# Effects of Triple Antiplatelet Therapy (*Aspirin*, *Clopidogrel*, and *Cilostazol*) on Platelet Aggregation and P-Selectin Expression in Patients Undergoing Coronary Artery Stent Implantation

Bong-Ki Lee, MD, PhD[a], Seung-Whan Lee, MD, PhD[a], Seong-Wook Park, MD, PhD[a,*], Se-Whan Lee, MD[a], Duk-Woo Park, MD[a], Young-Hak Kim, MD, PhD[a], Cheol Whan Lee, MD, PhD[a], Myeong-Ki Hong, MD, PhD[a], Jae-Joong Kim, MD, PhD[a], Seongsoo Jang, MD, PhD[b], Hyun-Sook Chi, MD, PhD[b], and Seung-Jung Park, MD, PhD[a]

**The purpose of this study was to determine the effect of the addition of cilostazol to aspirin plus clopidogrel on platelet aggregation after intracoronary stent implantation. Twenty patients who underwent coronary stent placement were randomly assigned to therapy with aspirin plus clopidogrel (dual-therapy group, n = 10) or aspirin plus clopidogrel plus cilostazol (triple-therapy group, n = 10). A loading dose of clopidogrel (300 mg) and cilostazol (200 mg) was administered immediately after stent placement, and clopidogrel (75 mg/day) and cilostazol (100 mg twice daily) were given for 1 month. Platelet aggregation in response to adenosine diphosphate (ADP; 5 and 20 μmol/L) or collagen and P-selectin (CD-62P) expression was assayed at baseline, 2 hours, 24 hours, 1 week, and 1 month after stent placement. Inhibition of ADP-induced platelet aggregation was significantly higher in patients receiving triple therapy than those receiving dual therapy from 24 hours after stent placement, and inhibition of collagen-induced platelet aggregation was significantly higher in the triple-therapy group beginning 1 week after stent placement. P-Selectin expression was significantly lower in the triple-therapy than dual-therapy group at 1 week and 30 days. In conclusion, compared with dual antiplatelet therapy, triple therapy after coronary stent placement resulted in more potent inhibition of platelet aggregation induced by ADP and collagen. These findings suggest that triple therapy may be used clinically to prevent thrombotic complications after coronary stent placement.**  **(Am J Cardiol 2007;100:610–614)**

Cilostazol is a drug with a different mechanism of action than clopidogrel. Cilostazol inhibited activity of the enzyme cyclic adenosine monophosphate (cAMP) phosphodiesterase in platelets and the production of platelet-derived growth factor in endothelial cells.[1] In randomized trials, cilostazol was as effective as ticlopidine[2–4] or clopidogrel[5] in preventing stent thrombosis after coronary stent placement, and triple antiplatelet therapy with aspirin and cilostazol plus clopidogrel or ticlopidine was more effective in preventing stent thrombosis than dual therapy with aspirin plus clopidogrel or ticlopidine.[6] However, less is known about the effects of these treatments on platelet function test results. We therefore compared the effects of triple (aspirin plus clopidogrel plus cilostazol) and dual therapy (aspirin plus clopidogrel) on platelet aggregation and P-selectin (CD-62P) expression in patients undergoing coronary intervention.

## Methods

The 20 patients awaiting elective coronary stent implantation were randomly assigned to treatment with aspirin plus clopidogrel (dual-therapy group, n = 10) or aspirin plus clopidogrel plus cilostazol (triple-therapy group, n = 10) after coronary angiography. All patients received aspirin (200 mg/day) for ≥1 week before coronary intervention. Loading doses of clopidogrel (300 mg) and cilostazol (200 mg) were administered immediately after stent placement, and patients were maintained on clopidogrel 75 mg/day, cilostazol 100 mg twice daily, and aspirin 200 mg/day for 30 days.

