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Inhibitory effects of hydrogen on in vitro platelet activation and in vivo prevention of thrombosis formation

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ABSTRACT

Aims: Hydrogen (H_2) has antioxidant effects. The pharmacologic function of H_2 in platelets is not yet clear. Therefore, in this study we sought to investigate the inhibitory effects of H_2 on in vitro platelet activation and in vivo prevention of thrombus formation.

Main methods: After platelets were incubated with H_2-rich saline (HRS), platelet adhesion in whole human blood was assessed in fibrinogen-coated perfusion chambers, while rat platelet aggregation induced by ADP, collagen and H_2O_2 was detected through light transmission aggregometry. The level of P-selectin, thromboxane B_2, nitric oxide (NO), malondialdehyde, reactive oxygen species (ROS), cGMP, extracellular signal-regulated kinases 1 and 2 (p-ERK1/2), and fibrinogen binding to platelets were evaluated in vitro. Besides, the in vivo effects were examined in arterio-venous shunt thrombosis, $FeCl_3$-induced artery thrombus formation, and tail bleeding time in mice and rats.

Key findings: HRS prolonged tail bleeding time in mice and rats, decreased thrombus weight and prolonged the time to occlusion in rat and mouse thrombosis models in vivo and inhibited platelet adhesion as well as aggregation in vitro. Additionally, HRS decreased P-selectin expression, release of thromboxane B_2, ROS, and fibrinogen binding, but enhanced NO levels in H_2O_2-exposed platelets. HRS also decreased malondialdehyde levels in plasma of the rat arterial thrombosis or H_2O_2-exposed platelet model. Moreover, HRS increased cGMP level, decreased p-ERK1/2 (diminished with KT5823) in the platelets stimulated by H_2O_2.

Significance: These results suggest that H_2 has antithrombotic effects, which may be due to its antioxidant property and subsequent inhibition of platelet activation via NO/cGMP/PKG/ERK pathway.

1. Introduction

Thrombosis in cerebral or coronary arteries is the most frequent cause of morbidity and mortality worldwide. Thus, prevention and treatment of thrombotic disorders are of global importance [1]. Platelets exert a crucial role in thrombus formation after blood vessel injury. Platelets recruit additional platelets, adhere to the injured vascular walls, and are activated. Then, the activated platelets make conformational changes in the platelet glycoprotein IIb/IIIa (integrin αIIbβ3). Subsequently, platelet aggregation may be formed, which is regulated by interaction between glycoprotein IIb/IIIa and fibrinogen or von Willebrand factor. Finally, an increasing number of platelets is recruited and a platelet-rich thrombus is formed [2,3]. In contrast, endogenous inhibitors such as prostacyclin and nitric oxide (NO) limit

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platelet activation in vivo [2].

Studies have shown that many cardiovascular diseases are linked to an excessive activation of platelets [4]. Increasing evidence has indicated that reactive oxygen species (ROS), either exogenous or platelet-derived, could modulate platelet activity [5]. The release of ROS from both resting platelets and oxidative stress or agonist-stimulated platelets was reported, which includes the superoxide anion, ·OH, and hydrogen peroxide (H_2O_2) [6,7]. Supplementation with antioxidants, such as vitamin C and vitamin E has been reported to inhibit platelet aggregation and benefit cardiovascular diseases via their antioxidant activity [8,9].

As the lightest and most abundant chemical element, hydrogen (H_2) is considered a novel antioxidant [10,11], and has come to the forefront of therapeutic medical gas research. At present, kinetics of hydrogen have been studied in human and mice. In the human body, endogenous H_2 is mainly produced by the anaerobic bacteria via hydrogenase in the large intestine, which may spread to the whole body, and is excreted through the intestines, lungs and skin [12]. In mice, after an aqueous H_2-containing solution, H_2-rich saline (HRS), was injected intraperitoneally, the hydrogen concentration reached a maximum value of 20 μM in the liver and 15 μM in the kidney at about 5 min and reduced to initial concentration about 40 min after the injection [13]. When HRS was injected intravenously to human volunteers, the hydrogen concentration in blood peaked at about 15 min and fell immediately after the injection was stopped [14].

Accumulating evidence proves that H_2 administration can be a feasible therapeutic strategy in many disease models or states, such as pulmonary hypertension [11,15], acute myocardial infarction [16], metabolic syndrome [17], atherosclerosis [18], hypercholesterolemia [19], anxiety-like behaviors [20], bladder outlet obstruction [21] and chronic dialysis [22]. In Takeuchi's study [7], H_2 had an effect of inhibiting platelet aggregation induced by collagen. However, it is unclear whether hydrogen (at different concentrations/doses) inhibits platelet aggregation induced by other agents except collagen, affects platelet fibrinogen binding and P-selectin expression, and has an antithrombotic effect. Therefore, we hypothesized that H_2 may inhibit experimental thrombosis associated with a decrease in platelet activation via its antioxidant activity. So, the aim of this study was to investigate the effects of HRS on thrombosis formation and platelet activation, as well as to explore the underlying mechanisms.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 220–250 g and male C57BL/6 mice weighing 20–22 g were purchased from Charles River Company (Beijing, China). Kunming mice were obtained from Taian Taibang Biological Products Co. Ltd. (Taian, China). Animals were housed in a controlled environment (humidity 40%–60%, 22 ± 2 °C) with a natural day/night cycle, and free access to food and water. Animals were acclimated for 1 week before any experimental procedures. All experiments were conducted following the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China and approved by the laboratory animals' ethical committee of Shandong First Medical University & Shandong Academy of Medical Sciences.

2.2. Reagents

HRS was prepared as described previously [15,23]. Fibrinogen binding and P-selectin expression were monitored with fluorescein isothiocyanate (FITC)-conjugated fibrinogen and FITC-conjugated CD62P (P-selectin) (BD Biosciences, San Jose, CA). Apyrase, prostaglandin E_1, human fibrinogen, ADP, bovine serum albumin (BSA), 2′,7′-dichlorofluorescein diacetate (H2DCF-DA), 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM DA), N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 3-isobutyl-1-methylxanthine (IBMX) and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO, US). KT5823, β-actin, and goat anti-rabbit conjugated to horseradish peroxidase antibody were from Abcam (Cambridge, MA, USA). Rabbit polyclonal antibodies against phosphorylated (Thr202/Tyr204) extracellular signal-regulated kinases 1 and 2 (p-ERK1/2) and ERK1/2 were from Cell Signaling Technology, Inc (Danvers, MA, USA). Collagen was purchased from the Chrono-Log Corporation (Havertown, PA, USA). Malondialdehyde (MDA), superoxide dismutase (SOD) and NO assay kits were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TXB_2 and cyclic guanylate monophosphate (cGMP) Enzyme Linked Immunosorbent Assay (ELISA) kits were from Shanghai Lang Dun Biotechnology Co., Ltd. (Shanghai, China). Pentobarbital sodium was from Merck (Darmstadt, Germany). All other reagents were of the highest quality available.

2.3. Tail bleeding time assay

Twenty Kunming mice and eighteen Sprague-Dawley rats were divided randomly into two groups respectively: the HRS group and Control group, with ten mice or nine rats in each group. On the day of the experiment, animals in these two groups were administered HRS (10 ml/kg body weight) or an equal volume of normal saline (NS) intraperitoneally (i.p.). The in vivo dose of HRS and the administration frequency used in this study were according to our previous studies [15,16]. Thirty minutes after a single injection, tail bleeding time was measured as described previously [24]. Briefly, anesthetized animals were placed in a domestic tube with an opening, from which the tail protruded. The tail was transected at 3 mm from the tip and the distal portion was immediately vertically immersed into 37 °C NS. The duration of time for the bleeding to stop (including episodes of rebleeding) was recorded. No animal was allowed to bleed for > 30 min.

