA) was measured using 0.9% saline
in the absence of an agonist, whilst AA-induced aggregation
was produced by the addition of 0.25 μM AA (Chronolog,
Havertown, PA, USA).

Soluble P-selectin was measured by ELISA with a
commercially available kit (R&D systems, Oxon, UK). The
assay was performed according to the manufacturer’s
instructions using a fully automated technique (Best 2000,
Biokit ELISA systems, Barcelona, Spain).

Statistical analysis

Data were analysed using Minitab (release 13.1) and Prism
(version 3.03). Deviations from Gaussian distribution were
assessed using the Anderson–Darlington test. For inter-
group analysis, a Wilcoxon signed-rank test was used for
data which were non-parametrically distributed, and the
results expressed as the median value with the inter-
quartile range (IQR).

Results

In this study, 22 patients were recruited. Risk factors and
demographics for PAD and medication are summarised in
Table 1. Two patients were unable to complete the treat-
ment due to headaches. One of these patients was identi-
fied to be treated with the placebo and the other with
cilostazol. One patient failed to attend the treadmill test
and withdrew from the study. Hence, 19 patients were able
to complete the trial and were included in the analysis. The
median (range) age was 64 (53–81) years and the ratio of
men to women was 16:3. All data were non-parametrically
distributed.

Surface P-selectin expression (Fig. 1)

A significant increase in P-selectin expression (p = 0.023)
was seen in the aspirin–placebo group 1 min post-exercise
(1.8%; IQR: 1.1–3.2% positive cells) compared to the pre-
exercise value (1.4%; IQR: 0.7–2.7% positive cells). The 1-
min post-exercise was also significantly higher (p = 0.018)
than the 40-min post-exercise (1.3%; IQR: 0.7–2.4% positive
cells). The same effect was not seen in the aspirin–
cilostazol group.

Glycoprotein Ib-IX-V receptor expression (Fig. 2)

Platelet activation is indicated by a reduction in the
expression of the GP Ib-IX-V complex. This is a consequence
of receptor internalisation following binding of the anti-
CD42b antibody. Only with the aspirin–placebo treatment
was the reduction in RMF 1 min post-exercise (41.1; IQR:
34.9–54.3) statistically significant (p = 0.015) from the
pre-exercise value (55.7; IQR: 46.6–70.4). Following
aspirin–cilostazol treatment, there was a marginal drop in
the RMF at 40 min (56.3; IQR: 45.3–77.0) which was found
to be significantly lower (p = 0.046) than the pre-exercise
value (57.3; IQR: 33.4–67.3). No statistically significant
difference was identified between the two treatments at
any time interval.

Platelet–monocyte aggregate expression (Fig. 3)

The 1-min post-exercise result for the aspirin–placebo
treatment (22.3%; IQR: 19.5–34.1% positive cells) was
significantly higher (p = 0.015) than the pre-exercise result
(20.5%; IQR: 17.3–28.6% positive cells). No statistically
significant difference was identified between the two treatments at
any time interval.

AA-induced aggregation (Fig. 4)

The pre-exercise aspirin–placebo treatment (19.6%; IQR:
13.4–23.3% aggregation) displayed significantly higher
(p = 0.026) aggregation than the aspirin–cilostazol treat-
ment (10.7%; IQR: 6.4–16.5% aggregation). At the 1-min
post-exercise interval, aggregation with the aspirin–
placebo treatment (19.9%; IQR: 16.1–25.5% aggregation)
remained significantly higher (p = 0.002) than that seen
with the aspirin–cilostazol treatment (15.0%; IQR: 9.7–
20.3% aggregation). This difference between the two
treatment groups persisted at 40-min post-exercise times

