Effects of aspirin, clopidogrel and dipyridamole administered singly and in combination on platelet and leucocyte function in normal volunteers and patients with prior ischaemic stroke

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Summary

The aim of this study was to assess whether triple antiplatelet therapy is superior to dual and mono therapy in attenuating platelet and leucocyte function. Aspirin (A), clopidogrel (C), and dipyridamole (D) were administered singly and in various combinations (A, C, D, AC, AD, CD, ACD), each for two weeks (without washout) to 11 healthy subjects and to 11 patients with previous ischaemic stroke in two randomised multiway crossover trials. At the end of each two-week period platelet aggregation, platelet-leucocyte conjugate formation and leucocyte activation were measured *ex vivo* blinded to treatment. Platelets were stimulated with collagen; additional measurements were made with adenosine diphosphate (ADP), platelet activating factor (PAF), adrenaline and the combination of, ADP, PAF and adrenaline. Results show that in the presence of collagen, ACD was superior to all antagonists or combinations, except AC, in reducing aggregation, platelet-leucocyte conjugate formation, and monocyte activation (all $p<0.05$). ACD was also more potent than other treatments, except AC, in inhibiting the aggregation and platelet-monocyte conjugate formation induced by the combination of ADP, PAF and adrenaline. The effects were similar in both volunteers and stroke patients. No serious adverse events or major bleeding events occurred. Triple antiplatelet therapy did not appear to be more effective than combined aspirin and clopidogrel in moderating platelet and leucocyte function. Any additional clinical benefit provided by dipyridamole may be through other mechanisms of action.

Keywords

Aspirin, clopidogrel, dipyridamole, platelets, ischaemic stroke

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Introduction

Antiplatelet agents are now established in secondary prevention after ischaemic stroke and transient ischaemic attack (1). Aspirin, clopidogrel and dipyridamole each reduce recurrent vascular events by about 20% in patients with prior ischaemic cerebrovascular disease (1–3). Evidence is now accruing that combining antiplatelet agents with different modes of action increases their effectiveness in reducing vascular events. The 'Second European Stroke Prevention Study' (ESPS-2) showed that the relative risk reduction for stroke was doubled with combined aspirin and dipyridamole as compared with either agent alone (3). Similarly, the combination of aspirin and clopidogrel was superior to aspirin alone in reducing vascular events in patients with acute coronary syndromes in the 'Clopidogrel in Unstable Angina to Prevent Recurrent Events' (CURE) trial, and those having percutaneous coronary intervention in the 'Clopidogrel for the Reduction of Events During Observation' (CREDO) trial (4, 5). These beneficial findings were in spite of a 30% increase in major bleeding events (significant in CURE, non-significant in ESPS-2 and CREDO) as seen in each trial.

If two antiplatelet agents offer improved prophylaxis, then three agents might be better still, providing the bleeding risk does not increase disproportionately. We have previously assessed the pharmacological effects *in vitro* of combining the three antiplatelet agents, aspirin, dipyridamole and AR-C69931 (6), on platelet aggregation, platelet-leucocyte conjugate formation and leucocyte activation, processes which are thought to be involved in vascular disease progression (7–17). AR-C69931, a direct acting $P2Y_{12}$ receptor antagonist was used in place of clopidogrel since the latter is a pro-drug and is ineffective *in vitro*. Whilst combined aspirin and AR-C69931 was as effective as all three agents used together in inhibiting platelet aggregation, the formation of platelet-leucocyte conjugates and leucocyte acti-

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vation were most inhibited when all three antiplatelet agents were present (18). Overall, the findings suggested that the combination of three antiplatelet agents with different modes of action was superior to any single agent alone, or pairs of agents, in modifying platelet activity and heterotypic cell adhesion and leucocyte activation.

The purpose of the present randomised crossover trial was to compare the effects of aspirin, clopidogrel and dipyridamole administered singly and in various combinations on platelet and leucocyte activation, and platelet-leucocyte conjugation, in normal volunteers and in patients with a prior history of ischaemic stroke. The primary aim was to assess whether triple antiplatelet therapy was superior to dual and mono therapy in attenuating collagen-induced activation of platelets, leucocytes, and their conjugation. Collagen was used as the primary agonist because this is the material that is exposed following vascular damage (19, 20). Other agonists investigated were ADP, adrenaline and PAF, which were studied singly and in combination. These are agents that are known to synergise to promote platelet aggregation and also influence leucocyte function (19–22).