2.4. Experimental thrombosis models

2.4.1. Arterio-venous shunt thrombosis model

Sixteen healthy male Sprague-Dawley rats were divided randomly into two groups: the HRS group and Control group, with 8 rats in each group. HRS (10 ml/kg body weight) or an equal volume of NS was given i.p. daily for 1 week. Two hours after the last dose, rats were anesthetized with pentobarbital sodium (40 mg/kg body weight, i.p.) and fixed in a supine position. The toe pinch reflex, muscular relaxation, and respiration rates were monitored to determine that adequate anesthesia was administered. An arterio-venous shunt tube was inserted between the left jugular vein and right carotid artery. The shunt was assembled from two 4-cm polyethylene tubes (0.6- and 0.9-mm inner and outer diameter, respectively) connected to a central tube (6 cm long, 0.9-mm inner diameter) containing a 5-cm-long cotton thread. The central tube was filled with NS and the 4-cm polyethylene tubes were filled with a heparin saline solution (25 U/ml). Extracorporeal circulation was maintained for 15 min, and a thrombus adhered to the thread during this period. Then the shunt was removed and the thread with its associated thrombus was withdrawn. The thrombus dry weight was determined 6 h later at room temperature by subtracting the weight of the dry 5 cm thread measured previously [3,25].

2.4.2. Ferric chloride-induced arterial thrombosis model

Both 30 male Sprague-Dawley rats and 30 male Kunming mice were divided randomly into three groups: the Control group, Model group and HRS group. Animals in the HRS group received HRS at a dose of 10 ml/kg body weight i.p. daily for 1 week, while animals in the other two groups received an equal volume of NS using the same procedure. Two hours after the last dose, a ferric chloride ($FeCl_3$)-induced carotid artery thrombosis model was established as described previously [26].

Briefly, anesthetized rats or mice were fixed in a supine position, and a segment of the right common carotid artery was exposed. A miniature Doppler flow probe (PeriFlux System 5000, Perimed AB, Järfälla, Sweden) was attached to the carotid artery to monitor blood flow. Thrombus formation was induced in the Model and HRS groups by wrapping a piece of Whatman filter paper (1 × 3 mm) saturated with 5% (for mice) or 40% (for rats) $FeCl_3$ solution around the carotid artery, in contact with the adventitial surface of the vessel. Carotid arteries of rats or mice in the Control groups were wrapped in filter paper saturated with distilled water. A small piece of sealing film was placed below the artery prior to the application of $FeCl_3$ to avoid injury of the adjacent tissue. Three minutes later, the filter paper was removed and carotid blood flow was continuously monitored after $FeCl_3$ application at the distal end. The time to occlusion was determined by measuring the time (in min) from removal of the $FeCl_3$ saturated filter paper until the blood flow was stable below 100 Perfusion Unit (PU) for 2 min.

2.5. Perfusion studies

Blood was collected from healthy donors receiving no medication for at least 2 weeks, and anti-coagulated with 3.8% trisodium citrate (9:1, v/v). When trisodium citrate acts as the anticoagulant, a ratio (9:1, v/v) of blood to 3.8% trisodium citrate is common and optimal with little effect on platelet function [27]. Then blood was incubated with NS (Control) or HRS with a volume ratio of 9:1 for 20 min at 37 °C. The blood temperature was maintained at 37 °C during the perfusion study. The procedures of the study were approved and carried out in accordance with the guidelines of the Ethics Committee of Shandong First Medical University & Shandong Academy of Medical Sciences. All donors gave written informed consent before participating in this study.

Platelet adhesion was evaluated in vitro using a well-defined perfusion chamber as previously described [28,29]. Briefly, coverslips were cleaned thoroughly, coated with 100 μg/ml fibrinogen for 1 h, and incubated in 1% human albumin to prevent non-specific protein binding. Then the coverslips were sealed reversibly to the chamber by vacuum force. Blood was aspirated through the coverslip in the chamber by a syringe pump at shear rate of 1000 s^{-1}. Each perfusion lasted for 5 min. After perfusion, the coverslips were removed, rinsed, fixed in 0.5% glutaraldehyde, dehydrated with methanol and stained with May-Grunwald-Giemsa. Platelet adhesion to the coverslip was measured with a light microscope (BX 51, Olympus, Tokyo, Japan) coupled to an image analyzer system (Image–Pro Plus v6.0, Media Cybernetics, USA). The platelet adhesion status was evaluated as the percentage of the surface area covered with platelets. For each coverslip, five visual fields were randomly selected and averaged to obtain the results. All perfusions were performed in triplicate for each condition. The results are presented as the mean from three different donors.

2.6. Platelet aggregation detection

Blood was collected from the hearts of male Sprague-Dawley rats that had been fully anesthetized and, anti-coagulated with 3.8% trisodium citrate (9:1, v/v). Samples were centrifuged at 100 ×*g* for 10 min at room temperature, and the platelet-rich plasma (PRP) supernatant was obtained. Then the pellet was centrifuged at 1000 ×*g* for 10 min at room temperature to obtain platelet-poor plasma (PPP). Platelet aggregation was measured as described previously [1]. Briefly, platelets in each PRP sample were counted using an automatic blood cell analyzer (PE-6800VET, Zibo, China) and adjusted to 250 × 10^9 platelets per liter with NS. The corresponding PPP of each PRP was diluted at the same proportion using NS [30]. The maximum aggregation rate of platelets was recorded within 5 min by turbidimetry using a LBY-NJ4 aggregometer (Pulisheng, Beijing, China). In the following procedure, platelet count was maintained at 250 × 10^9 platelets per liter. A total of 300 μl of PRP was added to an aggregation cuvette.

In the first series, four different volumes (10 μl, 20 μl, 30 μl, and 50 μl) of HRS or NS (Control) were added to the aggregation cuvette containing PRP and incubated for 5 min at 37 °C. ADP (10 μl, 10 μM final concentration) or collagen (10 μl, 15 μg/ml final concentration) was used as agonist. Then platelet aggregation was measured following the procedure described above.

In the second series, HRS (10 μl, 20 μl, 30 μl, 40 μl, and 50 μl) was added to PRP simultaneously with H_2O_2 at a final concentration of 25 mM. PRP exposed only to NS or H_2O_2 was used as Control or Model, respectively. After a 5-min incubation, platelet aggregation was measured.

2.7. Preparation of washed platelets

For preparation of washed platelets, blood was collected from the hearts of anesthetized C57BL/6 mice, anti-coagulated with 97 mM sodium citrate, 71 mM citric acid, and 111 mM dextrose, pH 6.5 (ACD, 5:1, v/v), and centrifuged at 80 ×*g* for 10 min. PRP was collected and further centrifuged at 750 ×*g* for 10 min containing 2 U/ml heparin, 0.2 U/ml apyrase and 1 μM prostaglandin E_1. The platelet pellet was then washed three times with Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose, 5 mM HEPES, and 0.35% BSA, pH 7.35, freshly prepared) with inhibitors (prostaglandin E_1, apyrase or heparin). The volume of Tyrode's buffer was the same as that of PRP. Finally, platelets were resuspended in Tyrode's buffer [31,32].

2.8. Flow cytometry analysis of P-selectin exposure and platelet fibrinogen binding

Washed mouse platelets were allowed to equilibrate for 30 min before beginning the following experiments. Then washed platelets were divided into three groups for the following flow cytometry analysis: (1) platelets incubated with NS (Control), (2) platelets incubated with H_2O_2 (final concentration 1 mM) and NS (Model) and (3) platelets incubated with H_2O_2 (final concentration 1 mM) and HRS (the same volume as H_2O_2) (HRS). Platelets were allowed to incubate for 5 min at 37 °C.