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics of claudicants.</th>
<th>n = 19</th>
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<tbody>
<tr>
<td>Age, years (Range)</td>
<td>64 (53–81)</td>
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<tr>
<td>Gender (M/F)</td>
<td>16/3</td>
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<tr>
<td>Ankle–brachial pressure index (range)</td>
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<tr>
<td>Risk factors</td>
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<tr>
<td>Smoking, n (%)</td>
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</tr>
<tr>
<td>Hypercholesterolaemia, n (%)</td>
<td>11 (58)</td>
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<tr>
<td>Hypertension, n (%)</td>
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<tr>
<td>Ischaemic heart disease, n (%)</td>
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<td>Medications</td>
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<td>Calcium channel blockers, n (%)</td>
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<td>Statins, n (%)</td>
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<td>Diuretics, n (%)</td>
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<tr>
<td>Nitrates, n (%)</td>
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</table>

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Figure 1  Platelet P-selectin expression in patients treated with cilostazol and with placebo, before and after exercise. The box extends from the 25th percentile to the 75th percentile, with a horizontal line to represent the median. The whiskers show the highest and lowest values (*$p < 0.05$, Wilcoxon signed-rank test).

Figure 2  Glycoprotein Ib-IX-V expression in patients treated with cilostazol and with placebo, before and after exercise. The box extends from the 25th percentile to the 75th percentile, with a horizontal line to represent the median. The whiskers show the highest and lowest values (*$p < 0.05$, Wilcoxon signed-rank test).

Figure 3  Platelet–monocyte aggregate expression in patients treated with cilostazol and with placebo, before and after exercise. The box extends from the 25th percentile to the 75th percentile, with a horizontal line to represent the median. The whiskers show the highest and lowest values (*$p < 0.05$, Wilcoxon signed-rank test).
with the aspirin–cilostazol treatment (11.4%; IQR: 8.4–19.8% aggregation) remaining below that of the aspirin–placebo group (17.6%; IQR: 13.1–23.5% aggregation). There were no significant changes in aggregation following exercise in either treatment groups.

Spontaneous platelet aggregation (Fig. 5)

There was no significant difference between the treatments at any time interval. There was no significant change in SPA following exercise for either of the treatments.

Soluble P-selectin (Fig. 6)

Cilostazol treatment was associated with a reduction in the release of soluble P-selectin following exercise. The cilostazol median pre-exercise value (65.5 pg ml$^{-1}$; IQR: 53.7–85.3) was significantly higher than the 1-min post-exercise value (53.6 pg ml$^{-1}$; IQR: 48.3–69.0; $p = 0.031$) and the 40-min post-exercise (56.7 pg ml$^{-1}$; IQR: 50.8–65.2; $p = 0.046$). Interestingly, the cilostazol 1-min value was also lower than the placebo 1-min value (60.4 pg ml$^{-1}$; IQR: 51.8–77.7; $p = 0.046$). The same effect was not seen between the cilostazol and the placebo pre-exercise values (64.98 pg ml$^{-1}$; IQR: 50.4–75.8; $p = 0.848$).

Discussion

Previous multicentre trials have shown cilostazol to improve claudication distance. This study focused on the effects of cilostazol on platelet activation only. The individual effects of aspirin and cilostazol on markers of platelet activation have been studied in various populations. Benefits in secondary cardiovascular prevention have been shown amongst patients with coronary and cerebrovascular disease. The antiplatelet effect of combination therapy with cilostazol has been studied in patients with ischaemic heart disease (IHD), and two
studies have previously shown that combined aspirin and cilostazol therapy inhibited platelet aggregation to a greater degree than aspirin monotherapy.\textsuperscript{15,16} To date, no work has been published documenting the effect of combination therapy on platelets in PAD patients subjected to exercise.