Methods

Design

Two randomised, outcome-blinded, multiway, crossover trials of antiplatelet therapy were performed in normal volunteers and patients with prior ischaemic stroke (Fig. 1). Platelet and leucocyte measures were performed as previously (18).

Subjects

The study protocol was approved by the local Research Ethics Committee. All subjects gave written informed consent and the trials were performed according to the Helsinki Declaration and the principles of Good Clinical Practice. Eleven healthy volunteers without any history of vascular disease were recruited from hospital or university staff or their friends. Eleven stable patients with a prior history of ischaemic stroke (on clinical and neuroimaging criteria) within 5 years (23) were recruited from the stroke service at Nottingham City Hospital; each patient was receiving aspirin for vascular prophylaxis. Subjects were screened clinically and a full blood count and electrocardiogram were obtained before participation. Subjects were excluded if they: had a history of cerebral haemorrhage, gastrointestinal bleeding, peptic ulcer, anaemia, thrombocytopenia, any severe concomitant medical condition, or hypersensitivity or intolerance to any of the study drugs; were taking anticoagulation or a non-steroidal anti-inflammatory drug; had a blood pressure > 180/110 mmHg; or were pregnant or lactating women.

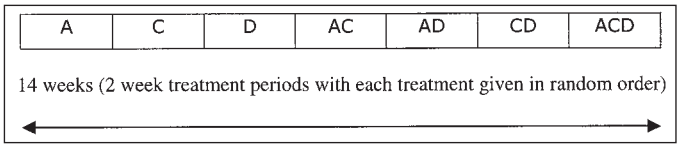

Figure 1: Crossover study design: applied to both volunteer and patient studies, two week treatment periods with each treatment given in randomised order without washout. A, aspirin; C, clopidogrel; D, dipyridamole.s.

Interventions

All subjects received two week periods of open-label aspirin (A, 75mg daily), clopidogrel (C, 75mg daily) or modified release dipyridamole (D, 200mg twice daily). No washout periods were used since the length of treatment (14 days) exceeded the lifespan of platelets (~10 days). The drugs were given either singly (A, C, or D), in pairs (AC, AD, or CD) or all three together (ACD) in random order. Normal volunteers, but not patients, were also studied off all therapy. Randomisation was performed using a computer and the treatment codes held by the hospital's Pharmacy to ensure concealment of allocation. Laboratory tests (see below) were assessed at the end of each two-week treatment phase. Adverse events (headache, gastrointestinal symptoms, rash) were similarly assessed blinded to treatment. Subjects who withdrew from treatment were replaced to ensure that complete data were present for each person; laboratory data from withdrawing subjects are not included in the statistical analyses.

Laboratory study

Blood was collected 1–2 hours following drug ingestion. The subjects were seated for 10–15 minutes and blood samples were then collected, usually from the right arm, by venepuncture with minimal stasis with a 19-gauge needle from an antecubital vein and anticoagulated with hirudin (Rhone-Poulenc-Rorer, UK, final concentration 7.2μM). The processing of blood samples was started within 5 minutes of venepuncture. Aliquots (480 μl) of blood were then stirred at 1000 rpm in a Multi-Sample Agitator (University of Nottingham, UK) for 2 min at 37°C. Either 20 μl collagen (Horm Chemie, UK, final concentration 2 μg/mL), ADP (Sigma, UK, 3 μM), PAF (Sigma, 1 μM), adrenaline, (Sigma, UK, 10 μM), or the combination of a low concentration of ADP, PAF and adrenaline (1μM, 0.33 μM and 3.33 μM respectively) were then added and the samples stirred for 10 min.

Platelet aggregation

Platelet aggregation was assessed by counting the number of single platelets using an Ultra-Flo 100 Whole Blood Platelet Counter. Aliquots of 15 μl of blood were transferred into polypropylene tubes containing 30 μl fixing solution (150 mM NaCl, 0.16% w/v formaldehyde, 4.6 mM disodium EDTA, 4.5 mM/L Na_2HPO_4 and 1.6 mmol/L K_2HPO_4 pH 7.4). Platelet aggregation was determined after comparison with the initial platelet count.