The effects of HRS on single platelet activation were monitored by flow cytometry. Briefly, the prepared platelets (1 × 10^7/ml) were incubated with FITC-fibrinogen (final concentration 0.1 mg/ml) and FITC-conjugated CD62P (P-selectin, final concentration 0.025 mg/ml) or their isotype controls (BD Biosciences, San Jose, CA) in the dark at room temperature for 20 min, respectively, and they were finally fixed in 1% paraformaldehyde in PBS at 4 °C. Samples were measured using a FACScalibur flow cytometer (Beckton Dickinson, San Jose, CA, USA) and data were analyzed with FlowJo V. 7.6.2 (Tree Star, Inc., Ashland, OR). Platelet fibrinogen binding and P-selectin expression were reported as the percentages of fibrinogen binding-positive and CD62P-positive cells in the platelet population, respectively [31,33].

2.9. Determination of levels of ROS and NO in platelets

Levels of ROS and NO in platelets were detected with the fluorescent probes H2DCF-DA and DAF-FM DA, respectively. Mouse platelets of the above three groups were incubated with H2DCF-DA (final concentration 2 μM) and DAF-FM DA (final concentration 0.5 μM) at 37 °C for 20 min. Samples were then fixed in 1% paraformaldehyde at 4 °C, measured and analyzed. Platelet ROS and NO levels were reported as mean fluorescence intensity (MFI).

2.10. Measurement of levels of MDA, NO and SOD

After $FeCl_3$-induced arterial thrombosis was determined, rat blood was obtained by cardiac puncture and plasma was prepared by centrifugation. Levels of MDA and SOD in plasma were measured according to the manufacturer's instructions.

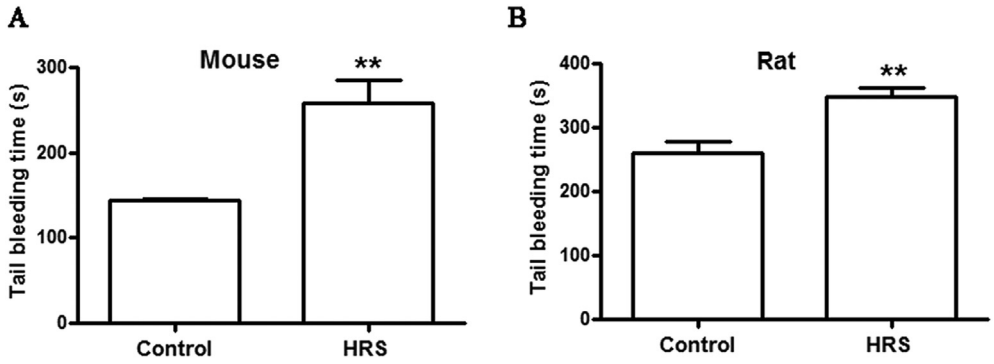

Fig. 1. HRS prolonged tail bleeding time in mice (panel A) and rats (panel B). Data are represented as means ± SD, $n = 9–10$. $^{**}P < 0.01$ versus Control.

After aggregation of rat platelets exposed to H_2O_2, incubated with or without HRS, samples were removed from the aggregation cuvette and subjected to two freeze-thaw cycles. The samples were then disrupted with ultrasound until intact platelets could no longer be detected under the microscope. Levels of MDA and NO in each sample were measured according to the kit instructions.

2.11. Measurement of release of TXB_2 from platelets and cGMP formation

Rat PRP, PPP and adjustment of platelet number were prepared as above. PRP (4.5×10^8/ml) were divided into three groups: (1) platelets incubated with 100 µl of NS (Control), (2) platelets incubated with 50 µl H_2O_2 (final concentration 1 mM) and 50 µl NS (Model) and (3) platelets incubated with 50 µl of H_2O_2 (final concentration 1 mM) and 50 µl of HRS. NS and/or H_2O_2 and/or HRS were added to the aggregation cuvette containing 200 µl PRP. Platelets were allowed to incubate for 6 min at 37 °C.

Because TXA_2 is very unstable and can rapidly turn into TXB_2, a more stable metabolite, TXB_2 was measured instead of TXA_2. For measurement of TXB_2 content, 2 mmol/l EDTA and 50 µmol/l indomethacin were added to the cuvette after incubation. Supernatant was obtained after rat platelets were centrifuged at 1760 ×*g* for 10 min at 4 °C. TXB_2 levels of the supernatants were measured using ELISA kit according to the procedure described by the manufacturer.

For measurement of cGMP level, rat platelet suspensions in above three groups were incubated with IBMX (100 µmol/l) simultaneously during the 6 min. Then the solution was immediately boiled for 5 min. After centrifugation, 50 µl of supernatant was used to determine the cGMP contents with an ELISA kit [34].

2.12. Western blot analysis of p-ERK1/2

Washed mouse platelets (0.4 ml, 25×10^6/ml) were incubated with NS, H_2O_2 (final concentration 1 mM), and HRS with or without the presence of KT5823 (final concentration 30 µM) at 37 °C for 5 min. Then samples were centrifuged and proteins of pellets were isolated using RIPA buffer (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Equal amounts of platelet protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, United States). The membrane was then incubated with the primary antibody (1:5000 for anti-ERK1/2; 1:2000 for anti-p-ERK1/2) and β-actin (1:1000) overnight at 4 °C, followed by incubation with the secondary antibody (1:2000) for 2 h at room temperature. The band with peroxidase activity was detected by enhanced chemiluminescence detection reagents (ECL+ system; Amersham Pharmacia Biotech, Buckinghamshire, UK). Densitometric analysis of specific bands was performed with a Photo-Print Digital Imaging System (IP-008-SD) with analytical software (Bio-1Dlight, V 2000).

2.13. Statistical analysis

Data are expressed as means ± SD. All statistical analyses were performed using GraphPad Prism, version 4 (GraphPad Software, Inc., La Jolla, CA, USA). A non-parametric Mann–Whitney *U* test was used to analyze tail bleeding time and time to occlusion in the $FeCl_3$ injury model. The student's *t*-test was used for comparing two samples and one-way analysis of variance (ANOVA) was used for comparing more than two experimental groups. A *P*-value < 0.05 was considered significant.

3. Results

3.1. HRS significantly prolonged tail bleeding time in mice and rats

To investigate the effects of HRS on thrombosis, we measured mouse and rat tail bleeding time 30 min after a single injection of HRS. As shown in Fig. 1, after HRS injection, the tail bleeding time increased significantly in both mice and rats.

3.2. HRS decreased thrombus weight and prolonged the time to occlusion in thrombosis models

To evaluate antithrombotic activities of HRS in vivo, a rat arteriovenous shunt thrombosis model was used. As shown in Fig. 2A, HRS treatment resulted in a decreased thrombus dry weight compared with the control.

The $FeCl_3$-induced arterial thrombosis model is a simple and well-established model that is sensitive to antiplatelet drugs [35]. When thrombosis is produced in rats, the concentration of $FeCl_3$ may range from 10%–80% [36]. In a mouse model of arterial thrombosis, 3.5%, 5% or 10% $FeCl_3$ is used [37,38]. In order to further examine in vivo antithrombotic activities of HRS, the concentration of 5% $FeCl_3$ for mice and 40% $FeCl_3$ for rats was considered optimal with avoidance of too severe or too mild thrombosis. Our results show that HRS prolonged the time to occlusion in both rats and mice (Fig. 2B, C and D).

3.3. HRS attenuated platelet adhesion to fibrinogen

After vascular injury, the adherence of platelets to the endothelium and exposed media is the initial step of platelet activation. This results in platelet–platelet interaction, which leads to the formation of aggregates [39]. Using an extracorporeal blood perfusion system, we mimicked blood flow in blood vessels and observed platelet adhesion to fibrinogen-coated coverslips. Our results show that HRS significantly attenuated human platelet adhesion to fibrinogen compared with controls at a shear rate of 1000 s^{-1} (Fig. 3).

