

## Effect of polysaccharide sulfate on rat thrombosis: in vitro and ex vivo study

Liao Wensheng<sup>1</sup>, Gao Yuan<sup>2\*</sup>, Nie Yali<sup>2</sup>, Ma Feifei<sup>2</sup>

<sup>1</sup> Orthopaedic Department, First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan, 450052, China

<sup>2</sup> Department of Pharmacology, School of Medicine, Zhengzhou University, Zhengzhou, Henan, 450052, China

E-mail: [manman2002521@hotmail.com](mailto:manman2002521@hotmail.com)

**Abstract Objective** In this study, Polysaccharide sulfate (PSS) was investigated for its anti-thrombosis activity *in vitro* and *ex vivo*. **Methods** The platelet-rich plasma (PRP) was prepared according to a previously described procedure. PRP was incubated with PSS (12.5-150 µg/ml) or ASA (1.15 µg/ml) for 5 min before adding ADP (final concentration, 5 µM). Aggregation was measured with a platelet aggregometer. MDA contents, SOD activities, GSH contents, NO synthesis were detected by microplate reader. TXB<sub>2</sub>, 6-keto-PGF<sub>1α</sub>, cAMP, cGMP levels were measured with ELISA kits. Measurement of [Ca<sup>2+</sup>]<sub>i</sub> was performed using a fluorescence spectrophotometer. Prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT) and fibrinogen (FIB) content were observed *ex vivo*. **Results** PSS significantly inhibited ADP-induced rat platelet aggregation. Meanwhile, PSS decreased MDA contents, the ratio of TXB<sub>2</sub> to 6-keto-PGF<sub>1α</sub> and [Ca<sup>2+</sup>]<sub>i</sub>. To the contrary, PSS elevated SOD activities, GSH contents, NO synthesis, cAMP level in washed platelets. In addition, PSS increased cGMP productions in non-stimulated platelets. PSS inhibited coagulation function of normal rats by prolonging APTT and TT. **Conclusions** Our study suggested that PSS may be a good agent in the treatment of thrombosis diseases.

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### 1. Introduction

Platelets in circulating blood are in nonadhesive state. However, platelets could come in contact with agonists such as ADP, collagen, thrombin, thromboxane, calcium ionophore and PAF following vascular injury [1]. ADP which is released from erythrocytes, endothelial cells and platelet dense granules plays a major role in haemostasis and the pathogenesis of arterial thrombus by activating platelets. Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) formation which contributes to an increase in cytosolic free Ca<sup>2+</sup> level ([Ca<sup>2+</sup>]<sub>i</sub>) is of important in the mechanism by which ADP induces platelet aggregation [2]. Liu et al [3] reported that the level of cyclic AMP (cAMP) was one of crucial mediators in regulating platelet activation. The homeostasis between the rate of synthesis regulated by adenylate cyclase and the rate of degradation mediated by cAMP phosphodiesterase maintained the level of intracellular cAMP. Therefore, increasing the intracellular cAMP could cause many inhibitory effects in platelet aggregation, ATP release, thromboxane formation and agonist-elevated intracellular free calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) [4]. Other research also showed that cyclic GMP (cGMP) was produced via the activation of guanylate cyclase in the presence or absence of nitric oxide (NO). NO was synthesized in platelets and also decreased agonist-elevated [Ca<sup>2+</sup>]<sub>i</sub>, thus inhibited platelet activation [5,6]. On the other hand, activated platelets generated reactive oxygen species (ROS).

These ROS, such as O<sub>2</sub><sup>-</sup>, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and the hydroxyl radical (OH<sup>•</sup>), cause lipid peroxidation which result in platelet aggregation. Antioxidant defense system, such as SOD, glutathione peroxidase (GPx) and catalase, prevents superoxide-induced platelet activation. However, its protective antioxidant mechanisms are multifactorial and complex.