Unfractionated heparin was administered intravenously to all patients before percutaneous coronary intervention to achieve an activated clotting time >300 seconds. Heparin therapy was not continued after coronary stent placement. Glycoprotein IIb/IIIa inhibitors were not given, as specified by the research protocol. Patients with a contraindication to antiplatelet agents were excluded, including those with thrombocytopenia (<100,000 platelets/μL), history of bleeding diathesis, illicit drug or alcohol abuse, acute myocardial infarction (within 2 weeks), or recent use of an antiplatelet agent other than aspirin. The study protocol was

[a]Departments of Medicine and [b]Laboratory Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea. Manuscript received November 22, 2006; revised manuscript received and accepted March 13, 2007.

This work was supported by the Cardiovascular Research Foundation, Seoul, Korea, and Grant No. 0412-CR02-0704-0001 from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Seoul, Korea.

*Corresponding author: Tel.: 82-2-3010-3150; fax: 82-2-486-5918.

*E-mail address:* swpark@amc.seoul.kr (S.-W. Park).

0002-9149/07/$ – see front matter 
doi:10.1016/j.amjcard.2007.03.070

approved by the hospital institutional review board and performed in accordance with international ethical regulations. Participants gave written informed consent.

Blood samples were obtained just before percutaneous coronary intervention and at 2 hours, 24 hours, 1 week, and 1 month after intervention using the double-syringe technique, in which the first 5 ml of blood was drawn into an empty syringe, which was discarded. Blood samples were drawn into sodium citrate–containing tubes (Vacutainer, Becton-Dickinson, Inc., San Jose, California) for platelet aggregation studies and ethylenediaminetetraacetic acid–containing tubes (Vacutainer) for flow cytometry.

Platelet aggregation tests were started within 60 minutes after blood sampling. People performing platelet aggregation and flow cytometric tests were blinded to patient randomization. Platelet-rich plasma was prepared immediately by centrifuging blood samples at 200 g for 10 minutes, and platelet-poor plasma was prepared by centrifugation at 1,600 g for 10 minutes. As agonists, we used adenosine diphosphate (ADP) at final concentrations of 5 and 20 $\mu$M (mol/L) and collagen at a final concentration of 2 $\mu$g/ml. Platelet aggregation was assessed at 37° using a PACKS-4 aggregometer (Helena Laboratories Corp., Beaumont, Texas) and expressed as maximal percentage of change in light transmittance from baseline platelet-rich plasma, using platelet-poor plasma as a reference.

Blood samples were incubated with ADP 20 $\mu$M at room temperature for 10 minutes to stimulate platelet activation. Samples were subsequently incubated for 20 minutes at room temperature with phycoerythrin-conjugated anti–P-selectin monoclonal antibody (Pharmingen, San Diego, California), a marker of platelet activation, and Peridinin Chlorophyll Protein-conjugated anti-CD41a (Pharmingen) as control. Each sample was diluted 10-fold in phosphate-buffered solution containing 1% paraformaldehyde to fix the cells. Platelet surface expression of P-selectin was analyzed using FACScan flow cytometry (Becton-Dickinson, Inc.) within 4 hours of cell fixation while the epitopes were still stable. Platelets were identified by their characteristic light-scattering profiles. Results were expressed as percentage of specific antibody-positive platelets, defined as those with a fluorescence intensity >99% of control platelets.

Continuous variables are presented as mean ± SD and compared using Wilcoxon's signed-rank test or Mann-Whitney U test. Categorical variables are presented as number or percentage and compared using chi-square test or Fisher's exact test. Statistical significance is defined as $p < 0.05$.

## Results

There were no significant differences in patient characteristics between the 2 groups (Table 1). No major adverse cardiovascular events, including death, myocardial infarction, stroke, or stent thrombosis, occurred during the 1-month study period. In addition, there were no serious adverse reactions causing drug discontinuation and no major bleeding episodes requiring transfusion.

Platelet aggregation profiles are shown in Table 2 and Figure 1. At baseline, platelet aggregation induced by ADP and collagen were similar between the 2 groups. Inhibition of ADP-induced platelet aggregation gradually increased over time in both groups. At 24 hours after stent placement, platelet aggregation induced by both concentrations of ADP (5 or 20 $\mu$mol/L) significantly decreased compared with baseline in both groups. Beginning 24 hours after stent placement and at all further time points, inhibition of platelet aggregation induced by both concentrations of ADP was significantly higher in patients receiving triple therapy than in those receiving dual therapy.