Platelet-leucocyte conjugate formation and leucocyte CD11b expression

Platelet-leucocyte conjugate formation and leucocyte CD11b expression (a measure of leucocyte activation) were determined by flow cytometry. 100 μl aliquots of the blood were treated with 2.0 ml Erythrolyse solution (Serotec, UK) for 10 min at room temperature. The samples were then centrifuged at 380 g for 10 min and the pellets were washed with FACSflow (Becton Dickinson, UK), and then re-centrifuged. The pellets were finally resuspended in 100 μl Dulbecco's PBS containing 10% (v/v) new born calf serum. A 30 μl aliquot was incubated at 4°C for 30 min in the dark with saturating concentrations of anti-CD14:PE (Becton Dickinson) to identify the monocytes and anti-CD42a:FITC (Serotec) to identify platelets bound to monocytes

and/or neutrophils. Platelet-monocyte and platelet-neutrophil conjugates were then quantified by flow cytometry. A further 30 µl aliquot was incubated with anti-CD14:PE and anti-CD11b:FITC (Serotec) at 4ºC for 30 min in the dark to detect leucocyte activation. In some experiments an irrelevant isotype-matched IgG1:FITC (Serotec) was used for non-specific membrane immunofluorescence.

Flow cytometry

Platelet-leucocyte conjugates and leucocyte CD11b expression were quantified using a FACScan flow cytometer (Becton Dickinson) equipped with a 5 W laser operating at 15 mW power and a wavelength of 488 nm, and connected to an Apple Mac G3 computer. Leucocytes were monitored using forward light scatter and side light scatter and fluorescence. Linear modes were used for light scatter and log modes for fluorescence. A total of 10,000 leucocyte events were recorded per sample. Monocytes were differentiated from other leucocytes by their CD14:PE positivity. Platelet-leucocyte conjugates are reported as the median CD42a fluorescence of the leucocyte populations. CD11b expression is reported as the median CD11b:FITC fluorescence of the leucocyte populations.

Safety

The subjects were monitored for adverse events every two weeks. Each of the three antiplatelet agents has a known profile of adverse events and these were specifically assessed: aspirin may promote bleeding and gastrointestinal disturbance; clopidogrel can cause bleeding and rash; and dipyridamole may cause bleeding, headache and gastrointestinal disturbance.

Statistical analysis

The phase I (volunteer) and phase II (patient) trials assessed the effect on blood cell activity *ex vivo* of triple antiplatelet therapy (ACD) as compared with mono and dual treatment using a randomised multiway crossover design. We did not make multiple other comparisons, e.g. between dual and mono therapies, to minimise the risk of false positive results (type I error). In the absence of existing published data, an initial samples size of 12 volunteers and 12 patients was chosen. An interim analysis relating to collagen effects and blinded to treatment allocation after 6 volunteers had been enrolled (data not shown), suggested that 10–12 subjects would be required in each study (power 0.8, alpha 0.05). Collagen was chosen as the primary platelet agonist since it is key in mediating platelet interactions with the damaged vessel wall, as occurs during atherothrombosis. Secondary analyses assessed the effect of antiplatelet therapy on ADP, PAF, adrenaline and combined ADP/PAF/adrenaline activated cells. Analyses were also performed comparing samples on and off each drug irrespective of the presence of any other drug: A versus no A, C versus no C, and D versus no D. Baseline data, i.e. off all antiplatelet agents, in the volunteer group was not included in the statistical analyses since there was no equivalent data for the patients with prior cerebrovascular disease. The results are expressed as mean (standard deviation). The data were analysed as a crossover trial using a least squares analysis fitting treatment, period and volunteer or patient as fixed effects. A fixed effect analysis was used since the numbers of subjects were small and no missing data were present. Pair wise comparisons were performed for triple therapy versus all other drug. The SAS statistical package (Windows, version 6.12, SAS Institute, Maidenhead, UK) was used.

Table 1: Baseline characteristics of subjects. Mean (standard deviation) or frequency.