Many life-threatening diseases including cerebrovascular thrombosis, myocardial infarction, coronary heart disease, and atherosclerosis and tumor metastasis are caused by disorders of platelet function [7]. Several anti-platelet drugs, including acetyl salicylic acid (ASA) prevent platelet aggregation. Polysaccharide sulfate (PSS) is a new type of heparinoid compound isolated from phylum phaeophyta known to dilute blood, lower blood viscosity, ameliorate hypercoagulation and modulate dyslipidemia [8-9]. There were many researches about PSS on ischemic diseases in China [10-11]. Yoon *et al.* [12] showed that PSS protected the vascular endothelial cell and make the role of anti-platelet activation. Han *et al.* [13] fractionated PSS through ultrafiltration and found the anticoagulant of sulfated polysaccharides is related to the molecular weight markedly. In this study, we first provided evidence that PSS prevented ADP-induced platelet aggregation *in vitro*, and examined normal rat coagulation function *ex vivo*.

### 2. Material and Methods

## 2.1 Agents

PSS was purchased from Haier Group (Qingdao, China, No. 20080601) (Purity: 98.5%). Acetylsalicylic acid (ASA) was product of National Institutes for Food and Drug Control. ASA were freshly prepared as a stock solution in dimethylsulfoxide (DMSO) and diluted with Tyrode-HEPES buffer before the experiment. Fura 2-AM and ADP was products of Sigma (St. Louis, MO, USA). Malondialdehyde (MDA), superoxide dismutase (SOD), nitric oxide (NO), glutathione (GSH) assay kits were products of Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Thromboxane B<sub>2</sub> (TXB<sub>2</sub>), 6-keto-prostaglandin F<sub>1α</sub> (6-keto-PGF<sub>1α</sub>), cAMP, cGMP enzymelinked immunosorbent assay kit (ELISA) were purchased from R&D System Co. (USA). Assay kits for prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT) and fibrinogen (FIB) were Siemns Healthcare Diagnostics products (German).

## 2.2 Animals

Male Sprague-Dawley rats (weight: 200-250 g) of SPF grade was used in the experiment. The animals were housed in a room with a 12h light: dark cycle. All experiments were performed in accordance with institutional guidelines of the Experimental Animal Center of the Chinese Academy of Medical Science.

## 2.3 Preparation of platelets

Rats were anesthetized with 10% chloral hydrate, and blood was collected from the abdominal aorta and anticoagulated with sodium citrate (3.8%; 1:9 v/v). Collected rat blood was centrifuged at 500 rpm for 6 min to prepare platelet-rich plasma (PRP). The remaining blood was further centrifuged at 4000 rpm for 10 min to prepare platelet-poor plasma (PPP) [14]. Rat platelets were pelleted by centrifugation at 2500 rpm for 10 min at room temperature. The platelet pellets were washed with modified Tyrode-HEPES buffer twice according to a previously described procedure [15]. (Tyrode-HEPES buffer: 129 mMNaCl, 2.8 mM KCl, 8.9 mM NaHCO<sub>3</sub>, 0.8 mM MgCl<sub>2</sub>, 0.8 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM EGTA, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4) The platelet concentration was adjusted to 4×10<sup>8</sup> platelets/ml for rat platelets.

## 2.4 Measurement of platelet aggregation

PRP was obtained by centrifuging blood at 500 rpm for 6 min at room temperature. The remaining blood samples were centrifuged at 4000 rpm for another 10 min to obtain PPP. The platelet pellets were washed with modified Tyrode-HEPES buffer and washed twice. The platelet concentration was adjusted to 4×10<sup>8</sup> platelets/ml for rat platelets. Then 0.3 ml of washed platelet was placed in a cuvette and stirred with rotor at 37°C for 5 min; The rat platelet

suspension was incubated with PSS (12.5 - 150 µg/ml) or ASA (1.15 µg/ml) for 5 min before adding ADP (final concentration, 5 µM). Aggregation was measured with a platelet aggregometer (LBY-NJ4, Pulisheng Instrument Co. Ltd., China). Platelet aggregation was recorded for 5min after the addition of ADP. The inhibition of platelet aggregation is expressed as % inhibition (X) using the following equation:  $X = [(A - B)/A] \times 100\%$ , where A=maximal aggregation of the control, and B= maximal aggregation of the PSS-treated sample.

