Dose-Related Effect of Aspirin on Laboratory-Defined Platelet Aggregation and Clinical Outcome After Coronary Stenting

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SUMMARY
Background: Acetylsalicylic acid (aspirin) is widely used in the secondary prevention of coronary artery disease. There is controversy regarding the prevalence of aspirin resistance in patients with coronary artery disease and the effect of dose on resistance. Our primary aims were to determine the degree of platelet responsiveness to aspirin, and to study the influence of dose on platelet inhibition and clinical outcomes after coronary stenting. Methods and Results: We prospectively studied the effect of aspirin on platelet function in 106 stable outpatients 6 months after successful percutaneous coronary angioplasty. Participants were randomized in a double-blind, double-crossover study (80 or 500 mg per day for 6 months). The platelet response to aspirin was determined by 10 μmol/L adenosine-5-diphosphate-induced aggregation with light transmission aggregometry. The clinical outcome was determined by single photon emission computed tomography with Tc-99m, and major adverse cardiac events were recorded (myocardial infarction, death, unstable angina or need for revascularization). In both groups 30.2% of the participants were resistant to aspirin. There was no significant difference between the dose of 80 mg compared to 500 mg aspirin in the incidence of aspirin resistance ($P = 0.3$). No correlation was found between aspirin resistance and clinical outcome ($P = 0.4$). Female sex and smoking were strongly associated with aspirin resistance. Conclusion: The frequency of aspirin resistance is not dependent on the dose of aspirin. Female sex and smoking were the strongest predictors of aspirin resistance. Aspirin resistance is not a predictor of poor clinical outcome in patients who received double antiplatelet therapy.

Introduction
Atherosclerotic coronary artery disease is a leading cause of mortality in the industrialized world. The primary factor in clot formation and the consequent acute coronary event is the role of platelets. These cells can be activated by several agents, including thromboxane A2, collagen, adenosine-5-diphosphate (ADP) and thrombin, each of which acts through different receptors. Antiplatelet therapy is thus a cornerstone of cardiovascular medicine [1].

Because of the central role of platelets in the pathophysiology of atherothrombosis, numerous medical therapies based on antiplatelet agents have been developed. The most frequently used platelet function inhibitor is acetylsalicylic acid (ASA), commonly known as aspirin [2,3]. Clinical trials have shown the efficacy of aspirin in both the primary and secondary prevention of myocardial infarction, stroke and cardiovascular death [4]. Meta-analyses of clinical trials have indicated that aspirin in patients with vascular disease is associated with a 25–44% reduction in adverse cardiovascular events [5,6]. Other metaanalyses of randomized trials have shown that antiplatelet therapy prevents serious vascular events [7], arterial occlusion [8], and venous thromboembolism [9].
among a wide range of patients at high risk for occlusive vascular events. A recent metaanalysis concluded that in the patient population at high risk for vascular events, aspirin therapy was associated with a 34% reduction in nonfatal myocardial infarction, a 25% reduction in non-fatal stroke and an 18% reduction in all-cause mortality [10]. Aspirin, therefore, qualifies as a successful and cost-effective antithrombotic that prevents acute events in cardiovascular disease.

Despite the clear benefit of aspirin’s antiplatelet properties, the absolute risk of recurrent vascular events among patients treated with aspirin remains relatively high at 8–18% after 2 years [4]. The failure of aspirin therapy in these patients suggests there may be heterogeneity in individuals’ responses to aspirin, and that an additional therapeutic agent may be necessary to block platelet function effectively. Recently, clinical and laboratory measurements of platelet function have confirmed that patients vary in their antithrombotic response to aspirin therapy [11–20].

Aspirin resistance is generally defined as the failure of aspirin to produce the expected biological effect (i.e., platelet inhibition) or failure of the drug to prevent an atherothrombotic event. The inability of aspirin to protect patients against vascular acute recurrences has been termed aspirin resistance, whereas the failure of aspirin to inhibit platelet reactivity has also been called biological aspirin resistance [21].

Although estimates differ widely among studies, aspirin resistance may affect between 5 and 45% of the population [4]. Because antiplatelet therapy is the cornerstone of cardiovascular medicine and ASA is the most commonly used drug, the potential impact of aspirin resistance is large. Therefore, identifying aspirin resistance in high-risk patients and finding alternative forms of antiplatelet therapy to augment platelet inhibition may have significant clinical impacts on the prevention of cardiovascular events. At this time, however, there is controversy regarding the prevalence of platelet resistance to aspirin in patients with coronary artery disease and the effect of different doses of aspirin on this resistance. The present study was designed to evaluate the dose-related effects of aspirin on biological ASA resistance and clinical outcomes in patients who had undergone percutaneous coronary angioplasty (stenting) 6 months previously were eligible for enrollment. They were randomly divided in two groups: group A received 80 mg ASA daily as part of their normal follow-up regimen, and group B received 500 mg ASA daily. Patients in both groups were followed for 6 months. After each dose the platelet response to aspirin was measured with the 10 μmol/L ADP-induced aggregation test, using light transmission aggregometry (Chronolog, Havertown, PA, USA).

