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Pretreatment with antiplatelet drugs improves the cardiac function after myocardial infarction without reperfusion in a mouse model

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Abstract

Background: *Reperfusion therapy is known to improve prognosis and limit myocardial damage after myocardial infarction (MI). The administration of antiplatelet drugs prior to percutaneous coronary intervention also proves beneficial to patients with acute MI (AMI). However, a good number of AMI patients do not receive reperfusion therapy, and it is not clear if they would benefit from antiplatelet pre-treatment.*

Methods: *Experimental C57BL/6 mice were randomly allocated to five groups: the sham group, control, post-treatment, pre-treatment, and pre- and post-treatment groups. Acetylsalicylic acid (15 mg/kg), clopidogrel (11 mg/kg), ticagrelor (27 mg/kg), and prasugrel (1.5 mg/kg) were intragastrically administered in the treatment groups. On day 7 post MI, cardiac function and cardiac fibrosis were evaluated using echocardiography and Masson's trichrome staining, respectively. Histopathological examinations were performed on tissue sections to grade inflammatory cell infiltration. Platelet inhibition was monitored by measuring thrombin-induced platelet aggregation.*

Results: *Left ventricular ejection fraction and fractional shortening improved significantly ($p < 0.01$) in the pre-treatment groups when compared to the post-treatment and control groups. A significant ($p < 0.01$) decrease in cardiac fibrosis was observed in the pre-treatment group, compared with the post-treatment and control groups. Inflammatory cell infiltration significantly decreased in the pre-treatment group compared with the control group ($p < 0.05$). Thrombin-induced platelet aggregation was significantly inhibited by antiplatelet drugs, but increased with the exposure to H_2O_2.*

Conclusions: *In the absence of reperfusion therapy, pre-treatment with antiplatelet drugs successfully improved cardiac function, reduced cardiac fibrosis and inflammatory cell infiltration, and inhibited oxidative stress-induced platelet aggregation after MI in the mouse model.* (Cardiol J 2021; 28, 1: 118–128)

Key words: antiplatelet drugs, pre-treatment, myocardial infarction, cardiac function, reperfusion therapy

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Introduction

Acute myocardial infarction (AMI) is the most severe manifestation of coronary artery disease [1]. Prompt recanalization of the culprit vessel and restoration of blood flow to the myocardium are the main therapeutic goals in AMI. Currently, percutaneous coronary intervention (PCI) and thrombolytic therapy are used for effective reperfusion, improving blood flow and preventing recurrent ischemia [2, 3]. Nevertheless, studies have suggested that there is a great variation in the provision of reperfusion therapy [4]. Although the rate of early reperfusion therapy for myocardial infarction (MI) has reached a fairly high level in many developed countries. In developing countries, such as China, during the decade 2001–2011, outcomes of in-hospital treatment for ST-segment elevation MI (STEMI) patients have not improved significantly: the proportion of patients receiving reperfusion therapy has not increased. The treatment of STEMI patients is still significantly delayed, and more than half of STEMI patients miss the opportunity of reperfusion therapy due to delayed consultation [5].

Antiplatelet drugs have been the cornerstone of secondary prevention in patients with coronary heart disease, and these agents have significantly reduced the mortality and have improved the prognosis. Double antiplatelet therapy (DAPT) is a pivotal treatment strategy for patients with AMI and has been recommended in many guidelines [2, 3, 6]. In recent years, assessment of the most appropriate timing for initiating the administration of antiplatelet drugs has been focused upon on with great interest. Many studies [7, 8] have shown the administration of antiplatelet drugs prior to PCI to be beneficial in patients with MI. Brener et al. [8] suggested that acetylsalicylic acid (ASA) pretreatment reduced the mortality at 30 days, especially in patients with non-ST-segment elevation acute coronary syndrome (NSTE-ACS). However, the benefits of pretreatment with antiplatelet drugs in patients with MI who do not receive reperfusion therapy are not clear. The aim of this study was to evaluate the effects of pretreatment with antiplatelet drugs on cardiac function after MI in a mouse MI model.

Methods

Reagents and materials

Apyrase and prostaglandin E1 were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Thrombin was obtained from Enzyme Research Laboratories (South Bend, IN, USA). CD45 antibody was obtained from Bio-Rad (Hercules, CA, USA). Biotinylated goat anti-rat antibody was obtained from Vector (Burlingame, CA, USA). ASA was obtained from Bayer Health Care (Leverkusen, Germany), ticagrelor was obtained from AstraZeneca PLC (Shanghai, China), clopidogrel was purchased from Sanofi Winthrop Industrie (Shanghai, China), and prasugrel was purchased from Bio tool (Houston, TX, USA). Prasugrel and clopidogrel active metabolites were purchased from Shanghai Race Chemical Co., Ltd. (Shanghai, China). The Masson Stain Kit was obtained from Shanghai Yeasen Biological Technology Co., Ltd. (Shanghai, China).