The effect of cilostazol on platelet surface P-selectin expression has previously been studied both \textit{in vitro} and \textit{in vivo}. Benefits have been identified in populations subjected to coronary stenting.\textsuperscript{17} Furthermore, cilostazol on its own and when administered with dipyridamole has been seen to reduce platelet-surface P-selectin expression in patients with arteriosclerosis obliterans.\textsuperscript{18} The ability of cilostazol to inhibit agonist-induced expression of P-selectin is also well documented in \textit{ex vivo} studies.\textsuperscript{4,19} The results from the current study failed to show a benefit in the baseline expression of P-selectin with combined aspirin and cilostazol treatment. However, a clear benefit was seen following exercise, whereby the exercise-induced P-selectin expression seen with aspirin/placebo therapy was prevented by cilostazol/aspirin combination therapy. The mechanism of exercise-induced platelet activation remains a topic of debate and is probably a combination of ischaemic–reperfusion injury, sheer stress as blood flows through stenotic lesions and catecholamine release.\textsuperscript{20} \textit{Ex vivo} studies have shown cilostazol to inhibit sheer stress-induced expression of P-selectin, and this would be consistent with the benefit following exercise noted in this study.\textsuperscript{21} At least one \textit{ex vivo} study has shown cilostazol to be superior to aspirin in suppressing P-selectin expression following sheer stress.\textsuperscript{21} Hence, the effect of combination treatment seen herein may be predicted by the results of \textit{ex vivo} studies and explained by the effect cilostazol has on sheer-induced platelet P-selectin expression.

The significant decrease found in GP Ib-IX-V receptor expression confirms platelet activation following exercise. The change was seen earlier, appeared to be greater and more strongly significant with aspirin compared to aspirin/cilostazol combination treatment. The effect of cilostazol on GP Ib-IX-V expression has not been previously studied. The results of this study suggest that some benefit may be gained, but further work is needed to define the role of cilostazol in the absence of aspirin. As with P-selectin, PMAs were seen to increase significantly following exercise in those treated with aspirin monotherapy. This effect was not seen with combination treatment. The similarities seen here with the P-selectin results are to be expected as P-selectin is an important ligand facilitating the platelet–monocyte interaction. The role and mechanism of cilostazol suppression of platelet–leucocyte aggregate formation have been suggested in at least one other study.\textsuperscript{22}

The effect of cilostazol on platelet aggregation has been previously studied, both in isolation and in combination with other antiplatelet agents. Cilostazol has been shown to inhibit the aggregation response to various agonists in blood from healthy individuals, CAD and PAD patients.\textsuperscript{18,23,24} Similar effects have also been described with aspirin.\textsuperscript{23,25} The effect of combination treatment with aspirin and cilostazol has been investigated in patients with IHD by Tanigawa et al. Aggregation studies demonstrated that combination treatment significantly inhibited AA-induced aggregation.\textsuperscript{15,16} However, Mallikaarjun et al. produced different results in a population of healthy individuals with no additive benefit seen on AA-induced aggregation.\textsuperscript{26} The results published by Tanigawa et al. are comparable to the baseline results seen in the population of PAD patients in the current study, with a significant improvement in AA-induced aggregation. The benefit seen in AA-induced aggregation was maintained at all time intervals. The presence of cilostazol may augment the inhibitory effect of aspirin upon the cyclo-oxygenase pathway, an effect which is not overcome by the exercise-induced stress.

In this study, combination treatment had no significant benefit over aspirin monotherapy on SPA. As previously mentioned, SPA represents the aggregation induced by \textit{in vitro} sheer stress. Other studies involving cilostazol have measured sheer stress using the free platelet counting method but with variations in the turbidometric methodology, thus making comparisons difficult. Sheer stress-induced platelet aggregation (SIPA) has been shown to be inhibited by individual aspirin and cilostazol treatment and
cicostazol/aspirin combination therapy.\textsuperscript{16,21} Neither of these studies used proven PAD patients, and comparisons with this PAD population are made difficult by the differences in methodology.

This study has shown that combination treatment with cilostazol and aspirin inhibits exercise-induced platelet aggregation (mediated by the AA pathway) significantly more than aspirin therapy alone. This benefit may represent a synergistic antiplatelet effect produced by combination therapy and has significant clinical implications. The increased risk of bleeding seen with clopidogrel and aspirin has not been demonstrated with cilostazol and aspirin.\textsuperscript{10}

Further benefits may include the prevention of myointimal hyperplasia by inhibiting vascular smooth muscle proliferation,\textsuperscript{6,10,27} Hence, combination therapy offers potential secondary prevention benefits without increased bleeding risk. Furthermore, combination therapy with aspirin and cilostazol may offer some benefit in preventing the platelet activation associated with repeated exercise.

Conflict of Interest and Funding

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