	Normal volunteers	Post ischaemic stroke
Number	11	11
Mean age (year)	39 (8)	62 (10)
Gender, male (%)	8 (73)	6 (55)
Hypertension (%)	0 (0)	8 (73)
Diabetes mellitus (%)	0 (0)	0 (0)
Smoking (%)		
Never	9 (82)	3 (27)
Past	1 (9)	5 (45)
Current	1 (9)	3 (27)
Blood pressure (mmHg)		
Systolic	130(14)	150(20)
Diastolic	85(4)	86(8)
Platelet count ($\times 10^9$.l)	257(35)	282(71)
Total cholesterol (mM)	5.0(0.7)	5.9(0.8)
LDL-C (mM^1)	3.4(0.6)	3.8(0.8)
HDL-C (mM)	1.0(0.2)	1.4(0.4)
Triglycerides (mM)	1.3(0.5)	2.2(1.5)

LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol

Results

Subjects

A total 22 subjects were recruited (11 healthy subjects and 11 patients with previous ischaemic stroke). The patients were older and had more vascular risk factors (smoking, hypertension, hyperlipidaemia) than the normal volunteers (Table 1). Patients were recruited median 2 (interquartile range 6, range 0.1–21.4) months post ictus.

Effects of the various platelet agonists

Stirred blood (saline with no antagonist) produced some platelet aggregation, platelet-monocyte conjugate formation, and monocyte activation, but little platelet-neutrophil conjugate formation or neutrophil activation (data not shown). Adding different platelet agonists to the blood enhanced platelet aggregation, platelet-monocyte conjugate formation, platelet-neutrophil conjugate formation, monocyte activation, and neutrophil activation. However, adrenaline inhibited stirring-induced monocyte activation.

Effects of the drug treatment

Non-agonist

There was a similar pattern for the effects of ACD on platelet and leucocyte parameters between healthy subjects and stroke patients. ACD was more effective than A, D and AD, but not C, AC and CD on inhibition of platelet aggregation. Overall, ACD had no greater effect than mono or dual therapy on platelet-mono-

Table 2: Effects of antiplatelet agents on basal platelet aggregation, platelet-leucocyte conjugate formation, and leucocyte activation in 11 volunteers and 11 stroke patients. Samples were stirred without platelet agonists. Mean (standard deviation); comparison of triple therapy (ACD) with mono and dual antiplatelet therapy, *p<0.05.

Subjects	Antiplatelet	Aggregation (%)	Platelet-monocyte (CD42a)	Platelet-neutrophil (CD42a)	Monocyte activation (CD11b)	Neutrophil activation (CD11b)
Volunteers	†	40(9)	27(8)	28(6)	70(34)	124(56)
	A	44(10)*	23(7)	28(4)	61(26)	113(63)
	C	18(12)	30(18)	27(8)	65(34)	125(48)*
	D	37(10)*	29(10)	28(7)	63(25)	114(59)
	AC	16(8)	21(6)	26(6)	65(32)	125(54)
	AD	45(9)*	27(9)	28(7)	59(22)	114(62)
	CD	20(8)	25(11)	31(8)*	68(39)	127(63)
	ACD	19(10)	21(13)	24(6)	59(29)	113(53)
Patients	A	34(16)*	(28)9	33(29)	59(24)	82(50)
	C	21(14)	23(9)	25(9)	59(19)	100(35)
	D	32(14)*	29(10)	34(21)	61(30)	102(36)
	AC	21(14)	27(10)	27(9)	61(27)	96(48)
	AD	38(19)*	27(8)	26(7)	55(28)	89(42)
	CD	22(14)	26(11)	27(13)	58(28)	97(50)
	ACD	22(14)	29(10)	30(11)	59(20)	101(38)

A: aspirin; C: clopidogrel; D: dipyridamole
† No comparison was made between ACD and no antiplatelet therapy for volunteers since no analogous data were available for patients

cyte, platelet-neutrophil conjugates and leucocyte activation (Table 2).

Collagen stimulation

The results in healthy subjects and stroke patients were qualitatively similar. ACD was superior to A, C, D, AD and CD, but not AC, at inhibiting collagen-induced aggregation, platelet-leucocyte conjugation, and monocyte and neutrophil activation (Table 3).