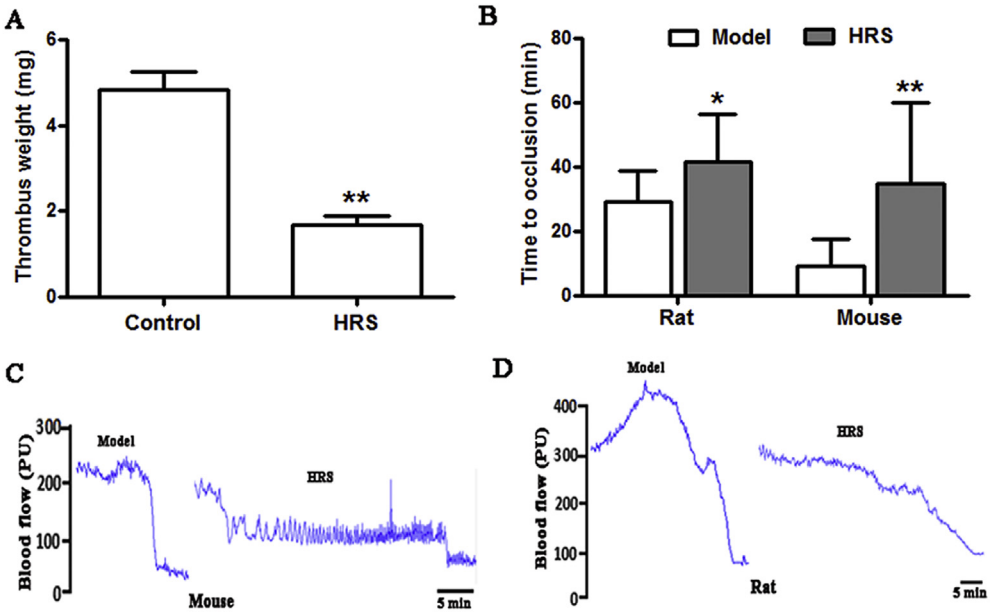

Fig. 2. Effects of HRS on thrombosis in mice and rats. (A) Thrombus weight in a rat arterio-venous shunt thrombosis model, $n = 8$. (B) Time till occlusion in a ferric chloride-induced arterial thrombosis model in mice and rats, $n = 10$. (C) and (D) Representative images of blood flow during thrombus formation in mice and rats, respectively. Data are represented as means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ versus Control.

3.4. HRS inhibited platelet aggregation induced by ADP, collagen and H_2O_2

To determine whether the antithrombotic effect of HRS was related to changes in platelet aggregation, we first observed platelet aggregation induced by agonists (ADP and collagen) in vitro. As shown in Fig. 4, HRS clearly inhibited ADP-induced rat platelet aggregation at doses of 10 μl, 20 μl, 30 μl, and 50 μl (Fig. 4A and B). Moreover, HRS significantly decreased collagen-induced rat platelet aggregation at doses of 20 μl, 30 μl, and 50 μl, whereas there was no significant difference in rat platelet aggregation induced by collagen between 10 μl HRS and NS (Fig. 4C and D).

As an ROS, H_2O_2 can modulate platelet activity [40–43]. So we investigated whether HRS exerted protective effects on H_2O_2 (25 mM)-induced platelet aggregation. We found that HRS at 20 μl, 30 μl, 40 μl and 50 μl inhibited H_2O_2-induced increases in rat platelet aggregation (Fig. 4E and F).

3.5. HRS reduced α-granule secretion and TXA_2 release in H_2O_2-exposed platelets

P-selectin expression reflects platelet α-granule secretion [31,44]. Our flow cytometry results show that H_2O_2 caused the increase of the percentage of mouse P-selectin-positive platelets. After treatment with HRS, the increase was significantly reduced (Fig. 5A and B). We also measured H_2 effect on mouse platelet P-selectin expression induced by thrombin. Again, it was found that HRS treatment decreased P-selectin-positive platelets (Supplementary Fig. S1). Thus HRS can effectively alleviate mouse platelet α-granule secretion.

H_2O_2 (1 mM) significantly increased TXB_2 level in rat PRP, suggesting promotion of H_2O_2-induced release of TXA_2. After treatment

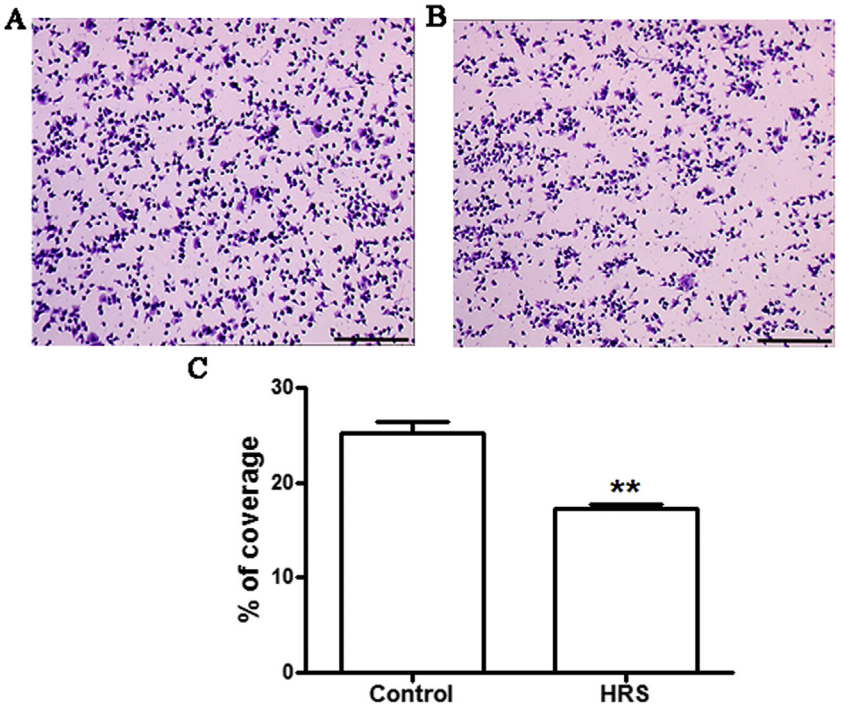

Fig. 3. HRS significantly attenuated platelet adhesion to fibrinogen. (A) and (B) Representative images of platelets adhering to fibrinogen of the Control group and HRS group at 1000 s^{-1}, respectively. Scale bar = 50 μm. (C) The platelet adhesion status was evaluated as the percentage of the surface area covered with platelets. Data are represented as means ± SD, $n = 3$. $^{**}P < 0.01$ versus Control.