In the dual-therapy group, there was no inhibition of collagen-induced platelet aggregation during the 1-month study period. However, in the triple-therapy group, collagen-induced platelet aggregation was significantly lower 1 week after stent placement than at baseline. Moreover, collagen-induced platelet aggregation was significantly lower at 1

Table 1
Patient characteristics at baseline

| Variable | Dual-Therapy Group (n = 10) | Triple-Therapy Group (n = 10) |
|---|---|---|
| Age (yrs) | 56.0 ± 10.2 | 56.6 ± 9.4 |
| Men | 5 | 6 |
| Hypertension | 5 | 5 |
| Diabetes mellitus | 3 | 2 |
| Smoker | 5 | 5 |
| Stent length (mm) | 46 ± 24 | 44 ± 21 |
| Platelet count (/$\mu$L) | 270,000 ± 51,000 | 228,000 ± 78,000 |

Values expressed as mean ± SD or number of patients.

Dual-therapy group treated with aspirin plus clopidogrel. Triple-therapy group treated with aspirin plus clopidogrel plus cilostazol.

There were no statistically significant differences between the 2 groups.

Table 2
Mean platelet aggregation over time

| Agonist | Dual-Therapy Group (n = 10) | Triple-Therapy Group (n = 10) | p Value |
|---|---|---|---|
| ADP (5 $\mu$mol/L) | | | |
| Baseline | 46.6 ± 14.0 | 40.9 ± 18.3 | 0.472 |
| 2 h | 40.8 ± 15.2 | 37.9 ± 19.5 | 0.693 |
| 24 h | 34.5 ± 17.0* | 20.2 ± 15.1* | 0.012 |
| 7 d | 34.5 ± 7.6* | 19.3 ± 12.3* | 0.004 |
| 30 d | 32.2 ± 7.4* | 13.4 ± 9.8* | 0.000 |
| ADP (20 $\mu$mol/L) | | | |
| Baseline | 67.6 ± 13.4 | 58.4 ± 20.9 | 0.226 |
| 2 h | 64.2 ± 11.4 | 61.5 ± 12.9 | 0.814 |
| 24 h | 51.7 ± 14.4* | 35.9 ± 18.9* | 0.044 |
| 7 d | 51.3 ± 9.1* | 34.2 ± 23.1* | 0.045 |
| 30 d | 49.4 ± 11.0* | 31.2 ± 15.0* | 0.008 |
| Collagen (2 $\mu$g/ml) | | | |
| Baseline | 66.7 ± 20.0 | 65.3 ± 15.5 | 0.753 |
| 2 h | 65.3 ± 15.5 | 60.7 ± 16.4 | 0.524 |
| 24 h | 63.9 ± 21.2 | 51.4 ± 21.7 | 0.212 |
| 7 d | 65.4 ± 12.2 | 31.4 ± 17.9* | 0.000 |
| 30 d | 55.9 ± 13.7 | 36.5 ± 13.4* | 0.007 |

All values expressed as mean ± SD.

Dual-therapy group treated with aspirin plus clopidogrel; Triple-therapy group treated with aspirin plus clopidogrel plus cilostazol.

* $p < 0.05$ compared with respective baseline values using Wilcoxon's signed-rank test.

