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ORIGINAL ARTICLE

Effects of various doses of aspirin on platelet activity and endothelial function

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Abstract Although aspirin has become an established medicine for cardiac and cerebrovascular diseases, the optimal dose remains unknown. We evaluated the optimal dose of aspirin on platelet activity and endothelial function by administering 11 healthy male volunteers (32 ± 6 years of age) doses of aspirin that were increased in a stepwise manner (0, 81, 162, 330 and 660 mg/day) every 3 days. Platelet activity was assessed as surface P-selectin expression (%) measured by flow cytometry and the platelet aggregation ratio. Endothelial function in the brachial artery was assessed by measuring flow-mediated dilation (FMD) before and after reactive hyperemia. Platelet aggregation and P-selectin expression were significantly and dose-dependently suppressed (81–660 mg), and the FMD ratio tended to increase from 0 to 162 mg, but decreased significantly at 660 mg. In conclusion, although aspirin suppressed platelet activity and even surface P-selectin expression, higher doses worsened endothelial-mediated arterial dilation.

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Introduction

Aspirin is established as a primary and secondary antiplatelet treatment for cardiovascular disease [1–5]. The recommended long-term daily dose of aspirin is 75–150 mg, which is considered to be at least as effective as higher doses [6]. Reports have indicated that higher doses of aspirin are ineffective because of bleeding complications [7–9] and impaired endothelial function [10–12]. Aspirin inhibits the synthesis of thromboxane A2 in platelets and of prostaglandin I2 in endothelial cells. Low dose aspirin only inhibits thromboxane A2 in platelets, whereas a high dose inhibits both. The inhibition of prostaglandin I2 synthesis in endothelial cells would increase the incidence of thromboembolic events [10–12]. However, the effects of optimal doses of aspirin on simultaneous platelet activation and endothelial function in humans remain obscure. One reason for this is that although platelet activation can be evaluated using the platelet aggregation test, a clinical method to evaluate prostaglandin I2 inhibition in endothelial cells remains to be established.

Endothelial function has recently been evaluated as flow-mediated dilation before and after reactive hyperemia [13], and endothelial dysfunction is apparent in patients with cardiovascular and metabolic diseases [14–19]. Although flow-mediated dilation after reactive hyperemia is mainly mediated by NO synthesized in the endothelia [20, 21], another pathway associated with prostacyclin and thromboxane A2 mediates vascular reactivity [22–24]. High dose of aspirin could affect this pathway, leading to vascular relaxation. The present study examines the effects

of various doses of aspirin on platelet activity and endothelial function in healthy humans to determine the optimal dose of aspirin required to suppress platelet aggregation and function.

Subjects and methods

Subjects

We enrolled 11 healthy male volunteers, aged 23–39 years (mean = 32 ± 6 years), with no evidence of heart disease according to a physical examination, standard 12-lead electrocardiography, chest radiography and echocardiography. None of the participants had hypertension, hypercholesteremia, diabetes mellitus or renal disease. All were in sinus rhythm and had not taken any medication for at least 14 days. Each of them provided written, informed consent to participate in the study, the protocol for which was approved by the Local Ethics Committee of Kochi Medical School.

Study protocol

Doses of aspirin (Bufferin 81 or 330 mg/tablet, Lion Co.) were increased (0, 81, 162, 330 and 660 mg) in a stepwise fashion every 3 days for 13 days. Platelet activation and endothelial function were measured at 11:00 a.m. before taking aspirin on the last day of each dose. The participants laid on a bed in a temperature-controlled quiet room, and venous blood was withdrawn from the left forearm vein. Endothelial function was measured in the right arm 30 min after blood collection. The participants did not exercise, consume beverages containing caffeine, high-fat foods or vitamin C, or use tobacco products for at least 4 h before the study.