Other agonists (Figures 2 and 3)

In secondary analyses, ACD was not apparently superior to the other antiplatelet strategies in inhibiting platelet aggregation or the formation of platelet-monocyte conjugates induced by ADP,

Table 3: Effects of antiplatelet agents on collagen-induced platelet aggregation, platelet-leucocyte conjugate formation, and leucocyte activation in 11 volunteers and 11 stroke patients. Mean (standard deviation); comparison of triple therapy (ACD) with mono and dual antiplatelet therapy, *p<0.05.

Subjects	Antiplatelet	Aggregation (%)	Platelet-monocyte (CD42a)	Platelet-neutrophil (CD42a)	Monocyte activation (CD11b)	Neutrophil activation (CD11b)
Volunteers	†	72(5)	155(53)	58(10)	143(46)	160(55)
	A	74(5)*	124(35)*	52(8)*	109(46)*	134(62)*
	C	71(9)*	163(49)*	58(11)*	122(45)*	142(48)*
	D	72(9)*	152(32)*	62(9)*	123(40)*	147(58)*
	AC	56(9)	76(25)	38(6)	102(43)	130(47)
	AD	70(9)*	122(49)*	50(9)*	112(43)*	133(63)
	CD	73(8)*	151(52)*	59(12)*	118(41)*	145(50)*
	ACD	55(10)	79(37)	42(8)	96(43)	122(49)
Patients	A	68(7)*	130(35)*	48(11)*	95(36)	107(44)
	C	70(6)*	151(39)*	55(11)*	101(25)*	116(31)
	D	70(6)*	161(45)*	56(10)*	102(35)*	112(35)
	AC	60(12)	93(27)	42(12)	84(32)	107(44)
	AD	72(13)*	129(37)*	53(14)*	91(29)	104(36)
	CD	72(9)*	146(51)*	51(15)*	104(43)*	116(50)
	ACD	56(12)	85(26)	42(9)	85(26)	106(30)

A: aspirin; C: clopidogrel; D: dipyridamole
† No comparison was made between ACD and no antiplatelet therapy for volunteers since no analogous data were available for patients

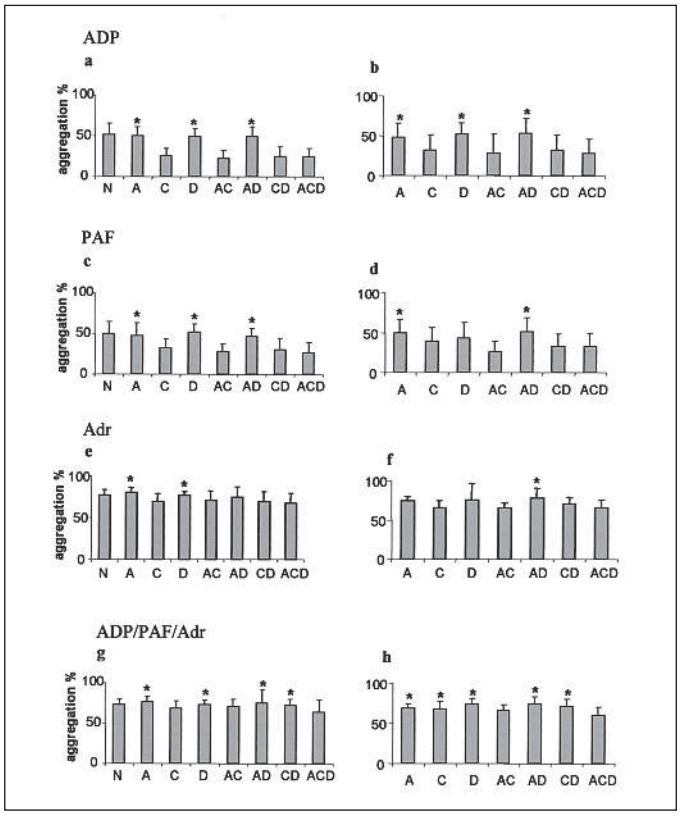

Figure 2: Platelet aggregation *ex vivo* following administration of antiplatelet agents. Left panel shows results obtained from healthy subjects; right panel from patients. N, no-antiplatelet agents; A, aspirin; C, clopidogrel; D, dipyridamole; Adr, adrenaline. (a,b), with 3μM, ADP; (c,d), with 1μM PAF; (e,f), with 3μmol/L adrenaline; (g,h) with 1μM ADP, 0.33μM PAF and 3.33μM adrenaline. Results are mean ± standard deviation. n = 11. *p<0.05, triple therapy against other therapies.