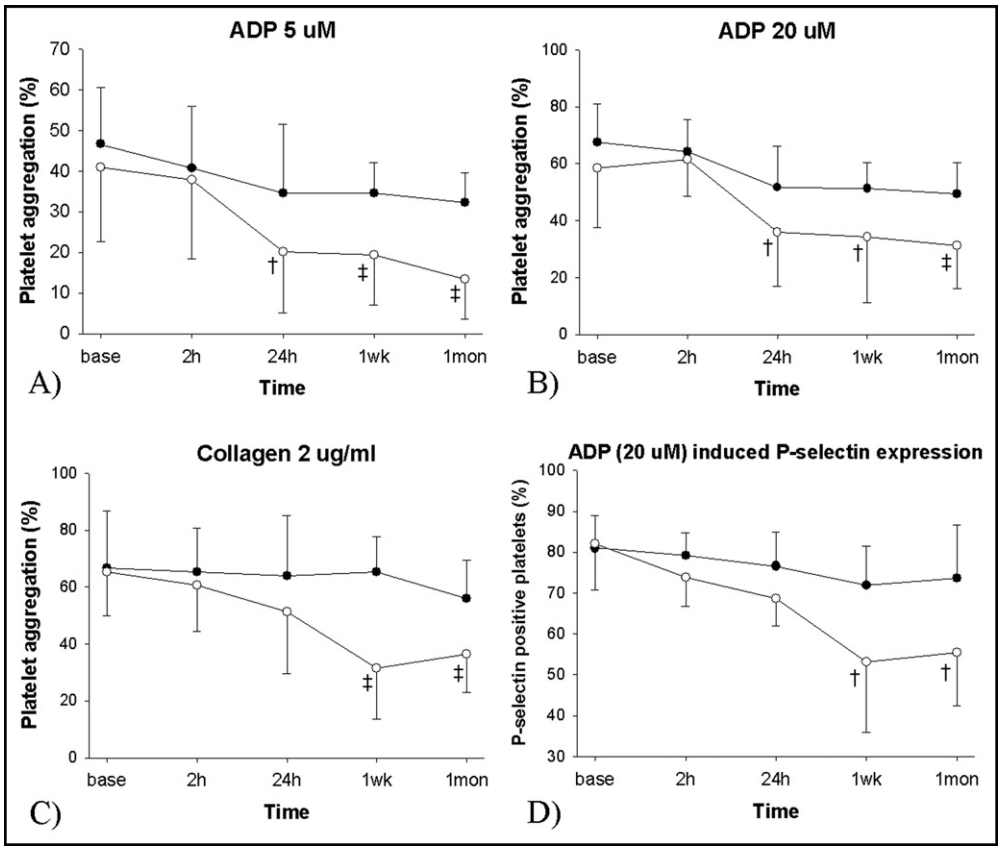


Figure 1. *(A to C)* Agonist-induced platelet aggregation and *(D)* percentage of P-selectin expression over 30 days. Percentage of platelet aggregation after stimulation with *(A)* ADP 5 $\mu$M, *(B)* ADP 20 $\mu$M, or *(C)* collagen 2 $\mu$g/ml. *(D)* Percentage of P-selectin–positive platelets after stimulation with ADP 20 $\mu$M. All results expressed as mean ± SD. $^{\dagger}p < 0.05$; $^{\ddagger}p < 0.01$ between the 2 groups (compared using Mann-Whitney U test). *Black circles*, aspirin plus clopidogrel; *white circles*, aspirin plus clopidogrel plus cilostazol.

Table 3
Percentage of P-selectin–positive platelets over time after stimulation with adenosine diphosphate 20 $\mu$mol/L

| | Dual-Therapy Group (n = 10) | Triple-Therapy Group (n = 10) | p Value |
|---|---|---|---|
| Baseline | 81.0 ± 10.2 | 82.1 ± 6.8 | 0.693 |
| 2 h | 79.2 ± 5.5 | 73.8 ± 7.1 | 0.472 |
| 24 h | 76.6 ± 8.4 | 68.7 ± 6.8 | 0.293 |
| 7 d | 71.9 ± 9.5 | 53.2 ± 17.2* | 0.042 |
| 30 d | 73.7 ± 12.9 | 55.5 ± 13.1* | 0.024 |

Dual-therapy group treated with aspirin plus clopidogrel; Triple-therapy group treated with aspirin plus clopidogrel plus cilostazol.

* $p < 0.05$ compared with baseline using Wilcoxon's signed-rank test.

week in the triple-therapy group than in the dual-therapy group. Platelet P-selectin expression after ADP (20 $\mu$M) stimulation is shown in Table 3 and Figure 1. Patients in the dual-therapy group showed no decrease in platelet P-selectin expression over 1 month. In contrast, platelets from patients in the triple-therapy group showed a significant decrease in P-selectin expression 1 week after stent placement versus baseline. In addition, ADP-induced P-selectin expression was significantly lower in the triple-therapy group than in the dual-therapy group at 1 week and 1 month after stent placement.