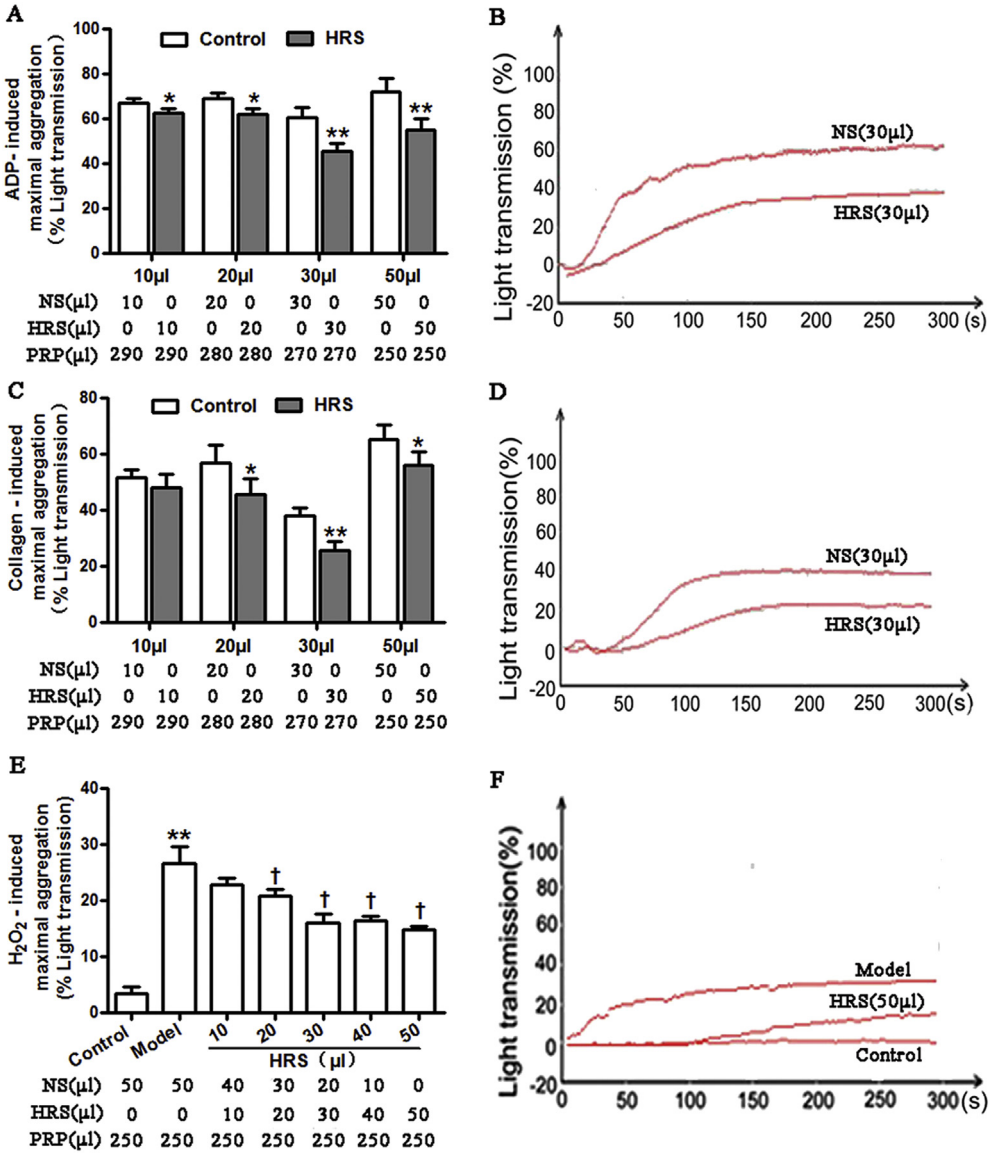

Fig. 4. HRS inhibited platelet aggregation in vitro. (A) and (B) ADP (10 μM) was used to induce platelet aggregation. (C) and (D) Collagen (15 μg/ml) was used to induce platelet aggregation. (E) and (F) HRS inhibited H_2O_2-induced increases in platelet aggregation. Data are represented as means ± SD, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Control, $^{\dagger}P < 0.01$ versus Model.

with HRS, the concentration of H_2O_2-induced TXB_2 decreased (Fig. 5C), indicating the inhibitory effect of HRS on the release of TXA_2 from rat platelets as induced by H_2O_2.

3.6. HRS reduced αIIbβ3 activation

Fibrinogen binding to platelets is an absolute requirement for aggregation, and platelet fibrinogen binding is regarded as a marker reflecting platelet aggregability or integrin αIIbβ3 activation [31,44]. H_2O_2 caused the increase of the percentage of mouse fibrinogen binding-positive platelets, while HRS significantly reduced positive ones (Fig. 6).

3.7. HRS improved oxidative status in mouse and rat platelets treated with H_2O_2 and in plasma of rats with $FeCl_3$-induced carotid arterial thrombosis

In mouse washed platelets exposed to H_2O_2, the MFI of ROS increased (Fig. 7A and B) and that of NO decreased (Fig. 7C and D) significantly compared with controls. After treatment with HRS, these changes were evidently ameliorated.

MDA is the ultimate product of unsaturated lipid peroxidation, and its content in blood may reflect excessive membrane injury generated by free radicals [15]. After aggregation, measurement of MDA and NO content in rat PRP showed that H_2O_2 significantly increased MDA levels and decreased NO levels. When HRS and H_2O_2 were added simultaneously to an aggregation cuvette containing rat PRP, HRS blocked the MDA increase (Fig. 7E) and NO decrease (Fig. 7F).

SOD is an important antioxidant enzyme in the regulation of oxidative tissue damage [15]. Levels of MDA and SOD in plasma of rats after $FeCl_3$-induced carotid arterial thrombosis were determined. As shown in Fig. 7G, the content of MDA in model rats increased significantly ($P < 0.01$) compared with the control rats, while HRS reduced MDA levels ($P < 0.05$) nearly to those of control rats. However, there were no significant differences in SOD level among the three groups (Fig. 7H). These results suggest that HRS may exert antithrombotic effects through its antioxidant properties.