## 2.5 Measurement of MDA productions, SOD activities, GSH contents and NO synthesis in Platelets

Blood was collected from the abdominal aorta and anticoagulated with sodium citrate (3.8%; 1:9 v/v). PRP was prepared as described above. The rat platelet suspension was preincubated with PSS (50, 100, 150 µg/ml) or ASA (1.15 µg/ml) for 5 min before adding ADP (final concentration, 5 µM) at 37°C for 5 min. MDA productions were determined by the microplate reader assay according to the procedures provided in the assay kits. Briefly, thiobarbituric acid reactive substances were assessed by measuring MDA concentration at 532 nm with the thiobarbituric acid method, which is based on the reaction of MDA with thiobarbituric acid to form a stable chromophoric product.

Activities of SOD, NO synthesis and GSH contents were determined by the microplate reader (Bio-Tek ELX800, USA) assay according to the procedures provided in the assay kits at 550 nm, 550 nm and 420nm respectively.

## 2.6 Determination of TXB<sub>2</sub> and 6-keto-PGF<sub>1α</sub> in platelets

Since TXA<sub>2</sub> and PGI<sub>2</sub> are unstable and can rapidly convert to TXB<sub>2</sub> or 6-keto-PGF<sub>1α</sub> respectively, the more stable metabolite, we measured the latter instead. Blood was collected from the abdominal aorta and anticoagulated with sodium citrate (3.8%; 1:9 v/v). PRP was prepared as described above. The rat platelet suspension was preincubated with PSS (50, 100, 150 µg/ml) or ASA (1.15 µg/ml) for 5 min and addition of agonist 5 µM ADP at 37 °C for 5 min. Subsequently, the reactions were terminated by adding ice-cold 2 mM Indometacin and 100 µM EDTA. After brief centrifugation in an eppendorf centrifuge at 14000 rpm for 2 min, TXB<sub>2</sub> and 6-keto-PGF<sub>1α</sub> levels in the supernatant were measured with ELISA kits according to manufacturer's instruction.

## 2.7 Platelet cAMP, cGMP level assay

This procedure was performed as described in previous publications (Willoughby et al., 1998). Washed rat platelets (4×10<sup>8</sup> platelets/ml) were preincubated for 5min at 37°C with PSS (50, 100, 150

$\mu\text{g/ml}$ ) or ASA (1.15  $\mu\text{g/ml}$ ), then 5  $\mu\text{M}$  ADP was added where required. The reaction was stopped by adding EDTA 10 mM followed by boiling for 3 min. After the mixture was cooled to 4°C, precipitated protein was sediment by centrifugation in an eppendorf microcentrifuge and the supernatant was collected to immediate assay. cAMP level in the supernatant were measured using an ELISA kit respectively according to the manufacturer's instructions. And we measured intracellular cGMP level in non-stimulated platelets using an ELISA kit respectively according to the manufacturer's instructions.

### 2.8 Determination of intracellular $[\text{Ca}^{2+}]_i$ in platelets

$[\text{Ca}^{2+}]_i$  was ascertained using a fluorescent  $\text{Ca}^{2+}$  indicator Fura 2-AM[16]. Briefly, platelets ( $4 \times 10^8$  platelets/ml) were incubated with Fura 2-AM (2  $\mu\text{M}$ ) for 30 min and then Fura 2-AM-loaded washed platelets were preincubated for 5 min at 37°C with PSS (50, 100, 150  $\mu\text{g/ml}$ ) or ASA (1.15  $\mu\text{g/ml}$ ), and then stimulated with 5  $\mu\text{M}$  ADP for 5min for evaluation of  $[\text{Ca}^{2+}]_i$ . Because Fura 2-AM is light-sensitive, the tube containing washed platelets was covered with aluminum foil during loading. Thereafter, centrifuged for 10 min at 800 rpm; the resultant pellet was washed with Tyrode-HEPES buffer. After centrifugation, platelets were gently resuspended in Tyrode-HEPES buffer containing 1mM  $\text{CaCl}_2$  to make a concentration of  $4 \times 10^8$  platelets /ml and were kept at room temperature. Measurement of intracellular free  $\text{Ca}^{2+}$  concentration was performed using a Hitachi F4500 fluorescence spectrophotometer. Two excitation wavelengths, 340 and 380nm, were used with an emission at 510 nm and the ratio was analyzed automatically by computer software. Platelets were lysed with 0.1% (v/v) Triton X-100 to determine the maximum fluorescence; 10 mM EGTA was added to determine the minimum fluorescence. The intracellular free  $\text{Ca}^{2+}$  concentration in platelets was calculated as follows:  $[\text{Ca}^{2+}]_i = \text{Kd} \times (\text{F} - \text{F}_{\text{min}})/(\text{F}_{\text{max}} - \text{F})$ , where Kd = 400nM at 22°C;  $\text{F}_{\text{min}}$  means the minimum

fluorescence;  $\text{F}_{\text{max}}$  means the maximum fluorescence; and F means the fluorescence of PSS-treated sample.