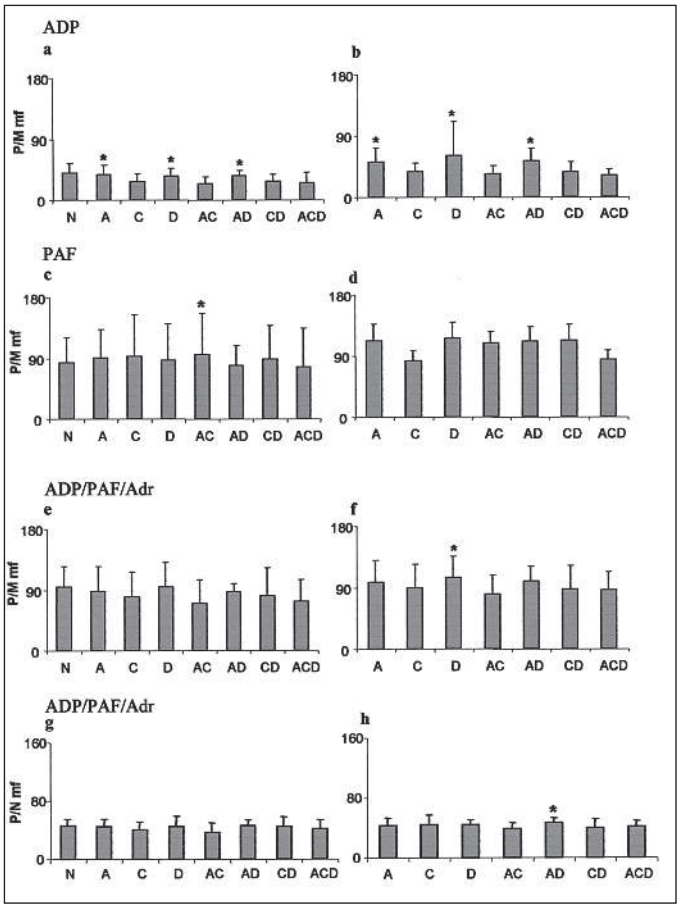

Figure 3: Platelet-monocyte and platelet-neutrophil conjugate formation *ex vivo* following administration of antiplatelet agents. Left panel shows results obtained from healthy subjects; right panel from patients. N, no-antiplatelet agents; A, aspirin; C, clopidogrel; D, dipyridamole; Adr, adrenaline. (a,b), with 3μmol/L ADP; (c,d), with 1μM PAF; (e,f and g,h), with 1μM ADP, 0.33μmol/L PAF and 3.33μM adrenaline. Results are mean ± standard deviation. n = 11. *p<0.05, triple therapy against other therapies.

PAF, adrenaline, and combined ADP/PAF/adrenaline. The one exception was aggregation in response to combined ADP/PAF/adrenaline where ACD was superior to all antiplatelet strategies apart from AC. Additionally, ACD was not significantly different from the other antiplatelet approaches with respect to for monocyte and neutrophil activation, irrespective of agonist (data not shown).

Individual effects of antiplatelet agents

In further analyses, the effects of each individual drug were studied on collagen-induced cell activation. Treatment phases involving aspirin (A, AC, AD, ACD) versus no aspirin (C, D, CD) had significant effect on platelet aggregation, platelet-leucocyte conjugates and leucocyte activation. Similarly, clopidogrel-based treatment (C, AC, CD, ACD) reduced platelet aggregation (in volunteers and patients), platelet-leucocyte conjugates (patients), but not leucocyte activation (volunteers and patients), in comparison with no clopidogrel (A, D, AD). There was no difference in these parameters when assessed by dipyridamole status (D, AD, CD, ACD versus A, C, AC) (Table 4).