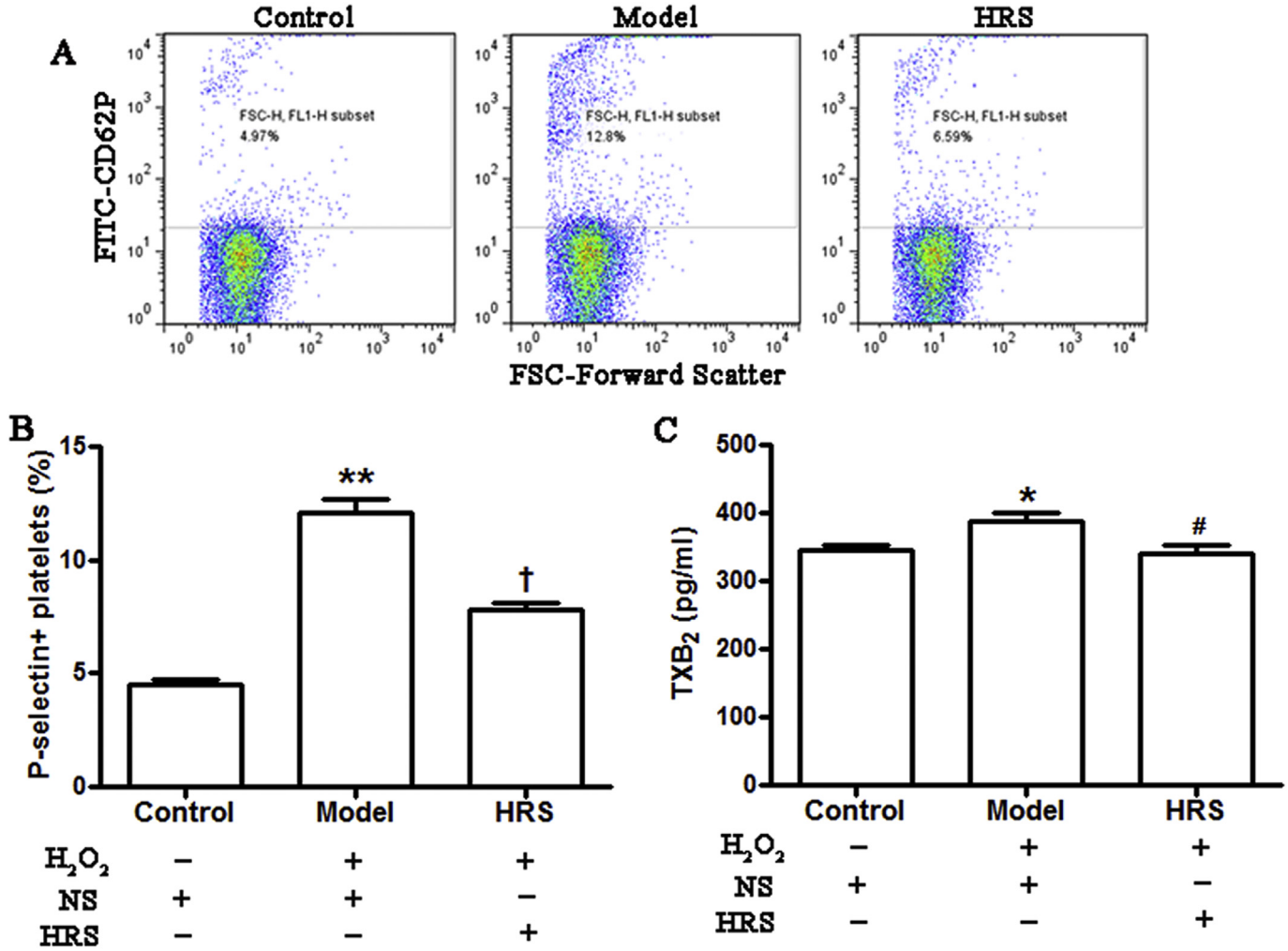

Fig. 5. HRS reduced α-granule secretion and TXB_2 release in H_2O_2-exposed platelets. (A) and (B) Flow cytometry analysis of P-selectin expression and quantification of CD62P-positive platelets, respectively. (C) TXB_2 level from H_2O_2-treated platelets. Data are represented as means ± SD, n = 8 for P-selectin, n = 3 for TXB_2. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Control, $^{\#}P < 0.05$, $^{\dagger}P < 0.01$ versus Model.

3.8. HRS increased cGMP formation and reduced phosphorylation of ERK1/2 in H_2O_2-induced platelets

As shown in Fig. 8, H_2O_2 was found to cause a decrease in cGMP formation in rat platelets and HRS mitigates this decrease (Fig. 8A). Furthermore, expression of p-ERK1/2 increased in washed mouse platelets exposed to H_2O_2 ($P < 0.01$), while HRS decreased p-ERK1/2 expression. When cGMP-dependent protein kinase (PKG) inhibitor KT5823 was added, phosphorylation of ERK1/2 again increased (Fig. 8B and C) compared with HRS treatment, indicating that ERK was the downstream of PKG.

4. Discussion

In this study, we demonstrated that: (1) H_2 prolonged bleeding time in mice and rats, and had antithrombotic activities in a rat arterio-venous shunt thrombosis model and FeCl3-induced artery thrombus formation in rats and mice; (2) H_2 reduced human platelet adhesion to fibrinogen, and collagen-, ADP-, and H_2O_2-induced rat platelet aggregation, as well as platelet release; (3) H_2 inhibited mouse αIIbβ3 activation induced by H_2O_2; (4) H_2 ameliorated oxidative status in mouse and rat platelets exposed to H_2O_2 and in plasma of rats with arterial thrombosis; (5) H_2 increased NO level and cGMP formation, but reduced pERK1/2 level (diminished with KT5823) in H_2O_2-treated platelets, suggesting involvement in NO/cGMP/PKG/ERK pathway.

Bleeding time is a reliable parameter of platelet function [45] and can comprehensively reflect antithrombotic activities of candidate agents in vivo [1]. Therefore, bleeding times were first investigated to estimate the antithrombotic activities of H_2. In our study, HRS significantly prolonged tail bleeding time in mice and rats (Fig. 1), which suggests possible antithrombotic activities of H_2.

Next, we used two thrombosis models to further determine the antithrombotic effects of H_2.The first was the rat model of arterio-venous shunt, which causes formation of a mixed platelet/fibrin thrombus. The second arterial injury model involved external application of $FeCl_3$ to induce vascular injury and thrombus formation, which was a modification of a similar injury model in rats and involves oxygen radical formation and specific damage to the endothelium to create a site for platelet adhesion and thrombus [46]. Our study found that HRS decreased thrombus weight in the rat arterio-venous shunt thrombosis model and prolonged time to occlusion in the $FeCl_3$-induced carotid arterial thrombosis model in both rats and mice (Fig. 2). We conclude that H_2 has antithrombotic effects. However, we did not find the inhibitory effect of HRS (at a dose of 5 ml/kg) on $FeCl_3$-induced thrombosis in rats and mice (data not shown), suggesting there needs a higher dose of HRS to control thrombosis.

Upon vascular injury, exposure of the subendothelial components provides an attractive surface for platelet adhesion and activation, resulting in platelet aggregation and release and thrombosis [47]. So, whether platelet adhesion, aggregation and release are affected by H_2

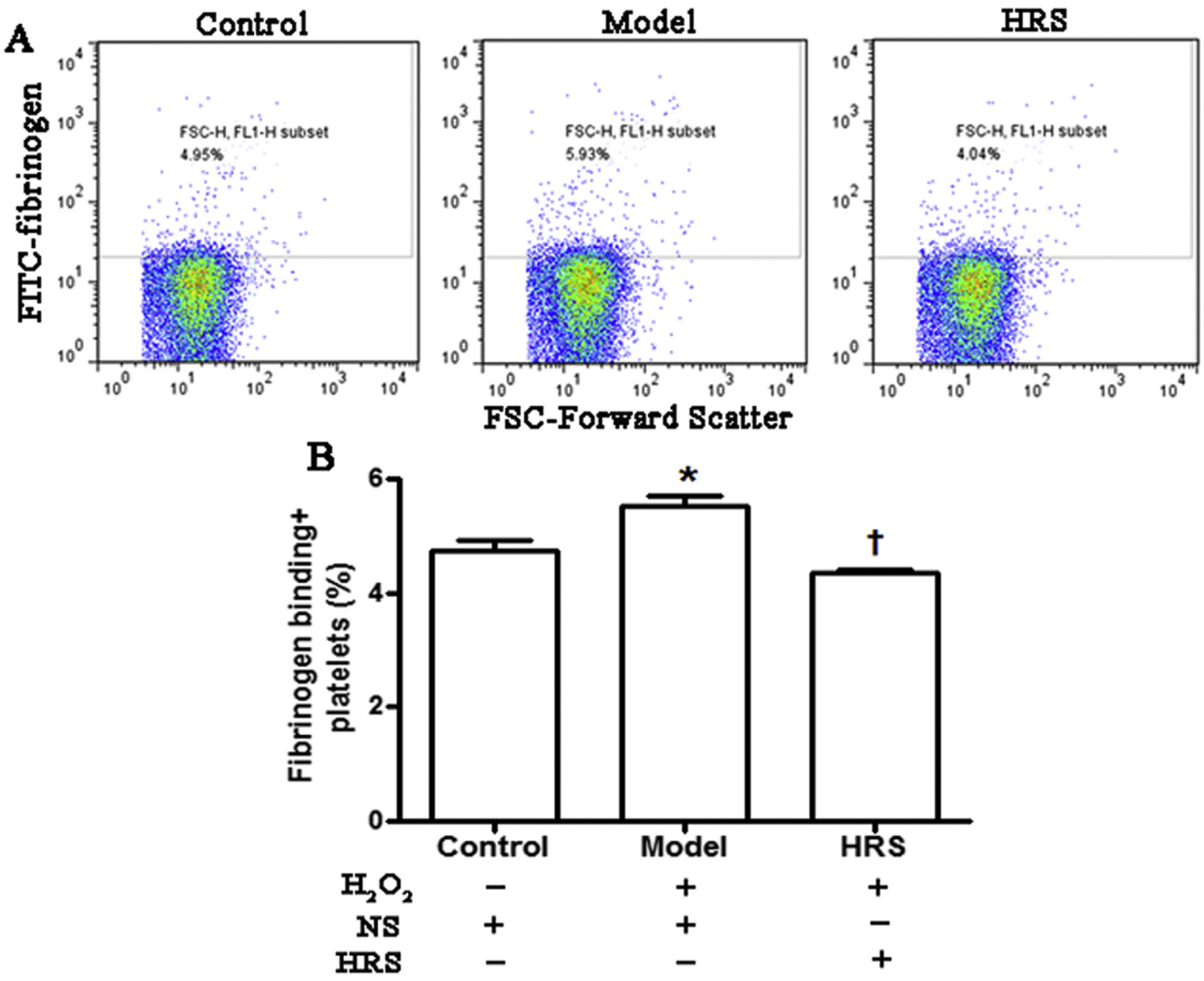

Fig. 6. HRS inhibited fibrinogen binding of platelet exposed to H_2O_2. (A) Flow cytometry analysis of fibrinogen binding. (B) Quantification of fibrinogen binding-positive platelets. Data are represented as means ± SD, n = 6. $^{*}P < 0.05$ versus Control, $^{\dagger}P < 0.01$ versus Model.

was investigated in our experiments.