Animals

C57BL/6 male mice (8 weeks old) were purchased from the Shanghai Slack Laboratory Company (Shanghai, China). The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and were then exsanguinated to retrieve blood and heart. Pentobarbital sodium (200 mg/kg, intraperitoneally) was used to euthanize the mice at the end of the experiments. The Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee approved the animal research protocol. All animal procedures conformed to NIH guidelines (Guide for the Care and Use of Laboratory Animals).

Experimental design

The mice were randomly divided into five groups: (1) sham operation group — a suture was passed under the left anterior descending (LAD) coronary artery without ligation; (2) control group — MI was induced using the model described below, and the same vehicle was administered intragastrically; (3) post-treatment group — after MI induction, mice were given ASA (15 mg/kg), clopidogrel (11 mg/kg), ticagrelor (27 mg/kg), or prasugrel (1.5 mg/kg) by intragastric administration for 7 days; (4) pre-treatment group — before MI induction, ASA (15 mg/kg), clopidogrel (11 mg/ /kg), ticagrelor (27 mg/kg), or prasugrel (1.5 mg/ kg) was orally administered to the mice for 7 days; (5) pre- and post-treatment group — for 7 days prior to and for 7 days after MI induction, ASA (15 mg/ /kg), clopidogrel (11 mg/kg), ticagrelor (27 mg/kg), or prasugrel (1.5 mg/kg) was orally administered to the mice. In each group, 10 mice were treated with each of the drugs. The drugs were dissolved in normal saline. For each drug, the dose selec-

tion was based on the human clinical dosage: ASA (100 mg/60 kg), clopidogrel (75 mg/60 kg), ticagrelor (90 mg/60 kg), or prasugrel (10 mg/60 kg).

Myocardial infarction model

The mice were housed in a temperature--controlled environment with 12-h light/12-h dark cycles. MI was induced by permanent ligation of the LAD coronary artery, as described previously [9]. Eight-week-old mice were anesthetized by isoflurane inhalation. A rodent ventilator (Model 683, Harvard Apparatus, Inc., Holliston, MA, USA) was used with 65% oxygen during the surgical procedure. The animals were kept warm using heat lamps and heating pads. A left thoracotomy was performed in the fourth intercostal space. The heart of the mouse was exposed, and the LAD coronary artery was ligated, 2 mm from its ostial origin, with a 7-0 silk suture. Regional ischemia was confirmed by changes in the electrocardiogram (ST-segment elevation). A sham operation involved the same procedure, but a suture was passed under the LAD coronary artery without ligation.

Echocardiography

Echocardiography was performed using a Vevo 770 high-resolution imaging system at day 7 after MI induction as described previously [9]. The animals were anesthetized with isoflurane inhalation and placed in the supine position. The chest was shaved, and parasternal short- and long-axis views were used to obtain two-dimensional and M-mode images by echocardiography. At least 10 independent cardiac cycles were obtained for each measurement.

Masson's trichrome staining

The hearts were harvested at the end of the study and were perfused and fixed in formalin. Parasternal short-axis sections were cut before staining with Masson trichrome reagent. The slides were analyzed and photographed with an AXIO ScopeA1 microscope (ZEISS Group, Jena, Germany). The area of cardiac fibrosis (blue collagen staining for scar tissue) was expressed as a percentage of the left ventricular (LV) surface area by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Platelet preparation and aggregation

The washed platelets from wild-type mice were prepared as previously described [10]. These were adjusted to a density of 3×10^8 platelets/mL. The platelets were evaluated for aggregation with reactive oxygen species (ROS)-induced stress with thrombin. They were incubated with H_2O_2 (200 μmol/mL) for 3 min and then with ASA, the clopidogrel active metabolite, ticagrelor, or the prasugrel active metabolite for 3 min, and platelet aggregation was induced by thrombin.

Immunohistochemistry

Myocardial tissue was fixed in formalin for 24 h and then embedded in paraffin. The parasternal short-axis sections were cut into 4 μm-thick slices. To quench the endogenous peroxidase activity the sections were deparaffinized and incubated with 3% hydrogen peroxide for 10 min at room temperature. The sections were layered with the anti-CD45 polyclonal antibody (1:500) at 4°C overnight and were subsequently washed 3 times with phosphate buffered saline (PBS) for 5 min each. The sections were incubated with secondary goat anti-rat antibody labeled with biotin for 30 min at 37°C and then visualized with diaminobenzidine using an AXIO ScopeA1 microscope (ZEISS Group, Jena, Germany). The CD45-positive cells were counted in 5 to 10 myocardial views per section with ImageJ software.