### 2.9 Antithrombotic activity *ex vivo*: Effect of PSS on the coagulation function of rats

To examine if PSS have an effect on coagulation in normal rats, we took the classical coagulant assays of prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT) and fibrinogen (FIB) content. Rats were randomly divided into five groups (Control group, PSS 2.5, 5, 10 mg/kg groups and ASA 57.5  $\mu\text{g/kg}$  group), each group consisting of 10 rats. PSS 5 mg/kg and ASA 57.5  $\mu\text{g/kg}$  are the equivalent molar dose. After intravenous injection of different dose of drugs for 7 days, rat was anesthetized with 10% chloral hydrate. The blood was immediately collected from the abdominal aorta and anticoagulated with sodium citrate (3.8%; 1:9 v/v). Blood plasma was obtained by 500 rpm for 10 min. PT, TT, APTT and FIB concentration was examined by kits according to the method of Jackson et al. [17] using blood plasma.

### 3.0 Statistical Analysis

Results were expressed as mean $\pm$ SEM. Significance of inter-group differences was evaluated by one-way ANOVA test. Differences were considered significant at  $P < 0.05$ . When appropriate, the  $\text{IC}_{50}$  values (the concentration able to inhibit 50% of the maximum effect observed) were determined by Probit analysis.

### 3. Results

#### 3.1 Effect of PSS on platelet aggregation *in vitro*

When washed rat platelets ( $4 \times 10^8$ /ml) were activated with 5  $\mu\text{M}$  ADP, the rate of aggregation was (56.45 $\pm$ 0.81) % (Tab.1). However, PSS (12.5-150  $\mu\text{g/ml}$ ) added to washed platelets 5min before stimulation by ADP 5  $\mu\text{M}$  inhibited platelet aggregation in a concentration-dependent manner (Fig.1). Washed rat platelets were preincubated with PSS (12.5 -150  $\mu\text{g/ml}$ ) for 5 min, and then exposed to ADP 5  $\mu\text{M}$  to stimulate platelet aggregation. Data is expressed as mean  $\pm$  SEM (n=5-6). <sup>aa</sup> $P < 0.01$  vs. Control group; <sup>bb</sup> $P < 0.01$  vs. ASA group.

**Table 1.** Effect of PSS on ADP-induced washed rat platelet aggregation

Group	Concentration ( $\mu\text{g/ml}$ )	Rate of platelet aggregation (%)	Percentage of inhibition of platelet aggregation (%)
Control		56.45 $\pm$ 0.81	
ASA	1.15	35.03 $\pm$ 1.04 <sup>aa</sup>	37.93
PSS	12.5	41.88 $\pm$ 1.40 <sup>aa</sup>	25.81
	25	35.97 $\pm$ 0.99 <sup>aa</sup>	36.28
	50	29.50 $\pm$ 1.71 <sup>aa bb</sup>	47.74
	100	23.45 $\pm$ 1.06 <sup>aa bb</sup>	58.46
	150	15.86 $\pm$ 1.77 <sup>aa bb</sup>	71.90

**Table 2.** Effect of PSS on ADP (5  $\mu$ M)-induced MDA productions, NO synthesis, SOD activities, and GSH contents in platelets