Endothelial function

Endothelial function was assessed in the brachial artery by measuring flow-mediated dilation before and after reactive hyperemia. Data acquisition and analysis proceeded as described [13]. Briefly, the brachial artery of the right arm was imaged by high-resolution ultrasound (Acuson Sequoia 512) with a 10-MHz linear probe supported by a stereotactic clamp. We selected the B mode longitudinal section of the distal brachial artery, and the M-mode image was magnified using a resolution box function. Vertical internal diameter at end diastole gated by ECG was measured at rest for baseline. A tourniquet placed distally around the ipsilateral forearm was inflated to 250 mmHg for 4.5 min to induce reactive hyperemia. After rapid release, the inner diameter was measured 55 s later. The diameter was measured before and after hyperemia. The FMD ratio (%) was calculated as: 100 × (after diameter − before diameter)/before diameter. Scans were stored and analyzed by two independent observers.

Platelet aggregation test

Platelet function was determined as maximal platelet aggregation rate; that is, platelet-rich-plasma (PRP) was prepared from each participant. To minimize platelet activation during blood collection, blood was withdrawn using a 21-G butterfly needle without a tourniquet. The first 2 ml of blood was discarded, and then 20 ml of blood was transferred into polypropylene tubes containing sodium citrate (3.8%, 1 volume for 9 volumes of blood). We then prepared PRP by centrifugation at $120\times g$ for 10 min at room temperature. Platelet-poor plasma for control to compare PRP aggregation was obtained after re-centrifugation at $1{,}710\times g$ for 15 min.

Platelet aggregation was induced by adenosine diphosphate (ADP) (final concentration of 1.0 or 5.0 μmol/l, MC Medical, Tokyo, Japan) or by collagen (final concentration of 0.25 or 2 μg/ml, MC Medical). The time course of % transmission was measured (MCM HEMA TRACER 212TM; MC Medical), and the maximal platelet aggregation rate was calculated [25, 26].

Measurements of P-selectin (CD62P) and PNC levels

Samples were prepared, and levels of platelet P-selectin (CD62P) and PNC were measured as described [27, 28]. The sample used was the same blood that was collected for the platelet aggregation test. After the first 2 ml of blood was discarded, 2 ml of blood was collected and immediately added to 200 μl of sodium citrate (3.13%). All antibodies were purchased as follows: Fluorescein isothiocyanate (FITC) labeled IgG1 anti-CD62P from Dainippon Pharmaceutical, Osaka; phycoerythin (PE) labeled IgG2a anti-CD42b and FITC labeled IgG1 anti-CD11b from Beckman Coulter, Fullerton. As negative controls, FITC labeled IgG1 (Beckman Coulter, Fullerton) and double-stained (FITC/PE) IgG1 and IgG2a (Dako, High Wycombe) irrelevant antibodies were included. Blood samples were analyzed (EPICS XL Profile Flow Cytometer, Coulter, Miami, FL) using either one or two fluorochromes.

To prepare samples for measurements of platelet CD62P levels, blood (5 μl) was added to round-bottomed polystyrene tubes containing 50 μl platelet buffer (10 mmol/l HEPES, 145 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgSO_4$, pH 7.4), and 5 μl of anti-CD62P or control IgG1 antibody. The samples were gently suspended and incubated in the dark at room temperature for 20 min without stirring. Then 250-μl fixative (Beckman Coulter, Fullerton,

9.25% formaldehyde) was added, and the tubes were incubated for a further 10 min. The samples were then diluted with 500 µl of buffer and analyzed by flow cytometry within 1 h of fixation. In flow cytometric analysis, the peaks emission intensity of FITC and phycoerythrin fluorescence were detected at 515 and 580 nm, respectively. After forward and side scatter was measured with the gain setting in logarithmic mode, platelet sized events were counted. CD62P positive platelets were defined as those with a fluorescence intensity exceeding that of 98% of the platelets stained with control antibody.

To prepare samples for the PNC measurements, blood (50 µl) was added to round-bottomed polystyrene tubes containing 5 µl each of anti-CD42b and anti-CD11b (platelet and neutrophil markers, respectively) or isotype control antibodies. The samples were gently mixed and incubated in the dark at room temperature for 10 min without stirring. Then 500 µl of fixative was added, and the tubes were incubated for a further 10 min. Flow cytometry proceeded within 1 h of preparation. After forward and side scatter were measured with the gain setting in linear mode, neutrophil-sized events were selected. Results were defined as positive when the fluorescence intensity exceeded that of the isotype matched (IgG1 and IgG2a) control antibody staining (98%). Both CD11b and CD42b positive events that were considered PNCs were expressed as ratios (%) of events with positive CD11b staining of those of whole neutrophils. We evaluated the ability of the platelets to be activated, i.e., platelet activation reserve in the presence of 5 µl of ADP (5 µmol/l).