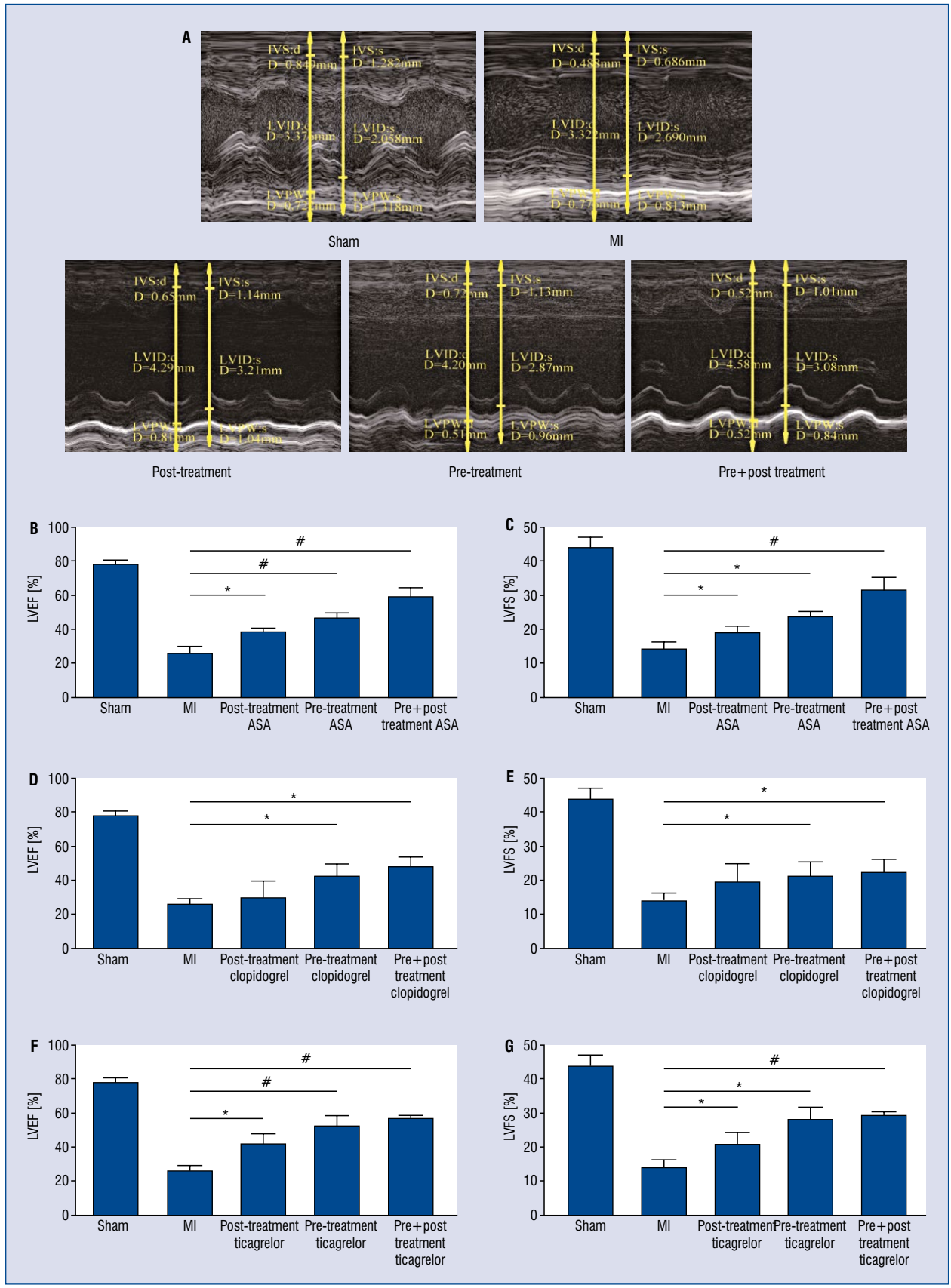
Statistical analysis

Data are presented as means ± standard error. The statistical significance of multiple treatments was determined using the GraphPad Prism Software Version 5.9 (San Diego, CA, USA) by the Student *t*-test or ANOVA (one-way and two-way), followed by either a Newman-Keuls or Bonferroni post hoc test, when appropriate. The values of $p < 0.05$ were considered statistically significant.

Results

ASA, clopidogrel, ticagrelor, and prasugrel pretreatment improves cardiac function after MI