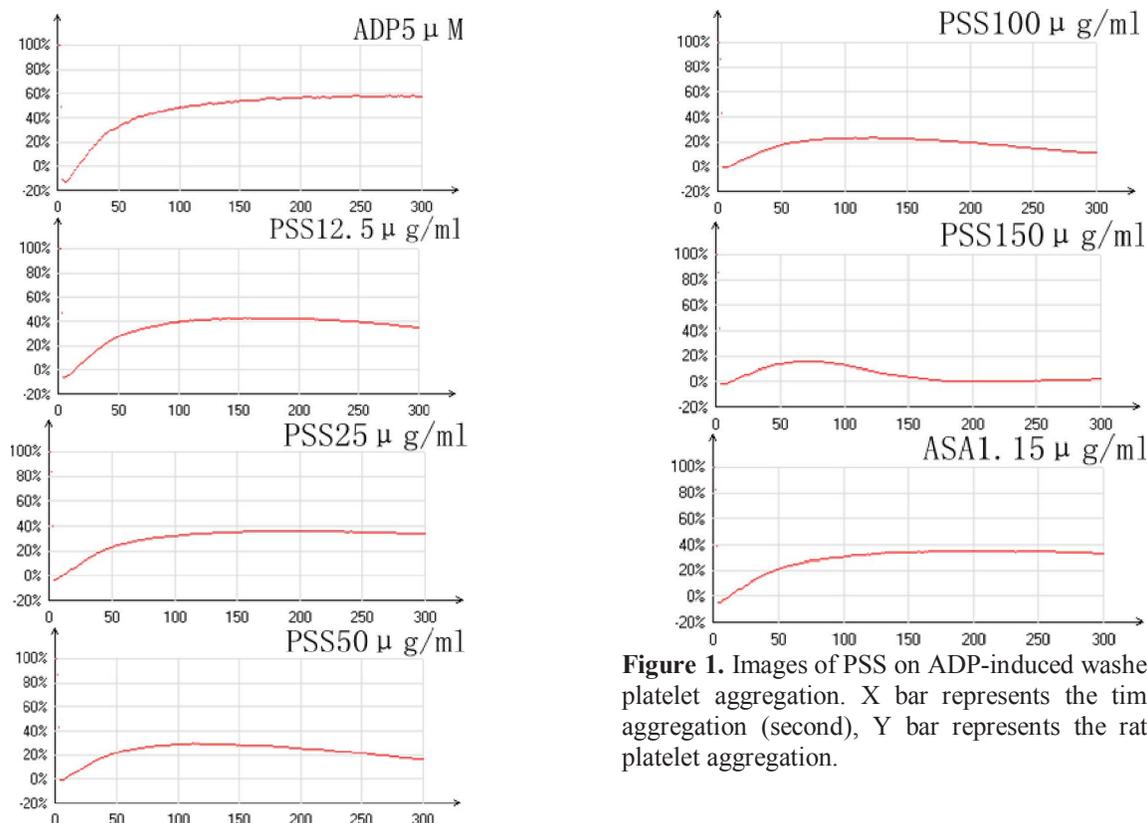
Group	Concentration ( $\mu$ g/ml)	MDA (nmol/ml)	NO ( $\mu$ M)	SOD (U/ml)	GSH (mg/l)
Control		5.65 $\pm$ 2.19	57.44 $\pm$ 15.02	66.15 $\pm$ 4.95	7.78 $\pm$ 1.67
ADP		11.80 $\pm$ 1.50 <sup>aa</sup>	7.69 $\pm$ 2.78 <sup>aa</sup>	46.18 $\pm$ 3.26 <sup>aa</sup>	1.84 $\pm$ 1.19 <sup>aa</sup>
ASA	1.15	8.58 $\pm$ 1.43 <sup>bb</sup>	42.27 $\pm$ 16.45 <sup>bb</sup>	53.36 $\pm$ 2.81 <sup>bb</sup>	5.12 $\pm$ 1.02 <sup>bb</sup>
PSS	50	9.33 $\pm$ 4.21	14.58 $\pm$ 9.11	51.79 $\pm$ 2.57 <sup>b</sup>	3.07 $\pm$ 1.18
	100	7.22 $\pm$ 3.18 <sup>bb</sup>	20.97 $\pm$ 8.02 <sup>bb</sup>	58.37 $\pm$ 3.22 <sup>bb</sup>	6.09 $\pm$ 0.75 <sup>bb c</sup>
	150	6.04 $\pm$ 2.45 <sup>bb c</sup>	23.14 $\pm$ 6.63 <sup>bb</sup>	63.14 $\pm$ 4.77 <sup>bb c</sup>	7.96 $\pm$ 1.22 <sup>bb cc</sup>

**Notes:** Effect of PSS on ADP-induced MDA productions, NO synthesis, SOD activities and GSH contents in platelets. Data were presented as mean  $\pm$  SEM (n=8). One-way ANOVA test was used to determine statistical significance. <sup>aa</sup>P<0.01 vs. Control group; <sup>b</sup>P<0.05, <sup>bb</sup>P<0.01 vs. ADP group; <sup>c</sup>P<0.05, <sup>cc</sup>P<0.01 vs. ASA group.