In addition, ADP-induced P-selectin expression was significantly lower in the triple therapy group than in the dual therapy group at 1 week and 1 month after stent placement. Figure 2 showed individual variability in reduction of agonist-induced platelet aggregation and P-selectin expression between baseline and 1 month. Most patients in the triple therapy group showed decreased agonist-induced platelet aggregation and P-selectin expression at 1 month compared with baseline activity.

## Discussion

We showed here that a triple-therapy regimen of cilostazol, clopidogrel, and aspirin showed more potent inhibition of ADP-induced platelet aggregation than dual therapy with aspirin and clopidogrel. This difference first appeared 24 hours after coronary stent placement and was maintained thereafter, suggesting that clopidogrel and cilostazol had a rapid and persistent action.[7–9] We also found that platelet P-selectin expression and collagen-induced platelet aggregation were not inhibited by dual antiplatelet therapy with aspirin and clopidogrel, which agrees with previous findings.[9] However, triple antiplatelet therapy with aspirin, clopidogrel, and cilostazol inhibited collagen-induced platelet aggregation and platelet P-selectin expression beginning 1 week after stent placement. P-Selectin, a marker of platelet activation expressed exclusively by activated platelets, promotes fibrin deposition, inducing leukocyte accumulation in

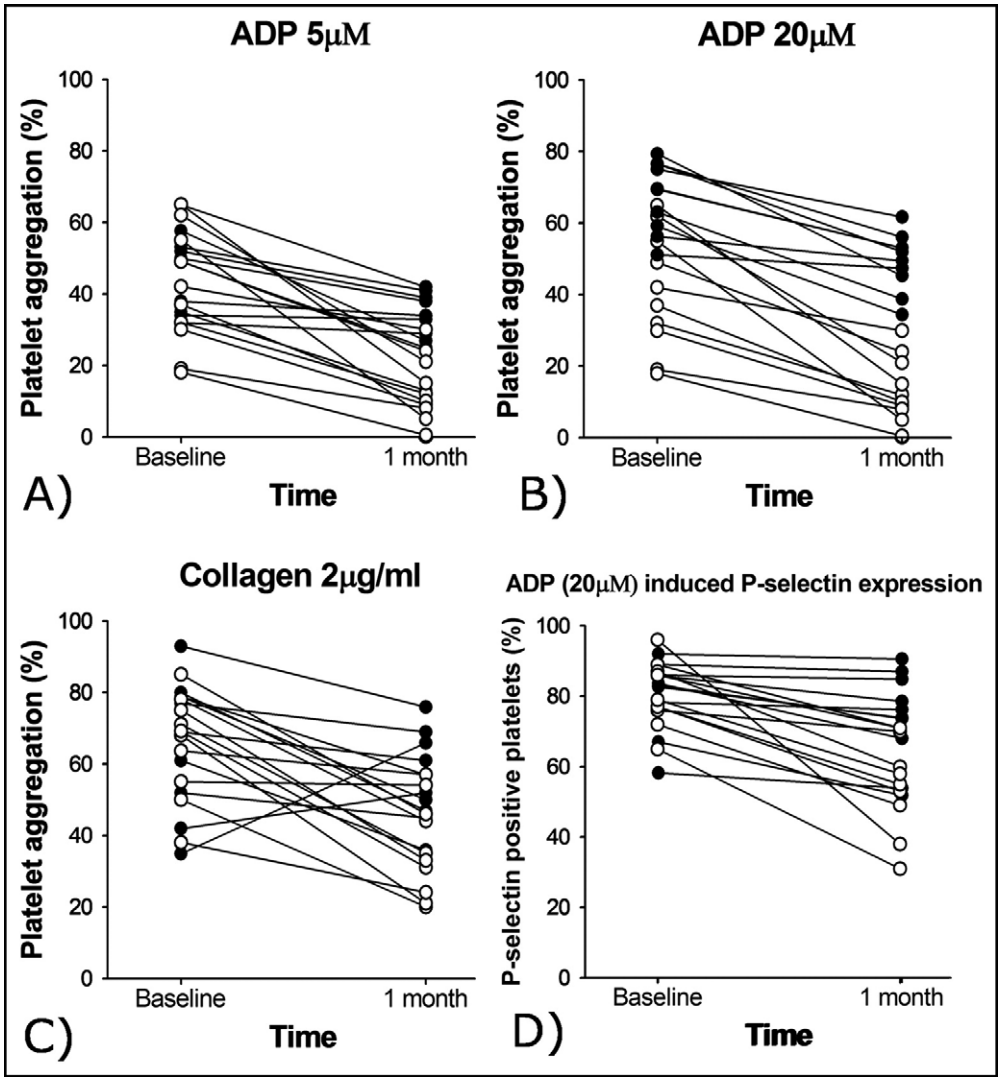


Figure 2. Individual comparison of *(Ato C)* agonist-induced platelet aggregation and *(D)* percentage of P-selectin expression over 30 days. Percentage of platelet aggregation after stimulation with *(A)* ADP 5 μM, *(B)* ADP 20 μM, or *(C)* collagen 2 μg/ml. *(D)* Percentage of P-selectin–positive platelets after stimulation with ADP 20 μM. *Black circles*, aspirin plus clopidogrel; *white circles*, aspirin plus clopidogrel plus cilostazol.

areas of vascular injury and arterial thrombogenesis.[10,11] Thus, P-selectin may be important during thrombogenesis after stent placement, and the addition of cilostazol to the conventional dual regimen may decrease thrombotic complications in patients at high risk of stent thrombosis. A recent clinical study involving 3,012 patients showed that triple therapy was more effective than dual therapy in preventing stent thrombosis, suggesting that the laboratory findings presented here may be translated to clinical settings.[6]