The representative M-mode echocardiograms, performed on day 7 after MI, from each group are shown in Figure 1A. Compared with the control group, significant changes in left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were observed in the pre-treatment, pre- and post-treatment, and post-treatment groups. In the groups that were pretreated with ASA, clopidogrel, ticagrelor, or prasugrel, the cardiac function was significantly preserved compared to that in the post-treatment and control groups (Fig. 1B–I). The differences between the control and pre-treatment groups were statistically significant ($p < 0.01$). Among the 4 drugs, clopidogrel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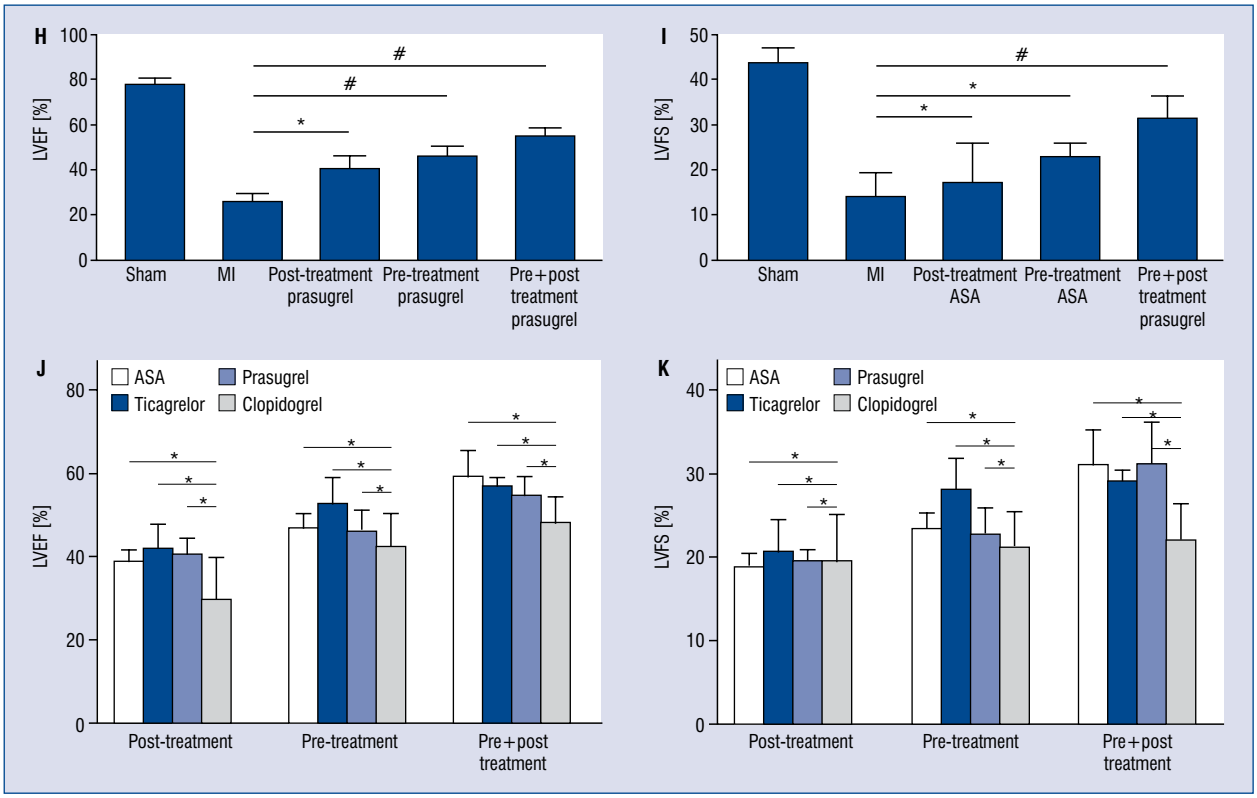

Figure 1. Acetylsalicylic acid (ASA), clopidogrel, ticagrelor, and prasugrel pretreatment preserved cardiac function after myocardial infarction (MI) in vivo; **A.** Representative M-mode echocardiograms for wild type, post-treatment, pre-treatment, and pre + post treatment mice on day 7 post MI; **B–I.** Echocardiographic quantification of left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS). Compared with the control group, the ASA, clopidogrel, ticagrelor, and prasugrel pretreated mice demonstrated improved LVEF and LVFS; **J, K.** Comparisons of the four different antiplatelet drugs; clopidogrel was less effective in improving mice cardiac LVEF and LVFS, when compared to ASA, ticagrelor, and prasugrel (mean ± standard error, n = 10, *p < 0.05, #p < 0.01 vs. control, t test for statistical analyses).

was less effective in improving the mouse cardiac LVEF and LVFS compared with ASA, ticagrelor, and prasugrel in the pre-treatment, pre- and post-treatment, and post-treatment groups (Fig. 1J, K); significant differences ($p < 0.05$) were observed in these cases.

ASA, clopidogrel, ticagrelor and prasugrel pretreatment decreases cardiac fibrosis after MI

After MI, the initial reparative fibrosis is crucial in preventing the rupture of the ventricular wall, but an exaggerated fibrotic response is detrimental and results in progressive impairment of cardiac function [11]. Cardiac fibrosis with the Masson trichrome staining on day 7 after MI was evaluated. Representative images of fibrotic areas in the different groups are shown in Figure 2A. Compared with the control group, treatment with ASA, clopidogrel, ticagrelor, and prasugrel decreased cardiac fibrosis. It was more significantly reduced in the group pretreated with antiplatelet drugs than in the posttreatment and control groups (Fig. 2B–E). The differences in cardiac fibrosis between the control and drug treatment groups were statistically significant ($p < 0.05$). In a comparison of different antiplatelet drugs, the clopidogrel group had a larger fibrotic area after MI compared with that in the ASA, ticagrelor or prasugrel group (Fig. 2F, $p < 0.05$); these differences were statistically significant.