The effect of HRS on platelet adhesion to fibrinogen was studied at first. During reperfusion of ischemic organs, fibrinogen accumulating on the endothelial surface is associated with the recruitment of platelets, and fibrinogen appears to support firm and irreversible platelet attachment [48]. Platelet adhesion at sites of vascular injury is regulated by blood flow, and increasing shear leads to increased thrombus growth [49]. In order to investigate the effects of H_2 on platelet adhesion under blood flow conditions, an in vitro flow-based assay was performed on a fibrinogen-coated surface at a shear rate of $1000s^{-1}$. The significant decrease of platelet adhesion to fibrinogen in the HRS group suggests that H_2 could weaken platelet adhesion (Fig. 3). Then we evaluated the inhibiting effect of HRS on platelet aggregation induced by ADP, collagen and H_2O_2. Our results indicate that HRS inhibited ADP-, collagen- and H_2O_2-induced platelet aggregation (Fig. 4). The inhibiting effect of HRS on collagen-induced platelet aggregation in our research is consistent with a previous study [7]. Besides, our research added new data that H_2 decreases ADP- and H_2O_2-induced platelet aggregation, suggesting H_2 inhibits platelet aggregation induced by multiple platelet agonists. Finally, platelet release reaction was observed with detection of TXA_2 release from platelets and P-selectin on the platelet surface. In this study, we found H_2O_2 (1 mM) significantly promoted the release of TXB_2 (reflecting TXA_2 change) from rat platelets (Fig. 5B), which is consistent with the previous studies [42,43]. Our data showed that HRS inhibited TXB_2 release from rat platelets as well as the expression of P-selectin on the platelet surface (Fig. 5). These results suggest H_2 inhibits platelet release reaction.

Together, these data showed a widespread inhibition effect of H_2 on platelet activation. It has been shown activated platelets promote fibrin formation in a factor XII-dependent manner in vitro [50]. Besides, activated platelets release polyphosphates. Polyphosphates, as the endogenous factor XII activator in vivo, link platelet activation (primary hemostasis) and fibrin production (secondary hemostasis) [51]. Based on our results of inhibitory effects of hydrogen on platelet activation, we speculate that hydrogen may inhibit secondary hemostasis.

Integrin αIIbβ3 is the receptor for fibrinogen and localizes on the platelet membrane and on α-granules. Once platelets are activated, αIIbβ3 undergoes conformational changes, becomes competent to bind fibrinogen [52] and then triggers a series of intracellular events. Fibrinogen binding to αIIbβ3 is the final pathway of platelet aggregation and the ultimate step in thrombosis [53,54]. To further study the underlying mechanism of the antithrombotic effect of H_2, we used washed platelets to observe the effect of HRS on fibrinogen binding. The results showed that HRS attenuated the percentage of fibrinogen binding-

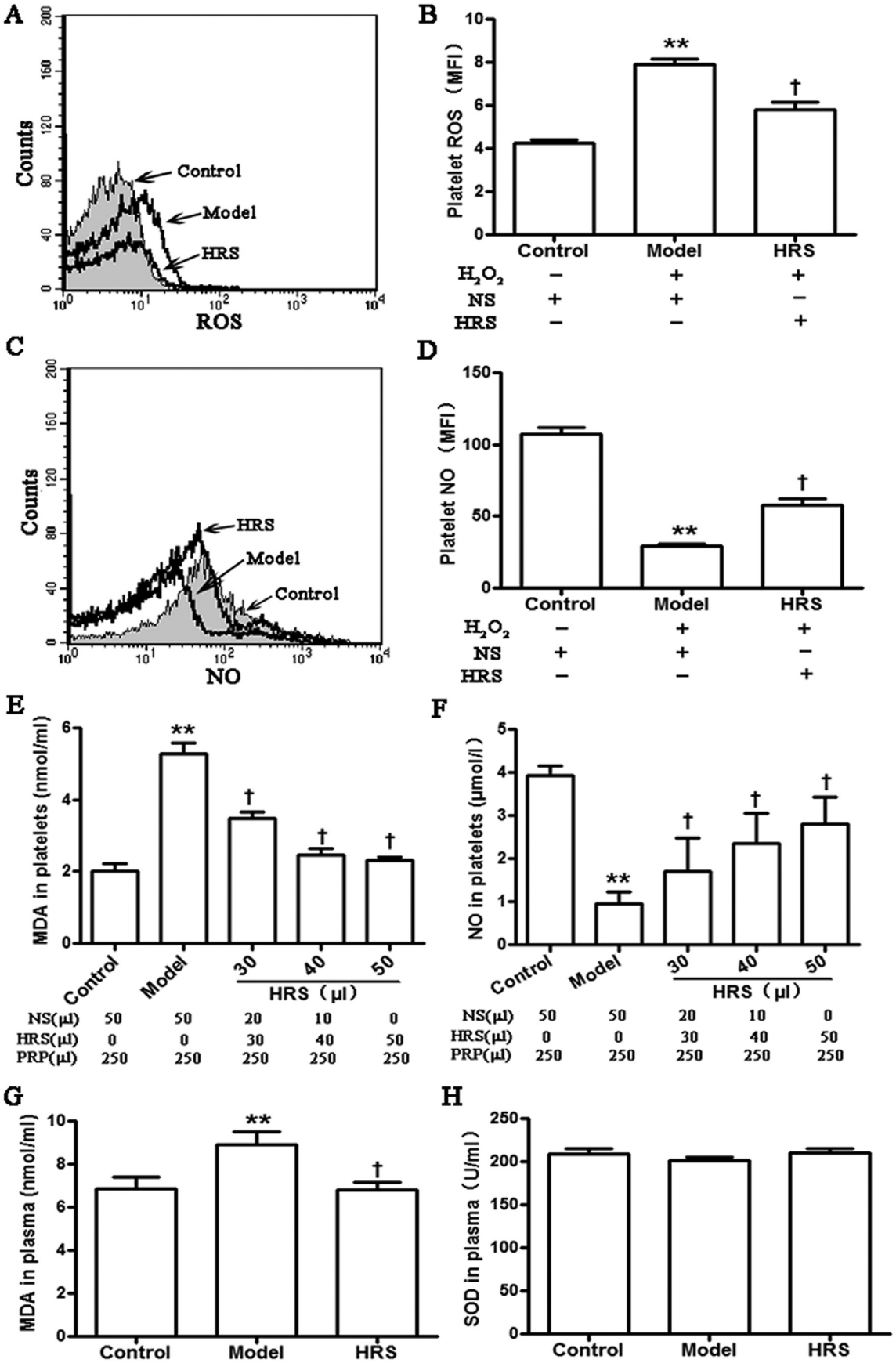

Fig. 7. HRS improved oxidative status in platelets and rat plasma. Flow cytometry of ROS and NO levels (A–D) in mouse washed platelets treated with H_2O_2, n = 6. (A) and (C) show representative fluorescence shifts of ROS and NO in platelets respectively. (B) and (D) are the corresponding MFI bar graphs. (E) and (F) Levels of MDA and NO in samples after platelet aggregation exposed to H_2O_2, n = 6. (G) and (H) Plasma levels of MDA and SOD in rats of the ferric chloride-induced arterial thrombosis model, respectively, $n = 10$. Data are represented as means ± SD, $^{**}P < 0.01$ versus Control, $^{\dagger}P < 0.01$ versus Model.