We also counted blood cells and measured coagulation factors of prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen.

Statistical analysis

Data are presented as means ± SEM. Group means for each parameter were determined and compared using the analysis of variance (ANOVA) repeated measures, with the post-hoc Tukey–Kramer test. A value of $p < 0.05$ was considered to represent statistical significance.

Results

Aspirin administration did not alter the white blood cells, red blood cells, hemoglobin, hematocrit or platelet counts in the blood. The coagulation factors, PT and APTT, did not change significantly before and after aspirin administration. However, maximal platelet aggregation rates in the presence of ADP (5.0 µmol/l) or collagen (2 µg/ml) were significantly reduced from the baseline level after all doses of aspirin (81, 162, 330 and 660 mg): ADP; 78 ± 3–67 ± 2, 66 ± 2, 65 ± 2, 66 ± 2%, all ps < 0.01; collagen; 85 ± 2–34 ± 6, 38 ± 6, 34 ± 5, 35 ± 6%, all ps < 0.01. The effects on P-selectin, P-selectin with ADP and PNC with ADP tended to be similar (Table 1; Fig. 1), whereas PNC showed no tendency. Therefore, aspirin of any dose over 81 mg/day suppressed platelet activity.

Blood pressure was 121 ± 8/68 ± 8 at baseline and 127 ± 9/71 ± 8 at the end of the study, showing no significant change. At any dose of aspirin, heart rate at each measurement of FMD did not change significantly. Although arterial diameter did not significantly change after hyperemia, the FMD ratio (%) tended to increase at a dose of 81 mg (1.68 ± 0.29–2.78 ± 0.47, $p = 0.08$) and significantly increased at 162 mg (3.67 ± 0.41, $p < 0.05$) compared with the baseline without aspirin. The FMD ratio then tended to decrease at a dose of 330 mg (3.30 ± 0.50, n.s.) from 162 mg, but tended to be higher compared with the baseline ($p = 0.07$). The FMD ratio further decreased at a dose of 660 mg (1.07 ± 0.34) compared with that at either 162 or 330 mg ($p < 0.01$, $p < 0.01$, respectively), and tended to be lower from baseline or at a dose of 81 mg (Table 1; Fig. 1). Therefore, the optimal dose of aspirin for endothelial function assessed by the FMD ratio was 162 mg per day.

Discussion

The major finding of this study was that aspirin at any daily dose over 81 mg suppressed platelet activity and that the optimal dose of endothelial function was 162 mg/day. However, 660 mg/day of aspirin worsened endothelial function.

Aspirin plays an important role in the primary and secondary prevention of cardiovascular events, and it has remained the most cost-effective clinical drug for over 3 decades [1–6]. Reports indicate that a daily dose of 75–150 mg is just as effective as higher doses. An initial loading dose of at least 150 mg aspirin might be required in acute settings, but the effects of daily doses of <75 mg have been less certain, and doses of >1,000 mg daily are not recommended due to bleeding side effects [7–9].

The recent findings of the Antithrombotic Trialists' Collaboration meta-analysis of patients with previous thrombotic events or other predisposing conditions showed that aspirin reduces the total risk for cardio-cerebral vascular events by 22% [6]. From the viewpoint of the daily aspirin dose, the proportional reduction in vascular events was 19% at 500–1,500 mg/day, 26% at 160–325 mg/day and 32% at 75–150 mg/day. Although the effects of aspirin doses of ≥ or <75 mg in a direct comparison did not significantly differ, doses of <75 mg/day seemed to have somewhat reduced

Table 1 Blood cell count, coagulation factor, platelet activity and endothelial function