ASA, clopidogrel, ticagrelor, and prasugrel pretreatment reduces infiltration of inflammatory cells in the myocardium

Inflammatory processes are known to result in myocardial injury and impair cardiac function after an MI. A histopathological examination was conducted of the tissue sections to evaluate the

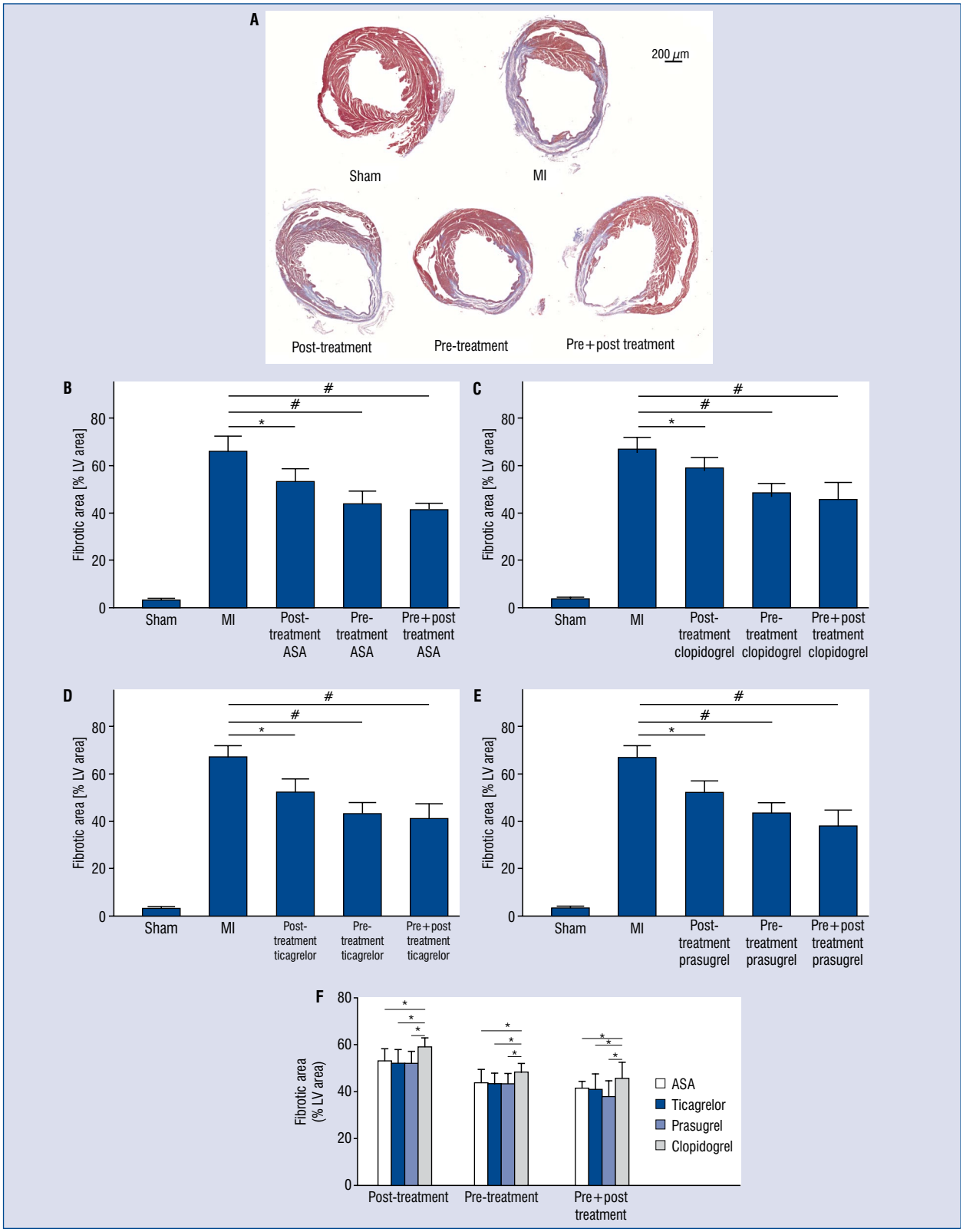

Figure 2. Pretreatment with acetylsalicylic acid (ASA), clopidogrel, ticagrelor, and prasugrel decreased cardiac fibrosis; **A.** Representative images of fibrotic area in different study groups stained with the Masson trichrome stain on day 7 post myocardial infarction (MI). Red represents viable myocardium and blue represents fibrosis; **B–E.** Quantification of Masson trichrome positive staining; **F.** Comparison of different antiplatelet drugs, clopidogrel had a larger fibrotic area after MI compared with ASA, ticagrelor and prasugrel (mean % of left ventricular [LV] area, n = 10/group, *$p \leq 0.05$, #$p < 0.01$ vs. control, t test for statistical analyses).

extent of infiltration of inflammatory cells. The representative images of CD45+ staining in the different study groups are presented in Figure 3A. A decreased infiltration of inflammatory cells was observed in ASA, clopidogrel, ticagrelor, and prasugrel groups compared with that in the control group (Fig. 3B–E). The groups pretreated with antiplatelet drugs demonstrated lesser infiltration of inflammatory cells than post-treatment and control groups. Differences in the infiltration of inflammatory cells in the control and experimental groups were statistically significant ($p < 0.05$). The number of CD45+ positive cells in the clopidogrel group was higher compared with that in the ASA, ticagrelor and prasugrel groups, and there were significant differences among the 4 drugs (Fig. 3F, $p < 0.05$).