The exact mechanism of beneficial effects of triple antiplatelet therapy remains uncertain. ADP-induced platelet activation and aggregation may have an important role in the pathogenesis of arterial thrombosis. Although clopidogrel and cilostazol inhibit ADP-induced platelet aggregation using different mechanisms of action, both increase intracellular cAMP, clopidogrel by inhibiting ADP-induced inhibition of adenylate cyclase and cilostazol by inhibiting phosphodiesterase III.[12,13] Intracellular cAMP was shown to correlate with degree of inhibition of platelet aggregation.[14] Thus, the additive antiplatelet effects of cilostazol and clopidogrel may be caused by their ability to increase cAMP using different mechanisms of action. The addition of cilostazol to an aspirin plus clopidogrel regimen resulted in additional suppression of platelet P-selectin expression and also showed an appreciable decrease of incidence of stent thrombosis after coronary stent placement.[6,15] This is especially important because of the recent finding of resistance to aspirin and clopidogrel in a substantial number of patients undergoing coronary stent placement. This resistance was associated with increased risk of stent thrombosis and other cardiac events.[16,17] Thus, the role of cilostazol in patients with resistance to aspirin and clopidogrel should be clarified. In addition, several large-scale recent publications and many clinicians currently use a 600-mg (or even 900-mg) loading dose of clopidogrel, and there are several reports on faster onset of platelet inhibition using a higher loading dose than a conventional loading dose of 300 mg.[18,19] If a higher loading dose of clopidogrel is used in triple therapy, it is supposed that a more rapid and potent antiplatelet effect could be achieved.

A few limitations need to be addressed. First, the sample size is small, but we found a significant difference in platelet aggregation between the triple and dual antiplatelet regimens. Second, study drugs were administered immediately after stent placement, which is not current standard care in patients undergoing coronary stent placement. Our study evaluated serial changes in platelet aggregation after coronary stent placement and was similar in design to previous studies.[20,21] Third, this study was not designed to show that a triple regimen could decrease platelet aggregation in patients who may be at particular risk of adverse events after percutaneous coronary intervention, such as those with high platelet reactivity after clopidogrel treatment. To provide a rationale for the use of a triple antiplatelet regimen after coronary stent placement in patients at high risk of thrombotic complications, specific tests should be performed in such patients. For such patients, higher daily dosing regimens of clopidogrel could also be beneficial in decreasing platelet aggregation.[22]


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