Aspirin dose (mg/day)	0	81	162	330	660
Blood cell count					
RBC ($\times 10^4/\mu l$)	490 ± 7	483 ± 10	483 ± 8	483 ± 8	486 ± 7
Hemoglobin (g/dl)	15.2 ± 0.1	15.2 ± 0.2	14.9 ± 0.2	15.0 ± 0.2	14.7 ± 0.2
Hematocrit (%)	44.5 ± 0.3	44.6 ± 0.5	43.8 ± 0.6	44.3 ± 0.6	43.1 ± 0.6
Platelet ($\times 10^4/\mu l$)	26.5 ± 1.4	25.1 ± 1.0	24.3 ± 0.9	24.6 ± 1.1	24.7 ± 0.9
WBC (μl^{-1})	5.70 ± 0.42	5.84 ± 0.34	5.84 ± 0.34	5.74 ± 0.34	6.29 ± 0.56
Coagulation factor					
PT (s)	10.4 ± 0.1	10.3 ± 0.1	10.3 ± 0.1	10.2 ± 0.1	10.1 ± 0.1
PT (%)	95.7 ± 4.0	98.3 ± 5.1	96.3 ± 3.3	99.7 ± 3.1	105.2 ± 5.4
INR	1.03 ± 0.03	1.02 ± 0.03	1.03 ± 0.02	1.01 ± 0.02	0.98 ± 0.03
APTT (s)	28.1 ± 0.8	27.8 ± 0.5	27.8 ± 0.6	27.7 ± 0.7	27.2 ± 0.6
APTT (%)	108.5 ± 5.3	109.6 ± 3.8	109.9 ± 4.6	113.0 ± 4.8	116.1 ± 4.3
Fibrinogen (mg/dl)	198.9 ± 14.8	203.8 ± 9.3	200.4 ± 10.2	193.6 ± 8.8	188.1 ± 12.1
Platelet surface marker					
PNC (%)	7.0 ± 0.8	6.4 ± 0.7	6.9 ± 0.7	6.6 ± 0.6	6.0 ± 0.8
PNC (ADP) (%)	17.8 ± 3.1	14.1 ± 2.0	15.5 ± 1.1	14.4 ± 2.5	15.9 ± 3.4
P-selectin (%)	12.9 ± 1.3	10.2 ± 0.4	11.3 ± 0.7	10.4 ± 0.7	10.5 ± 0.5
P-selectin (ADP) (%)	29.6 ± 2.7	26.6 ± 2.2	27.4 ± 2.4	26.2 ± 2.2	27.3 ± 2.2
Platelet maximal aggregation rate					
ADP (5 μmol/l) (%)	78 ± 3	67 ± 2**	66 ± 2**	65 ± 2**	66 ± 2**
ADP (1 μmol/l) (%)	31 ± 3	32 ± 4	33 ± 4	31 ± 4	33 ± 3
Collagen (2 μg/ml) (%)	85 ± 2	34 ± 6**	38 ± 6**	34 ± 5**	35 ± 6**
Collagen (0.25 μg/ml) (%)	23 ± 8	7 ± 2	8 ± 2	8 ± 1	7 ± 1
Endothelial function					
Diameter (before) (%)	0.367 ± 0.013	0.386 ± 0.014	0.375 ± 0.012	0.379 ± 0.013	0.379 ± 0.010
Heart rate (before) (bpm)	77 ± 10	76 ± 7	75 ± 8	72 ± 8	73 ± 7
Diameter (after) (%)	0.374 ± 0.013	0.396 ± 0.013	0.388 ± 0.013	0.391 ± 0.013	0.382 ± 0.009
Heart rate (after) (bpm)	77 ± 10	78 ± 9	73 ± 12	71 ± 8	73 ± 6
FMD ratio (%)	1.68 ± 0.29	2.78 ± 0.47	3.67 ± 0.41*	3.30 ± 0.50	1.07 ± 0.34$^{\#\$}$

Values are mean ± SE

RBC red blood cells, *WBC* white blood cells, *ADP* adenosine diphosphate, *PT* prothrombin time, *INR* international normalized ratio, *APTT* activated partial thromboplastin time, *PNC* platelet neutrophil complexes, *FMD* flow-mediated dilation