ASA, clopidogrel, ticagrelor, and prasugrel inhibit platelet aggregation in response to oxidative stress

Platelets are activated soon after an MI, and their activation depends on the duration of coronary occlusion and the extent of myocardial injury. Platelet aggregation was measured using washed mouse platelets stimulated with thrombin. In the control group, thrombin-induced platelet aggregation was increased with exposure to H_2O_2. Thrombin-induced platelet aggregation in the presence of H_2O_2 was significantly inhibited by ASA, clopidogrel, ticagrelor, and prasugrel (Fig. 4). Ticagrelor and prasugrel were used at lower doses than ASA and clopidogrel; nonetheless, they demonstrated similar inhibitory effects on thrombin-induced platelet aggregation (Fig. 4).

Discussion

The main findings of the present study were that ASA, clopidogrel, ticagrelor, and prasugrel pre-treatment improved cardiac function, reduced cardiac fibrosis, decreased infiltration of inflammatory cells into the myocardium after MI, in cases where reperfusion therapy was not performed, and inhibited platelet aggregation under oxidative stress.

In China, morbidity and mortality from AMI are increasing year by year. In the past two decades, the death rate of coronary heart disease in China has doubled to 1 million per year [12]. However, the provision of coronary reperfusion therapy varies greatly in patients with AMI. The China PEACE-Retrospective Acute Myocardial Infarction Study [13] showed that the proportion of patients who did not receive reperfusion was 44.8% in 2001 and 45.0% in 2011. The China AMI (CAMI) registry [14] showed that among patients with non-STEMI, 9.3% received an early invasive approach. Among patients with STEMI, only 43.0% were treated with primary PCI; besides, 9.9% received thrombolytic therapy. In many other developing countries this phenomenon may even be less optimistic. Recanalization with PCI and thrombolytic therapy are not always available, and other approaches have been taken to limit damage caused by MI. The clinical benefits of ASA, clopidogrel, ticagrelor, and prasugrel in STEMI patients undergoing reperfusion therapy have been previously confirmed in clinical trials [6, 15, 16]. Brener et al. [8] showed that pretreatment with ASA for 5–7 days reduced mortality at 30 days among patients with acute coronary syndromes; all patients accepted revascularization within 72 h. Nanhwan et al. [17] recently reported that pretreatment with ticagrelor protected against ischemia-reperfusion injury and limited the infarct size in rats. At present, primary prevention by ASA is highly controversial. Sanmuganathan et al. [18] showed that ASA treatment for primary prevention is safe and worthwhile at coronary event risk ≥ 1.5%/year, safe but of limited value at coronary risk 1%/year, and unsafe at coronary event risk 0.5%/year. Raju et al. [19] suggested that ASA prevents death, MI, and ischemic stroke. Moreover, it would help reduce hemorrhagic stroke as well as major bleeding when used in the primary prevention of cardiovascular disease. However, as shown in a recent research, Aspirin in Reducing Events in the Elderly (ASPREE) trial [20–22], the use of ASA in healthy elderly people who did not have known cardiovascular disease failed to prolong disability-free survival but led to a higher rate of major hemorrhage than placebo, which suggests that the use of ASA is not reasonable for primary prevention. In conjunction with the results of the present study, it was believed that primary prevention with antiplatelet drugs may bring significant clinical benefits for people with high risk factors of MI as well as for those who are not able to receive reperfusion therapy in time. In particular, the use of ASA will let the economically underdeveloped countries become key beneficiaries, as ASA does have a cost advantage and is easy to obtain.

In the current study, the cardiac function after an MI was significantly preserved on antiplatelet pre-treatment compared with that in the post-treatment and control groups. These results suggest a mechanism for reduction in 30-day mortality reported in previous studies [8]. Clopidogrel was