**Table 3.** Effect of PSS on ADP-induced TXB<sub>2</sub> level, 6-keto-PGF<sub>1 $\alpha$</sub>  formation and the ratio of TXB<sub>2</sub>/6-keto-PGF<sub>1 $\alpha$</sub>  in platelets

Group	Concentration ( $\mu$ g/ml)	TXB <sub>2</sub> (pg/ml)	6-keto-PGF <sub>1<math>\alpha</math></sub> (pg/ml)	TXB <sub>2</sub> /6-keto-PGF <sub>1<math>\alpha</math></sub>
Control		607.61 $\pm$ 45.13	54.14 $\pm$ 1.32	11.22
ADP		1031.93 $\pm$ 47.16 <sup>aa</sup>	44.14 $\pm$ 2.52 <sup>aa</sup>	23.38
ASA	1.15	875.68 $\pm$ 43.31 <sup>bb</sup>	45.02 $\pm$ 1.83	19.45
PSS	50	998.07 $\pm$ 24.78	43.15 $\pm$ 3.07	23.13
	100	960.34 $\pm$ 17.84	45.33 $\pm$ 2.24	21.19
	150	946.02 $\pm$ 38.13 <sup>b</sup>	51.54 $\pm$ 2.09 <sup>b</sup>	18.36

**Notes:** Blood was collected from the abdominal aorta and anticoagulated with sodium citrate (3.8%; 1:9 v/v). PRP was prepared as described above. The rat platelet suspension was preincubated with PSS (50, 100, 150  $\mu$ g/ml) or ASA (1.15  $\mu$ g/ml) for 5 min and addition of agonist ADP (final concentration, 5  $\mu$ M) at 37  $^{\circ}$ C for 5 min. The concentration of TXB<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  were determined with ELISA assay kits. Values are presented as mean  $\pm$  SEM. (n = 5); <sup>aa</sup>P<0.01 vs. Control group; <sup>b</sup>P<0.05, <sup>bb</sup>P<0.01 vs. ADP group.

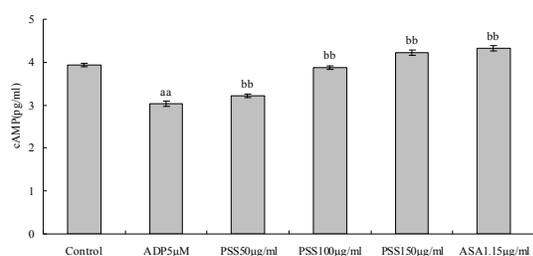


**Figure 1.** Images of PSS on ADP-induced washed rat platelet aggregation. X bar represents the time of aggregation (second), Y bar represents the rate of platelet aggregation.

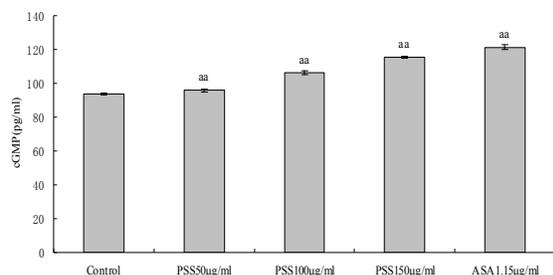
**Table 4** Effect of PSS on the coagulation function of rats

Group	Dose(mg/kg)	PT(sec)	TT(sec)	APTT(sec)	FIB(g/l)
Control		19.33±1.46	17.39±2.43	22.84±1.39	1.61±0.26
ASA	0.0575	21.12±2.11	18.89±3.31	23.90±3.07	1.70±1.02
PSS	2.5	19.59±0.77	18.44±2.01	24.66±1.18	1.53±0.41
	5	20.01±1.92	20.76±3.20	27.12±2.27 <sup>aa</sup>	1.44±0.55
	10	20.37±1.04	23.55±2.12 <sup>aa</sup>	28.25±2.71 <sup>aa bb</sup>	1.37±0.76