* $p < 0.05$ and ** $p < 0.01$ compared with baseline value (0 mg); $^{\#}$ $p < 0.01$ and $^{\$}$ $p < 0.01$ compared with values at 162 and 330 mg aspirin, respectively

effects (proportional reduction 13%). However, trials that examined doses of aspirin of ≥75 mg/day found a significant reduction in cardiovascular events, whereas three trials using doses of <75 mg/day did not. Similarly, a recent large trial of aspirin in primary prevention among women did not demonstrate a benefit of very-low-dose aspirin (100 mg every other day) [29]. No evidence supports the notion that daily aspirin doses of >1,000 mg is preferable for the prevention of serious vascular events among patients at high risk of stroke [7, 8]. One study found that the risk of the composite outcome of myocardial infarction, stroke or death within 3 months of carotid endarterectomy was significantly lower among patients taking 81 or 325 than 625–1,300 mg of aspirin daily [9]. In the present study, aspirin at doses above 81 mg suppressed platelet activity assessed by the aggregation test, P-selectin and PNC levels. This result is consistent with previous reports that show aspirin at even the low dose of 81 mg exerts antiplatelet effects.

Higher doses of aspirin can cause bleeding complication [7–9] and also impair endothelial function, which is known as the "aspirin dilemma" [10–12]. Aspirin inhibits a production of thromboxane A2 and cyclo-oxygenase enzyme, which synthesize thromboxane A2, a potent platelet aggregator in the platelets. Therefore, inhibiting thromboxane A2 is anti-thrombotic. Aspirin also inhibits cyclo-oxygenase enzyme in vascular endothelial cells, which is the source of prostaglandin I2, a vasodilator. A high dose of aspirin achieves both platelet inhibition