Clinical outcome was evaluated with single photon emission computed tomography myocardial perfusion imaging (SPECT MPI) with Tc-99m, and by recording major adverse cardiac events, that is, the composite of cardiovascular death, myocardial infarction and unstable angina requiring hospitalization. Cardiovascular death was defined as any death for which there was no clearly documented nonvascular cause. Myocardial infarction was defined according to European Society of Cardiology/American College of Cardiology criteria. Unstable angina was defined as angina pectoris or its equivalence with one of the following features: Canadian Cardiovascular Society (CCS) class IV, new-onset CCS class III, or increasing severity to at least CCS class III. Positive SPECT was considered as any evidence of ischemia in the stented vessel territory.

Aspirin concentration in blood was measured in patients with aspirin resistance to ensure that circulating aspirin levels were sufficient for treatment. Patients were excluded from the study if they had bleeding diathesis or a history of gastrointestinal bleeding, hemorrhagic stroke, illicit drug or alcohol abuse, coagulopathy, major surgery within 6 weeks before randomization, platelet count <100,000/mm³, hematocrit <25%, creatinine >4 mg/dL, or if they were using nonsteroidal anti-inflammatory drugs, anticoagulants or antiplatelet drugs other than aspirin. None of our patients used oral contraceptive pills.

Nonfasting blood samples were obtained between 9.00 and 12.00 a.m. (2 h after aspirin intake). They were drawn at room temperature (22–24 °C) by antecubital venipuncture with a 10-mL syringe, and the first milliliters of blood were discarded to avoid spontaneous platelet activation. Blood specimens were immediately transferred to three laboratory tubes: two plastic tubes containing 0.5 mL sodium citrate for platelet aggregation studies, and one plastic tube containing one drop of potassium oxalate for hematology assays. The first two tubes were filled with 4.5 mL of the patient’s blood to obtain a 1:9 concentration, and the third tube was filled with 1–2 mL of the patient’s blood. The samples were kept at pH 6.5–8.5 to prevent hemolysis due to ADP release from lysed red blood cells. All laboratory procedures

Materials and Methods

A total 106 patients were enrolled in this single-center, double-blind double-crossover study. All participants, investigators and study-related staff were blinded to randomization and treatment schedules. Patients who had
were done within 2–3 h after sampling to minimize environmental effects.

The hemoglobin assay and platelet counts were performed on a Sysmex KX-21 automated hematology analyzer (Sysmex Corp., Kobe, Japan). As normal laboratory values we used platelet count 150 to 450 × 10^3/μL, hemoglobin 13.5–17.5 g/dL in men and 12–16 g/dL in women.

Aggregation studies were done in 10.0–μM concentrations of ADP agonist (Helena Biosciences, Tyne and Wear, UK). A stock solution was prepared by gently mixing one vial (1 mL) with 1 mL distilled or deionized water until the reagent was completely dissolved. The working concentration was 20.0 μM, and the solution was further diluted with distilled water to a final concentration of 10.0 μM. Platelet aggregation specimens were kept at room temperature (22–24 °C) and processed within 1 h of blood collection. The instrument was turned on 30 min before specimens were loaded. One of the whole blood specimens from each participant was centrifuged at 800 rpm for 8 min to obtain platelet-rich plasma, and the other specimen was centrifuged at 4000 rpm for 20 min to obtain platelet-poor plasma (Eppendorf 5810R refrigerated centrifuge, Hamburg, Germany). Platelet counts were obtained with platelet-rich plasma, and were adjusted to 250 and 300 × 10^3 platelets per milliliter with platelet-poor plasma. Aggregation was performed with ADP (Helena Biosciences) at 10 μM with a Helena Laboratories PACKS-4 Platelet Aggregation Chromogenic Kinetics System (Beaumont, TX, USA). Assay time was 5 s for blank cuvettes filled with platelet-poor plasma and 3 min for test cuvettes. Aspirin resistance was defined as 70% ADP-induced aggregation evaluated by light transmission aggregometry [4].

The concentration of salicylic acid in blood in 32 patients with aspirin resistance (platelet aggregation >70%) was measured by high-performance liquid chromatography (HPLC). Blood samples were prepared with acetonitrile and HCI to precipitate the proteins; then 10 μL of the aliquot was injected with a 2-μL Hamilton loop. The compounds were separated on a reversed phase column (C18, 25 mm × 4.5 mm, 5 μm particle size, Shimadzu, Kyoto, Japan) with an isocratic mobile phase consisting of methanol, acetic acid and water (47.5:5:47.5 v/v). The mobile phase was eluted with a Waters 600 series HPLC pump controller system (Waters, Milford, MA, USA) at 1.5 mL/min. A Waters 2487 series ultraviolet absorbance detector was used at a setting of 280 nm. Salicylate level more than 100 mcg/mL was considered acceptable [22].