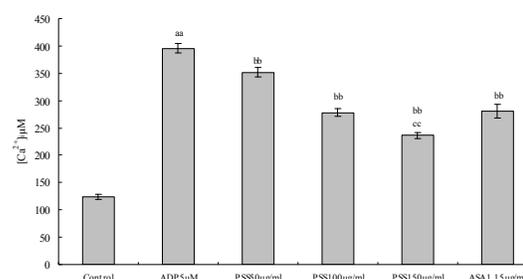
**Notes:** Rats were randomly divided into five groups (Control group, PSS 2.5, 5, 10 mg/kg groups and ASA 57.5 µg/kg group), each group consisting of 10. After intravenous injection of different dose of drugs for 20 days, rat was anesthetized with 10% chloral hydrate. Blood was collected from the abdominal aorta and anticoagulated with sodium citrate (3.8%; 1:9 v/v). The serum was obtained by 500 rpm for 10 min. The APTT, PT and TT was expressed in seconds and FIB concentration was expressed in g/l. <sup>aa</sup> $P < 0.01$  vs. Control. <sup>bb</sup> $P < 0.01$  vs. ASA group.



**Fig.2** Effect of PSS on ADP-induced cAMP level in platelet. Washed rat platelets ( $4 \times 10^8$  platelets/ml) were preincubated for 5 min at 37°C with PSS (50, 100, 150 µg/ml) ASA (1.15 µg/ml), then 5 µM ADP was added where required. Data were presented as mean ± SEM (n = 5). One-way ANOVA test was used to determine statistical significance. <sup>aa</sup> $P < 0.01$  vs. Control group; <sup>bb</sup> $P < 0.01$  vs. ADP group.



**Fig.3** Effect of PSS on cGMP level in non-stimulated platelets. Washed rat platelets ( $4 \times 10^8$  platelets/ml) were preincubated for 5min at 37°C with PSS(50, 100, 150 µg/ml) or ASA (1.15 µg/ml), then we measured cGMP productions in non-stimulated platelets by means of ELISA assay. Data were presented as mean ± SEM (n = 5). One-way ANOVA test was used to determine statistical significance. <sup>aa</sup> $P < 0.01$  vs. Control group.



**Fig.4** Platelets ( $4 \times 10^8$  platelets/ml) were incubated with Fura 2-AM (2 µM) for 30 min and then Fura 2-AM-loaded washed platelets were preincubated for 5min at 37 °C with various concentration of PSS or ASA, and then stimulated with 5 µM ADP for 5 min for evaluation of  $[Ca^{2+}]_i$ . Measurement of cytosolic  $Ca^{2+}$  concentration was performed using a Hitachi F4500 fluorescence spectrophotometer. Two excitation wavelengths, 340 and 380 nm, were used with an emission at 510 nm and the ratio was analyzed automatically by computer software. Data were presented as mean ± SEM (n =5). One-way ANOVA test was used to determine statistical significance. <sup>aa</sup> $P < 0.01$  vs. Control group; <sup>bb</sup> $P < 0.01$  vs. ADP group; <sup>cc</sup> $P < 0.01$  vs. ASA group.

### 3.2 Effect of PSS on ADP-induced MDA productions, NO synthesis, SOD activities, and GSH contents in platelets

As shown in Tab 2, after washed rat platelets were stimulated by ADP, the elevated productions of MDA and decreased NO synthesis, SOD activities and GSH contents ( $P < 0.01$ ) in platelets were observed, preincubation with PSS at different concentrations significantly attenuated MDA contents and increased SOD activities and GSH contents ( $P < 0.01$ ) in a concentration-dependent manner.

### 3.3 Effect of PSS on ADP-induced TXB<sub>2</sub> level, 6-keto-PGF<sub>1α</sub> formation and the ratio of TXB<sub>2</sub>/6-keto-PGF<sub>1α</sub> in platelets

We examined the effects of PSS on TXB<sub>2</sub> synthesis in platelets stimulated with exogenous ADP. PSS at different concentrations was found to inhibit ADP-induced TXB<sub>2</sub> synthesis in a concentration-dependent manner. It is well known that PGI<sub>2</sub> inhibit platelet aggregation, therefore, we further measured 6-keto-PGF<sub>1α</sub> contents (the more stable metabolite) after washed rat platelets were preincubated with PSS (50, 100, 150 μg/ml) for 5 min, and then exposed to ADP 5 μM to stimulate platelet. As shown in Tab. 3, we observed that PSS just at high concentrations increased ADP-induced 6-keto-PGF<sub>1α</sub> contents. However, PSS at different concentrations reduced the ratio of TXB<sub>2</sub> to 6-keto-PGF<sub>1α</sub> in a concentration-dependent manner. Interestingly, ASA was found to have no effect on ADP-induced 6-keto-PGF<sub>1α</sub> contents ( $P > 0.05$ ), only decreased TXB<sub>2</sub> synthesis significantly ( $P < 0.01$ ).