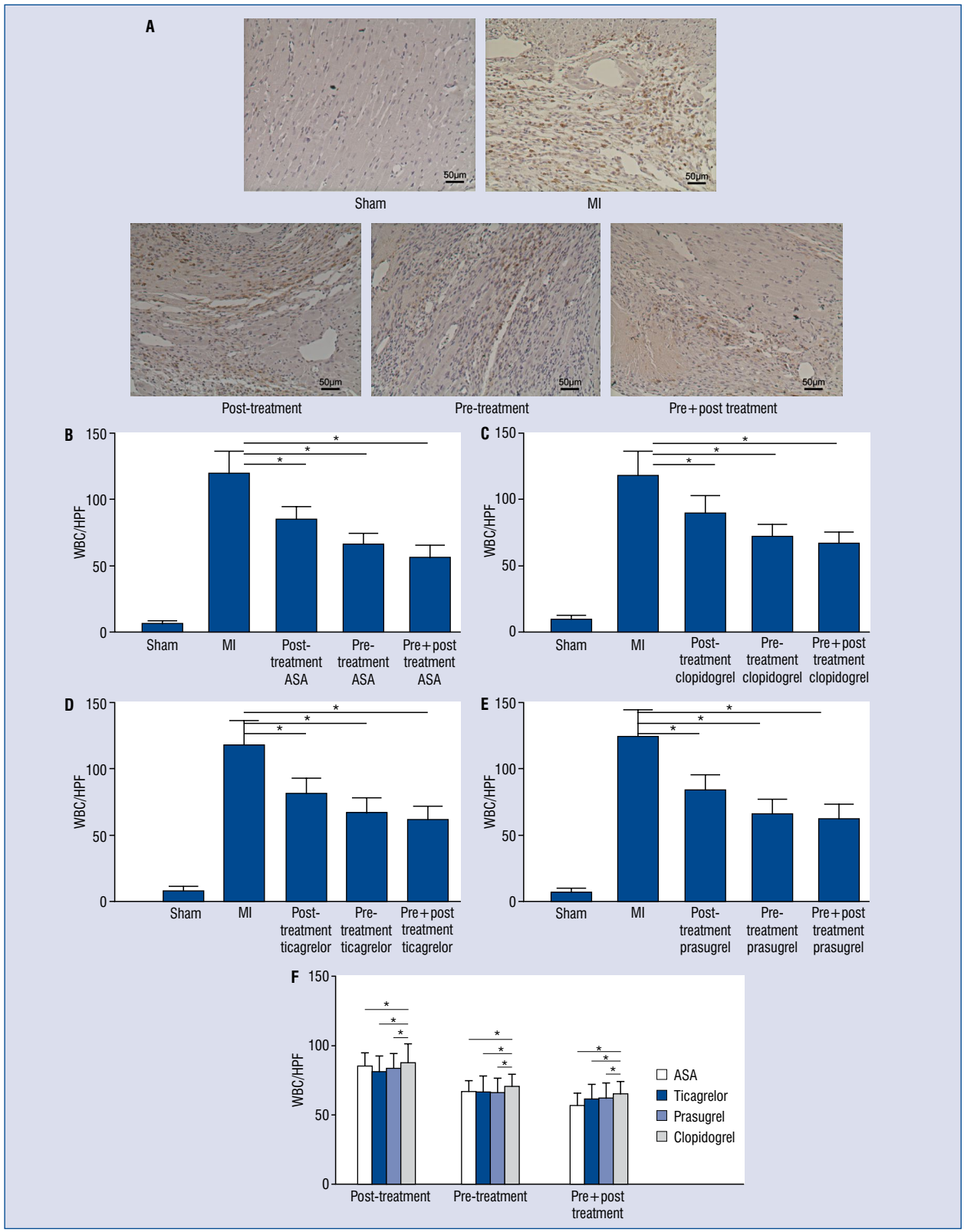

Figure 3. Myocardial white blood cell (WBC) infiltration. CD45+ staining on day 7 post myocardial infarction (MI) in the mouse model; **A.** Representative images of CD45+ positive staining in different groups; **B–E.** Quantification of CD45+ positive cells in the different study groups; **F.** Comparison of different antiplatelet drugs, CD45+ positive cells were more in the clopidogrel group compared with acetylsalicylic acid (ASA), ticagrelor and prasugrel. Immunohistochemical staining was quantitatively analyzed using ImageJ (magnification × 200). Data are shown as the mean white blood cell count per high powered field (mean ± standard error, n = 6; *p < 0.05, t test for statistical analyses); HPF — high power field.