Continuous (quantitative) variables are reported as means ± SD, and categorical (qualitative) variables as frequencies and percentages. Categorical variables were compared with the chi-squared test, Pearson’s chi-squared test, and Fisher’s exact test. For continuous variables we used analysis of variance with the t-test. Mann-Whitney, Wilcoxon, and Z sample tests were used to compare continuous variables if the sample size was smaller than expected. A P-value of <0.05 was considered statistically significant. All statistical analyses were done with Statistical Package for Social Sciences v. 15 software (StataCorp, College Station, TX, USA). The primary end-point of this study was the degree of ASA resistance in 80 and 500 mg dosing of ASA and secondary endpoint was poor clinical outcomes in ASA resistance and ASA sensitive groups. Concerning the small number of patients with poor clinical outcomes in both ASA resistant and sensitive groups the study may be underpowered for secondary endpoints.

All patients were informed about the aims of the study, and signed a written consent form prior to the study.

Results

Demographic data for the patients in the two groups are given in Table 1. There were no statistically significant differences between the groups. A total of 32 (30.2%) of the 106 enrolled patients were found to be aspirin resistant. Aspirin resistance developed in 15 patients (26.3%) in group A (80 mg aspirin) and in 17 patients (34.7%) in group B (500 mg aspirin). The difference in numbers was not statistically significant (P = 0.3, Odd ratio: 0.67). Problematic clinical outcomes (SPECT MPI-positive findings and major adverse cardiac events) developed in 6 patients (10.7%) in group A, but in no patients in group B, a difference that was statistically significant (P = 0.01, Odd ratio: 2.31). When we compared clinical outcomes between aspirin-resistant and aspirin-sensitive patients, we found that poor clinical outcomes appeared in 1 (3.1%) of the former and 5 (6.9%) of latter, a difference which was not significant (P = 0.4).

The incidence of aspirin resistance correlated with age, sex, diabetes status, smoking habit, hypertension, hyperlipidemia, family history, and stent size and type (drug-eluting or bare metal). In both groups, sex (P = 0.01, Odd ratio: 4.41) and smoking (P = 0.04, Odd ratio: 4.11) were significant predictors of aspirin resistance. There were no statistically significant differences in the association of other variables with aspirin resistance (Table 2).

Poor clinical outcome did not differ significantly by age (P = 0.9), sex (P = 0.3), hypertension (P = 0.7), hyperlipidemia (P = 0.2), smoking (P = 0.5), diabetes (P = 0.3), family history (P = 0.7), stent size (P = 0.056), or type of stent (P = 0.6). In two participants in group B, SPECT MPI was not done because they withdrew from the study; however, neither developed any major cardiac events.
Table 1  Demographic characteristics of 106 patients in 80 and 500 mg ASA group, Shiraz, Iran

<table>
<thead>
<tr>
<th>Variable</th>
<th>80 mg aspirin group (n = 57)</th>
<th>500 mg aspirin group (n = 49)</th>
<th>P-value/Odd ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57 ± 10.05</td>
<td>55 ± 10.22</td>
<td>0.3</td>
</tr>
<tr>
<td>Stent size (millimeter)</td>
<td>21 ± 8.2</td>
<td>21 ± 9</td>
<td>0.8</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>40:17</td>
<td>32:49</td>
<td>0.5/0.8</td>
</tr>
<tr>
<td>Smoker (P:N)</td>
<td>22:35</td>
<td>20:29</td>
<td>0.8/1.23</td>
</tr>
<tr>
<td>Diabetic mellitus (P:N)</td>
<td>8.49</td>
<td>5.44</td>
<td>0.5/1.44</td>
</tr>
<tr>
<td>Hypertension (P:N)</td>
<td>34:20</td>
<td>24:25</td>
<td>0.09/1.93</td>
</tr>
<tr>
<td>Family history (P:N)</td>
<td>8.49</td>
<td>14.35</td>
<td>0.09/0.41</td>
</tr>
<tr>
<td>Hyperlipidemia (P:N)</td>
<td>46.11</td>
<td>44.5</td>
<td>0.10/0.48</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD for age and stent size and frequency for other variables.

*P: Positive clinical marker, N: Negative clinical marker.

Chi-squared test was use for analysis of age and stent size in two groups and analysis of variance with t-test was used for comparison of other variables.