Safety

No serious adverse events or major bleeding episodes occurred. Treatment with dipyridamole was associated with headache of sufficient intensity such that 4 volunteers and 3 patients withdrew from the trial and had to be replaced with new subjects; their data are excluded from the above analyses. After 17 subjects had been recruited, we amended the protocol so that all subsequent recruits were given open-label dipyridamole prior to starting the randomised trial; 12 of these did not tolerate the drug, largely because of headache, and were excluded from further study. We did not use techniques to minimise headache such as weaning up the dose of dipyridamole to induce tolerance. Other adverse events included minor bleeding with aspirin and clopidogrel based therapy. We did not assess whether adverse events were modified by interaction between drugs, e.g. whether aspirin modified dipyridamole headache, in view of the small numbers of subjects.

Discussion

Increased platelet activation is well-documented in acute vascular syndromes (7). Studies have shown that platelet activation can lead to the formation of platelet-leucocyte conjugates and

Table 4: Effects of each antiplatelet agent individually on collagen-induced platelet aggregation, platelet-leucocyte conjugate formation, and leucocyte activation in 11 volunteers and 11 stroke patients. Mean (standard deviation); comparison of aspirin (A) with no aspirin (no A), clopidogrel (C) with no clopidogrel (no C), and dipyridamole (D) with no dipyridamole (no D); *p<0.05.

	Antiplatelet	Aggregation (%)	Platelet-monocyte (CD42a)	Platelet-neutrophil (CD42a)	Monocyte activation (CD11b)	Neutrophil activation (CD11b)
Volunteers	A vs no A	64(14)* 72(7)	100(43)* 156(44)	46(9)* 60(11)	105(42)* 121(41)	129(54)* 145(51)
	C vs no C	64(13)* 72(9)	117(57) 133(40)	50(13) 55(10)	109(43) 115(42)	134(48) 138(59)
	D vs no D	67(13) 67(12)	126(51) 121(51)	54(12) 49(12)	112(41) 111(44)	137(54) 135(51)
Patients	A vs no A	64(10)* 71(8)	109(37)* 152(44)	46(12)* 54(11)	88(30)* 102(34)	106(37)* 115(38)
	C vs no C	64(11)* 70(8)	119(47)* 140(41)	48(13)* 52(12)	93(32) 96(33)	111(38) 108(37)
	D vs no D	67(11) 66(9)	130(49) 125(41)	51(13) 48(12)	95(34) 93(31)	109(37) 110(39)

A: aspirin; C: clopidogrel; D: dipyridamole; vs: versus.

leucocyte activation (8, 9, 12). The latter has the potential to support thrombus formation via activation of the coagulation cascade with enhanced thrombin generation and fibrin production (10, 11, 13–17). This is the first study to assess the effects of combining three antiplatelet agents on platelet and leucocyte function ex vivo; previous studies have limited their comparisons to mono and dual antiplatelet therapy (24). Our study was neutral since we did not confirm that triple therapy is superior to dual or mono therapy, at least in respect of the platelet and leucocyte parameters we measured. In particular, ACD was not superior to AC in inhibiting collagen-induced platelet and monocyte activation, and heterotypic cell interactions. However, ACD was better than other dual combinations (AD, CD) and monotherapy. We chose collagen a priori as the focus of this study since it is a key agonist in mediating platelet interactions with the damaged vessel wall, as occurs during atherothrombosis. ACD was also superior to other treatments, apart from AC, in inhibiting platelet aggregation induced by combined ADP/PAF/adrenaline.

These *ex vivo* results contrast with our earlier *in vitro* study where we found the combination of ACD was superior to all dual and mono preparations, especially in inhibiting platelet-monocyte and platelet-neutrophil conjugate formation, and monocyte and neutrophil activation (6). Importantly, similar laboratory methods were used in the two studies. Two possibilities may explain this discrepancy. First, we used AR-C69931, a direct acting $P2Y_{12}$ receptor antagonist, in our *in vitro* study since clopidogrel is a pro-drug and therefore ineffective *in vitro*. Hence, differential efficacy between clopidogrel and AR-C69931 might explain the results. Second, the anti-aggregatory capacity of dipyridamole *in vitro* is concentration dependent and we used 10 μmol/L in our first study. However, modified release dipyridamole, given as 200 mg twice daily, has previously been shown to achieve maximal levels of ~3–4 μM after 4 days of treatment (25). Hence, reliable anti-aggregation concentrations of dipyridamole may not be achieved with conventional dosing, as used in our present trials.