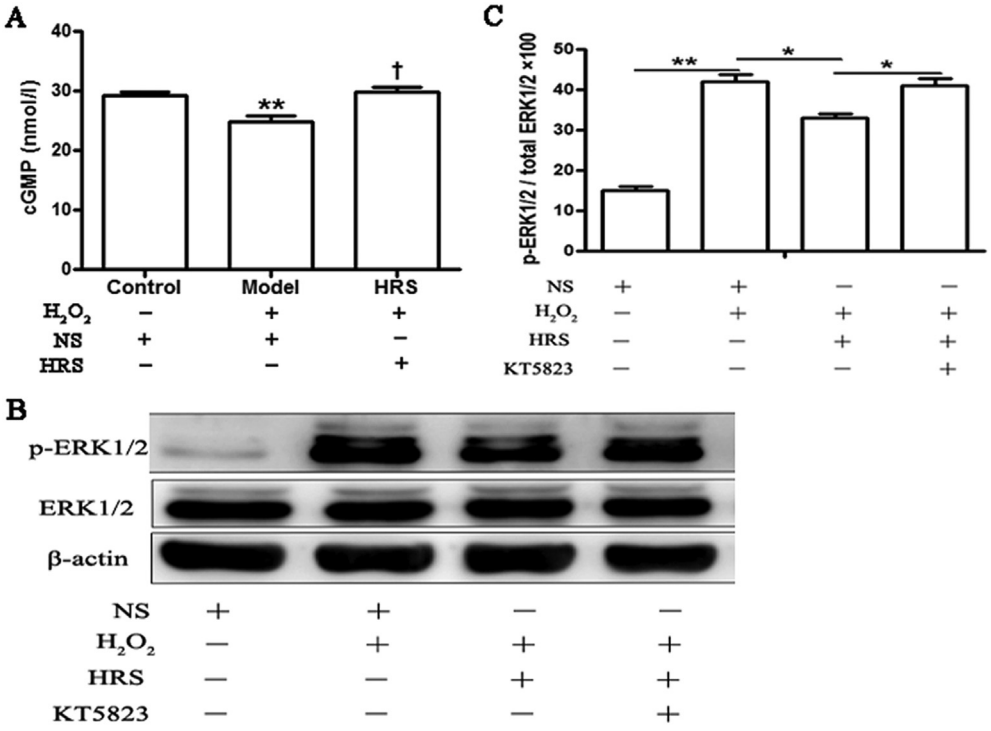

Fig. 8. Effects of HRS on cGMP formation and phosphorylation of ERK1/2. (A) Level of cGMP in H_2O_2-treated platelets; (B) and (C) expression of p-ERK1/2 in platelets exposed to H_2O_2. Data are represented as means ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Control; $^{\dagger}P < 0.01$ versus Model.

positive platelets in H_2O_2-exposed platelets (Fig. 6), suggesting inhibition of αIIbβ3 activation was responsible for the decreased platelet activation and thrombosis with H_2 treatment.

In the present study, we have shown that one mechanism of platelet inhibition by H_2 was involved in oxidative stress. The following evidence supports this conclusion. First, H_2O_2, a kind of ROS, induced platelet aggregation and release reaction. HRS inhibited platelet aggregation and release reaction induced by H_2O_2 (Figs. 4 and 5); Second, H_2O_2 activated αIIbβ3, whereas HRS inhibited αIIbβ3 activation (Fig. 6); Third, H_2O_2 increased the levels of ROS, whose overproduction may results in oxidant stress, but decreased NO levels in platelets. The decrease of NO level may be due to an increase of ROS [11,55]. HRS reversed this change of ROS and NO level in H_2O_2-treated platelets (Fig. 7A, B, C and D). Fourth, H_2O_2 treatment increased the content of MDA, an index of ROS generation [56], with decrease of NO content in platelets. HRS improved this change of MDA and NO content in H_2O_2-exposed platelets (Fig. 7E and F). Fifth, when oxidative stress is provoked, even without an accompanying aggregation, thrombi may tend to form [57]. There existed an increase of MDA content and a decrease of SOD level in plasma of a rat model of $Fecl_3$-induced arterial thrombosis. However, HRS inverted the change of MDA content and SOD level in plasma of the rat $FeCl_3$ model (Fig. 7G and H). Sixth, in Gordon's paper [58], the authors write that one of the products during synthesis of TXA_2 is MDA, a marker of oxidative stress. Oxidative stress may active platelets in turn. HRS decreased TXB_2 (Fig. 5B) and MDA (Fig. 7E) content in H_2O_2-treated platelets, suggesting H_2 may break the wretched cycle of TXA_2 and MDA. So H_2 could exert its effects mainly through opposing the oxidant system in this study.

In addition, we also have shown that another mechanism of platelet inhibition by H_2 was related to NO/cGMP/PKG/ERK pathway. At least three lines of evidence support this conclusion. Firstly, NO-cGMP-PKG pathway was detected in our study. The importance of NO-cGMP-PKG pathway as an inhibitor of platelet activation has been established [59–61]. In our manuscript, HRS increased NO level in H_2O_2-treated platelets (Fig. 7C, D and F). So we further investigated whether cGMP was influenced. The results showed that HRS increased cGMP level in platelets treated to H_2O_2 (Fig. 8A), suggesting the mechanism of NO-cGMP pathway. Secondly, when cells were exposed to high H_2O_2 levels as a model of oxidative stress, there existed strong and persistent activation of ERK1/2 [62,63]. In our study, activation of ERK1/2 was detected in platelets exposed to 1 mM H_2O_2. Indeed, we found that H_2O_2 increased expression of p-ERK1/2 (Fig. 8B and C), which is important in platelet activation [59,60]. Moreover, some substances exhibited anti-platelet effects through inhibition of ROS and ERK phosphorylation [64]. Hence, our results suggested ERK pathway was involved in the H_2O_2-induced platelet activation. When treated with HRS, expression of p-ERK1/2 was decreased in platelets stimulated with H_2O_2 (Fig. 8B and C), indicating ERK signal was associated with inhibitory effect of HRS on platelet activation. Thirdly, we investigated if PKG was related to inhibitory effect of HRS on ERK1/2 activation. In our study, KT5823 was used as an inhibitor of PKG [60]. The results showed that KT5823 diminished the inhibitory effect of HRS on expression of p-ERK1/2 (Fig. 8D). It has been reported that PKG inhibited ERK activation [60,65]. Therefore, we suggested ERK functioned as one of downstream effectors of PKG. Taken together, NO/cGMP/PKG/ERK pathway may be another mechanism underlying inhibitory effect of HRS on platelet activation.

5. Conclusion

In summary, our data suggest that H_2 effectively attenuates in vitro platelet activation and inhibits thrombus formation in vivo, which may be due to its antioxidant property and subsequent inhibition of platelet activation via NO/cGMP/PKG/ERK pathway, and H_2 might have the potential to be used as a novel antithrombotic agent. However, we cannot exclude other mechanisms, such as inhibition of inflammation and protection of the endothelium by H_2. Additionally, effect of hydrogen on secondary hemostasis, and the duration of HRS anti-platelet effect within the in vivo models should be investigated further.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116700.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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