Table 2  Correlation of demographic and clinical variables with aspirin resistance in 106 patients in Shiraz, Iran

<table>
<thead>
<tr>
<th>Variable</th>
<th>Aspirin resistance n = 32</th>
<th>P-value/likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57.09 ± 10.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Stent size (millimeter)</td>
<td>19.8 ± 7.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>14 (43.8%):18(56.3%)</td>
<td>0.001/4.41</td>
</tr>
<tr>
<td>Smoking (P:N)</td>
<td>26 (81.3%):6(18.8%)</td>
<td>0.004/4.11</td>
</tr>
<tr>
<td>Diabetes mellitus (P:N)</td>
<td>5 (15.6%):27(84.4%)</td>
<td>0.4/0.65</td>
</tr>
<tr>
<td>Hypertension (P:N)</td>
<td>23(71.9%):9(28.1%)</td>
<td>0.057/0.41</td>
</tr>
<tr>
<td>Hyperlipidemia (P:N)</td>
<td>3(9.4%):29(90.6%)</td>
<td>0.2/0.48</td>
</tr>
<tr>
<td>Family history (P:N)</td>
<td>7(21.9%):25(78.1%)</td>
<td>0.8/0.91</td>
</tr>
<tr>
<td>Stent type (B: D)</td>
<td>20(62.5%):12(37.5%)</td>
<td>0.5/1.27</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD (age and stent size) or as frequencies and percentages (other clinical variables).

*P: Positive clinical marker, N: Negative clinical marker.

Chi-squared test was use for analysis of age and stent size in two groups and analysis of variance with t-test was used for comparison of other variables.

None of patients in ASA resistant group had salicylate level below 100 μg/mL (mean salicylate level was 143 mcg/mL).

Discussion

Aspirin is the most widely used drug in the world [23], and ASA is the most commonly used antiplatelet agent in clinical practice. Although it reduces the risk of ischemic events by 22% in a broad spectrum of patients with atherothrombosis [6], the effects of aspirin may not be uniform in all patients. Our study of aspirin resistance and clinical outcome in people given two different doses of aspirin was motivated, in part, by the controversy that surrounds the relationship between aspirin dose and treatment effect [11,16,24].

It has been estimated that up to 45% of patients do not achieve an adequate antiplatelet response with aspirin [25–27]. Few prospective studies have focused on the link between laboratory-measured aspirin resistance and clinical outcomes after long-term follow-up in stable cardiovascular patients [27,28]. In recent years an increasing number of reports about aspirin resistance have led to growing concern among clinicians and patients about the efficacy of aspirin treatment [26]. Various studies that evaluated the antiplatelet effect of aspirin therapy found the prevalence of aspirin resistance to be between 0.4 and 35% [29,30]. However, these studies involved different doses of aspirin and used different methods to assess aspirin response.

In our study the incidence of aspirin resistance was 30.2%. We found no statistically significant difference in the incidence of aspirin resistance between the 80 and the 500-mg groups (P = 0.3). There was no correlation between aspirin resistance and clinical outcome (P = 0.4). Our results also confirmed that female sex and smoking were strongly associated with aspirin resistance.

Aspirin resistance correlated with worse clinical outcomes in previous studies [17,31–33], but this
correlation was not found in our study. This may be due to the coadministration of plavix with ASA for 6 months. Dual antiplatelet therapy in stented patients in first 6 months, a high-risk period for stent thrombosis, can abolish the effect of ASA resistance on clinical outcome. In general practice we have not found high resistance to ASA in patients who have received a stent, and this may be due to the absence of an effect of ASA resistance on clinical outcomes in these patients. ADP induced aggregation test by light transmission aggregometry measures COX1 independent mechanism of platelet aggregation but there are evidences that ASA can exert its antiplatelet action by COX1 dependent and independent mechanisms [34,35]. So there is no perfect test to measure ASA resistance, and laboratory defined ASA resistance have poor clinical correlation [35]. Although, this may be partly due to use of a dual antiplatelet regimen for stented patients in the high risk period of first six months. Astonishingly, the higher dose of ASA (500 mg per day) was associated with more ASA resistance. Although, it was not reach to clinical significance, this finding needs further study to determine whether higher doses of ASA are associated with more resistance. In addition, further research will be needed to determine the possible influence of aspirin resistance on outcomes in patients who undergo percutaneous coronary angioplasty with stenting concerning the small number of patients with poor clinical outcome in each group. It should be emphasized that this study is underpowered for its secondary endpoint which is ASA resistant, and larger study with more adverse clinical outcomes needed to evaluate effect of ASA resistance on clinical outcomes.

**Conclusion**

The frequency of aspirin resistance was not dependent on the dose of ASA. There was no correlation between aspirin resistance and clinical outcome. Female sex and smoking were the strongest predictors of ASA resistance.

**Study Limitation**

The number of patients in each group is small and more importantly the numbers of patients with poor clinical outcomes are small in both groups, which may affect the final comment of effect of ASA dose on clinical outcomes.

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**Conflict of Interest**

The authors have no conflict of interest.

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