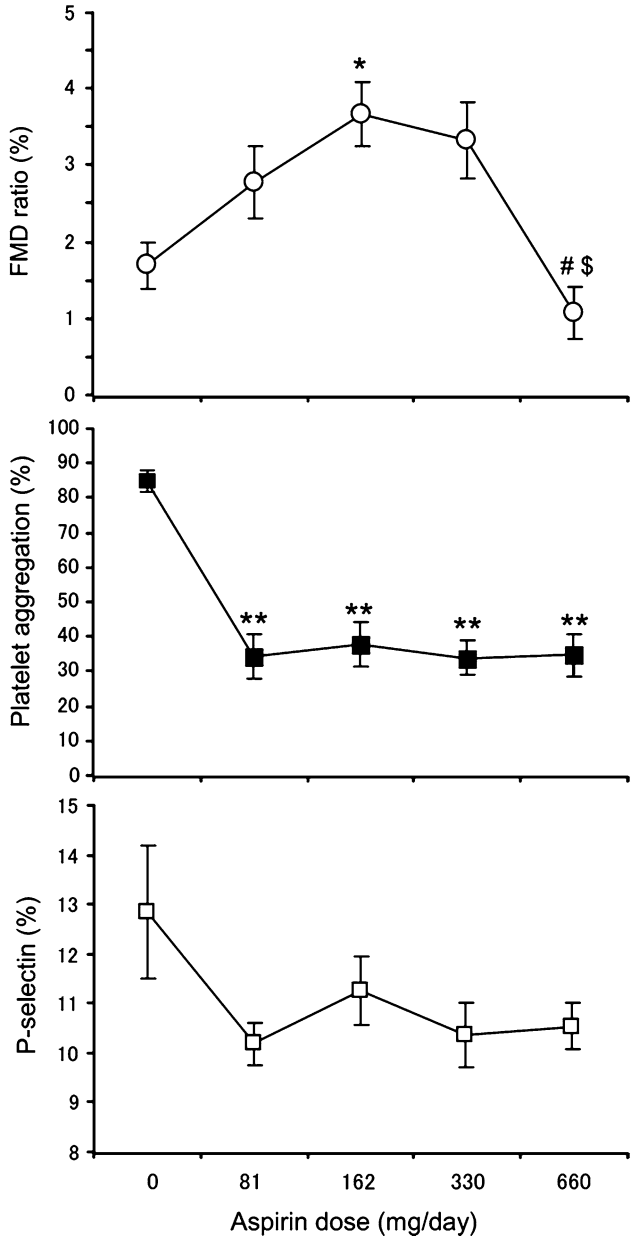

Fig. 1 FMD ratios (*upper panel*), maximal platelet aggregation rates (*middle panel*) and P-selectin levels (*lower panel*). Values are means ± SE. *FMD* Flow-mediated dilation. $^{*}p < 0.05$ from baseline (0 mg), $^{\#}p < 0.01$ from value at aspirin 162 mg, $^{\$}p < 0.01$ from value at 330 mg

and vasodilation, whereas a low dose spares endothelial cyclo-oxygenase activity and vasodilation. Therefore, the inhibition of prostaglandin I2 synthesis in the endothelial cells would increase the incidence of thromboembolic events. However, no clinical studies have examined endothelial function at various doses of aspirin, because endothelial function cannot be evaluated to determined platelet activity in a clinical setting. Endothelial function has recently been evaluated noninvasively as FMD to stratify patients according to cardiovascular risk, and endothelial dysfunction is associated with a poor prognosis [14–19]. Although a direct mechanism of decreased a FMD ratio at high dose of aspirin is uncertain, this could be due to high dose aspirin affecting vasodilation via endothelial cyclo-oxygenase inhibition.

Although FMD after reactive hyperemia is mainly mediated by NO synthesized in the endothelia [20, 21], another pathway associated with prostacyclin and thromboxane A2 mediates vascular reactivity [22–24]. Husain et al. [22] showed aspirin modulates acetylcholine-induced peripheral vasodilatation in patients with coronary atherosclerosis, and this effect may be due to the inhibition of vasoconstriction induced by one or more cyclo-oxygenases. Sun et al. [23] also showed that FMD is mediated by endothelium-derived prostanoids as it is blocked by indomethacin in eNOS knockout mice. Furthermore, Taubert et al. [24] showed a therapeutically relevant concentration of aspirin elicits NO release from the vascular endothelium independently of cyclo-oxygenase inhibition in in vitro study. These findings showed low dose aspirin could improve endothelial dysfunction and increase FMD. On the other hand, Gori et al. [30] reported 500 mg aspirin (once oral) did not affect the FMD ratio, but it significantly blunted low-flow-mediated constriction of the resting arterial tone. The difference from our results may be due to the dose and period of aspirin intake.

We found here that the FMD ratio increased at 162 mg from the baseline level without aspirin, and that this significantly decreased at a dose of 660 mg from that of 162 or 330 mg. Thus, low dose aspirin (<330 mg) did not deteriorate endothelial function, and 81 mg was sufficient to suppress platelet activity in healthy volunteers. However, platelets are more activated in patients with atherosclerosis than in normal individuals [31], and patients with atherosclerosis have endothelial dysfunction [14–19]. Moreover, several studies have indicated that patients at risk for atherosclerosis were less sensitive to the platelet inhibitory effect of aspirin [32–36]. Therefore, the optimal dose of aspirin might differ among patients with atherosclerosis compared with normal individuals, and aspirin doses of >81 mg might be required.

Platelet aggregation tests and FMD measurement might comprise an easy and noninvasive tool to determine the optimal dose of aspirin for such patients in a clinical setting. We applied the platelet aggregation test with light transmittance aggregometry and direct P-selectin measurements. Levels of platelet inhibition might be determined by evaluating several critical pathways [37]. That study used a new device based on light transmittance aggregometry that measures platelet activity more rapidly than classical devices and that could guide the choice of antiplatelet therapies. This method can evaluate patients

with aspirin resistance and optimize the dose, but cannot evaluate the extent to which various aspirin doses affect the endothelial function. On the other hand, FMD can evaluate the endothelial function affected by aspirin and generate useful information that could improve clinical outcomes.

Finally, the sample size in this study was small, and the subjects were normal male volunteers. Patients with atherosclerotic disease might have more activated platelets than normal individuals. Further studies are needed with a larger number of normal individuals and a cohort of patients with atherosclerotic disease.

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