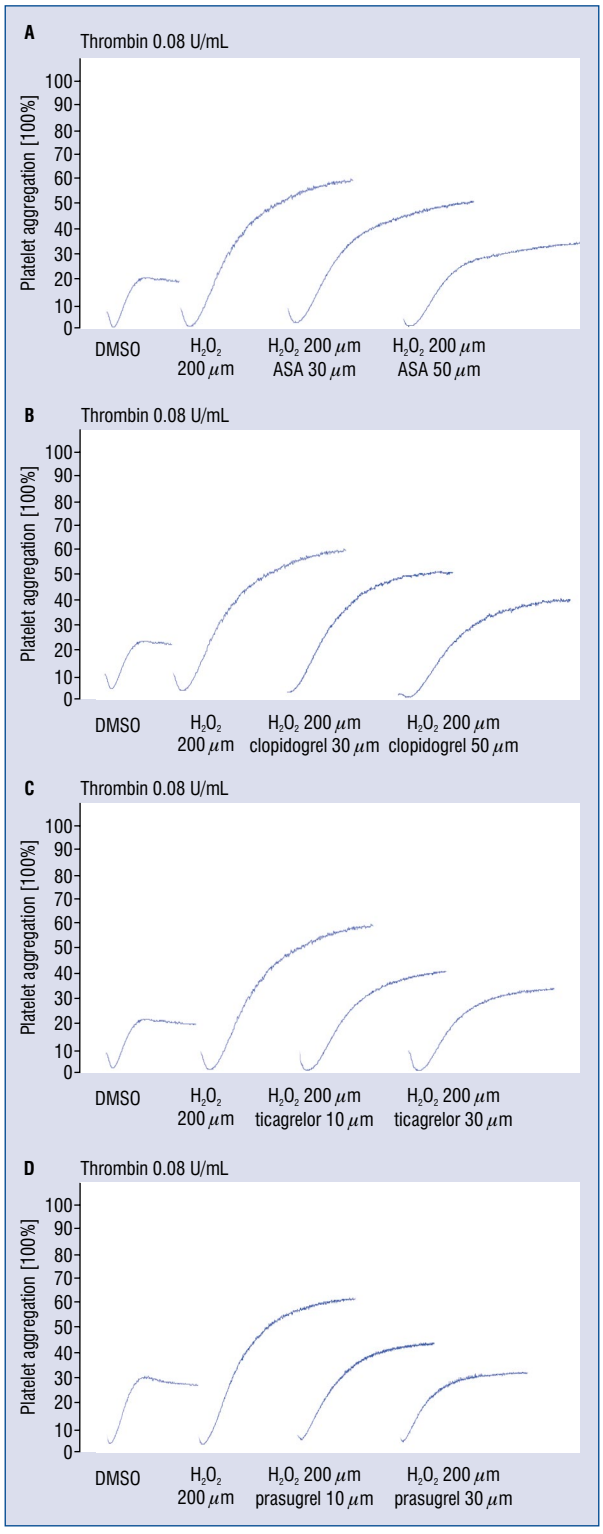

Figure 4. Acetylsalicylic acid (ASA), clopidogrel, ticagrelor, and prasugrel inhibited mouse platelet aggregation with oxidative stress; **A–D.** Washed mouse platelets were stimulated with H_2O_2 (200 μmol/L) for 3 min. Thrombin induced aggregation of mouse platelets was diminished by ASA, clopidogrel, ticagrelor, and prasugrel in a dose-dependent manner. At least five independent experiments were performed; DMSO — dimethyl sulfoxide.

less effective than ASA, ticagrelor, and prasugrel with regard to improving the LVEF and LVFS in mice in each of the treated groups. The dosage of each drug was based on human clinical dosage. Because different medications showed similar platelet inhibition, differences in the improvement of cardiac function with the use of the 4 drugs suggests that the protective effects might occur through mechanisms other than platelet inhibition. Vilahuret et al. [23] suggested that ticagrelor, but not clopidogrel, exerted cardioprotective effects via adenosine-dependent mechanisms.

The adult cardiomyocyte has a limited capacity to regenerate after injury, and the reparative scarring process after MI is critical for maintaining the structural integrity of the ventricular wall. Reparative scarring is followed by remodeling of the surrounding myocardium and eventually leads to impaired cardiac function [11]. It was reported [24] that activated platelets participate in the recruitment and activation of white blood cells in injured areas, release a large number of cytokines and tissue repair factors, and promote the occurrence of inflammatory response in injured areas, while the latter promotes the process of myocardial fibrosis. Antiplatelet drugs can effectively inhibit activation or aggregation of platelets, thus reducing the degree of myocardial fibrosis after MI. Some studies [25, 26] demonstrated that inhibition of platelet activation by clopidogrel prevented cardiac inflammation and fibrosis in response to angiotensin II-induced hypertension. In this study, compared with the control group, mice treated with ASA, clopidogrel, ticagrelor, or prasugrel demonstrated decreased cardiac fibrosis. This might be related to platelet inhibition as suggested in previous studies, and these findings are consistent with the effects of antiplatelet pretreatment on cardiac function after an MI.

An intense inflammatory response is triggered after MI, which has been implicated in the pathogenesis of post-infarction remodeling and heart failure [27]. The activated platelets not only bind to different subsets of leukocytes, but also secrete mediators that induce transendothelial migration [28]. In the present study, decreased infiltration of inflammatory cells was observed after MI in the ASA, clopidogrel, ticagrelor, and prasugrel treatment groups compared with that in the control group, especially in those mice pretreated with antiplatelet drugs.

When MI occurs, ROS are acutely produced in an ischemic microenvironment [29] and are major mediators of distal embolization, microvascular

obstruction, and inflammatory response [30]. It was reported [31] that H_2O_2 alone does not affect platelet aggregation. H_2O_2 dose-dependently promotes low-level thrombin-induced human platelet aggregation. To simulate oxidative stress after MI, platelets were incubated with H_2O_2. The effects of ASA, clopidogrel, ticagrelor, and prasugrel on platelet aggregation under oxidative stress was investigated in this study. Results herein showed that platelet aggregation, stimulated with H_2O_2, was inhibited significantly by these 4 drugs. These results suggest that inhibition of platelet activity was associated with limiting myocardial fibrosis and reducing inflammatory cell infiltration, thereby improving cardiac function.

Limitations of the study

There are several limitations of the present study. Firstly, the cardiac function was evaluated on the 7th day after MI, and therefore, there is a need for evaluating long-term effects of pretreatment with these drugs. Secondly, the European Society of Cardiology states that assessment in large animal models is an obligatory step before initiating human trials; thus, results of the current study should be verified in other animal models. Finally, the elucidation of mechanisms responsible for the improvement in cardiac function using these drugs need further investigations.

Conclusions

Pretreatment with ASA, clopidogrel, ticagrelor, and prasugrel can improve the cardiac function after MI. Pretreatment with these agents may reduce harm caused by MI in patients not receiving reperfusion therapy.

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Conflict of interest: None declared

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