Our data do not necessarily conflict with the findings of ESPS-II where the combination of dipyridamole and aspirin offered superior protection against stroke recurrence than using aspirin or dipyridamole alone, and where dipyridamole was superior to placebo (3). It is increasingly becoming clear that drugs exhibiting vascular protection are often multimodal in their action. For example, statins and angiotensin converting enzyme inhibitors have pleiotropic effects beyond their respective lipid and blood pressure lowering effects (26), although the relevance of these to their proven role in vascular prevention (23, 27, 28) remains to be established (29). Dipyridamole appears to act through a number of mechanisms: it elevates intracellular cyclic AMP and cyclic GMP concentrations via inhibition of types III and V phosphodiesterases respectively, and prevents red cell uptake of circulating adenosine, a platelet antagonist (30–33). It has been suggested that oral dipyridamole has mild hypotensive properties, probably mediated via its inhibition of types V phosphodiesterase (34–36). We did not find that dipyridamole (with or without aspirin and/or clopidogrel) lowered blood pressure (data not shown). In addition to its inhibitory effects on phosphodiesterases and adenosine uptake, dipyridamole also appears to stimulate the production of prostacyclin, inhibit lipid peroxidation and smooth muscle proliferation, and has antioxidant properties (34, 35). Finally, dipyridamole has mild hypotensive properties which might explain some of its stroke protective effects (36). Recently, the capacity of dipyridamole to inhibit platelet function induced by ATP has been established (37). This is under circumstances in which large amounts of adenosine are formed. This may be an important mechanism of thrombus formation *in vivo* that, so far, has not been adequately explored.

The potential downside of using multiple antiplatelet agents is that the risk of bleeding is increased. Our study involved treatment phases of only two weeks duration and definitive statements on the safety of ACD cannot be made. Nevertheless, no serious adverse events or episodes of major bleeding occurred. Dipyridamole was poorly tolerated in some subjects leading to early withdrawal from the trial. We did not perform formal statistical comparisons but treatment with dipyridamole was associated with headache (in some cases severe enough to lead to withdrawal from the study), and clopidogrel with minor bleeding.

These studies involved a relatively small number of subjects and we may have missed biologically important differences between ACD and dual therapy. However, several points suggest that this is unlikely. First, the use of a crossover design maximised the power of the study since each subject acted as their own control. We could not perform a sample size calculation before the trials since the studies were entirely novel and there were no available data. Nevertheless, a blinded calculation after 6 patients in each study suggested that biologically relevant differences would be detectable with a sample size of 10–12 subjects per trial. Second, the point estimates for ACD and AC were similar for most measures (although in some comparisons ACD was non-significantly superior to AC) such that a power calculation on the final data suggested that a trial involving at least 60 patients would be required to detect a difference between ACD and AC. The logistics of the study design would preclude enrolling this number of subjects. Third, we were able to detect meaningful differences between ACD and non AC treatments. Hence, we believe the study was large enough to pick up meaningful differences in cellular function.

Our study is also limited in that we could not assess all platelet and leucocyte measures for logistical reasons. Earlier studies have utilised other measures such a high shear tests and platelet activation, e.g. P-selectin (24). It is possible that other non-platelet tests relevant to atherothrombosis might have been altered more by ACD, particularly ones addressing the mechanisms given above by which dipyridamole may work. For example, dipyridamole appears to reduce plasma von Willebrand factor levels suggesting it may have a protective effect on endothelium (38).

In conclusion, these data do not support our original hypothesis, first shown *in vitro*, that triple antiplatelet therapy might be superior to dual therapy based on either aspirin and clopidogrel or aspirin and dipyridamole. Nevertheless, triple therapy offers a potential strategy for the acute treatment and prevention of cerebrovascular disease. We have used this combination in the management of a small number of complex patients suffering recurrent cerebrovascular events on dual therapy, and so far have found this approach to be both effective and safe (39). Further trials are warranted and we are currently comparing triple therapy with aspirin alone (to maximise our ability to detect potential safety issues, especially major bleeding) in two phase II randomised controlled trials, one involving patients with acute ischaemic stroke and the other including patients with prior ischaemic stroke or TIA.

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