### 3.4 Effects of PSS on intracellular cAMP, cGMP level in platelets

We have investigated that PSS affected on cAMP and cGMP productions in platelets by means of an ELISA assay. As shown in Fig.2, These results showed that PSS significantly enhanced cAMP level in a concentration-dependent manner. At the same time, PSS at different concentrations enhanced the cGMP level in non-stimulated platelets (Fig.3).

### 3.5 Effect of PSS on ADP-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation in platelets

As shown in Fig.4, The increased intracellular calcium triggered by 5 μM ADP in the Fura 2-AM loaded platelets could be detected by fluorescence spectrophotometer. However, pretreated with PSS (50, 100, 150 μg/ml) attenuated significantly these fluorescence signals in platelets. Therefore, PSS-induced platelet suppression is relevant to the mechanisms of calcium mobilization.

### 3.6 Effect of PSS on the coagulation function of rats

PT is used to describe the exogenous pathway of coagulation. The length of PT reflects the level of prothrombin, fibrinogen and blood coagulation factor V, VII, X in plasma. TT is mainly affected by the contents of fibrinogen and fibrin in plasma and coagulation activity, the length of which stands for the level of common pathway of coagulation. APTT is activated partial thromboplastin time and the length of APTT reflects the level of prothrombin, fibrinogen and blood coagulation factor V, X in plasma in endogenous pathway of coagulation. Moreover, fibrinogen is the main protein during the process of coagulation. As shown in Tab.4, PSS significantly

prolonged APPT and TT in a dose dependent manner ( $P < 0.01$ ). However, PT and fibrinogen content undergo no significant modification between PSS group and Control group. ASA group had no effect on coagulation function.

## 4. Discussions

Platelets play an important role in thrombosis formation via platelet activation, adhesion and aggregation [18]. Once blood vessels are damaged, platelets in circulating blood contacting with agonists may induce platelet aggregation, a process responsible for thrombus or hemostatic plug formation and wound healing. On the other hand, several articles have shown that platelets became more sensitive to their agonists and hyperactive, which is pathophysiological for arterial thrombosis [19, 20]. ADP may induce platelet shape change, exposure of fibrinogen binding sites, the influx and intracellular mobilization of Ca<sup>2+</sup>. In addition, ADP inhibits adenylyl cyclase activity stimulated by prostaglandins.

This study highlights PSS anti-platelet properties. We have shown that *in vitro*, PSS inhibited in a concentration-dependent manner ADP-induced platelet aggregation. Antioxidants can modify platelet stability, platelet aggregation and tendency to thrombosis. Saldeen et al [21] reported that antioxidants could inhibit monocyte adhesion and platelet activation. Article has shown that PSS possessed activities against oxidative stress [22]. In the present study, we examined the effect of PSS on ADP-induced MDA productions, SOD activities and GSH contents in platelets. The results exhibited that PSS at different concentrations not only inhibited ADP-induced MDA productions but also elevated SOD activities and GSH contents in platelets. Therefore, we thought that the mechanisms of PSS anti-platelet were related to its antioxidant properties. ADP-induced platelet aggregation needs influx of Ca<sup>2+</sup> and mobilization of Ca<sup>2+</sup> from the intracellular stores [23]. In order to realize possible mechanism of PSS-induced platelet suppression, we did further study on the interaction between calcium mobilization and platelet suppression. The results showed that PSS inhibited ADP-induced intracellular [Ca<sup>2+</sup>]<sub>i</sub> elevation in platelets. A complex signal transduction cascade reaction mediated by various stimulants induces platelet activation. Several potential mechanisms for inhibition of platelet aggregation including cAMP increase, synthesis of prostacyclin analogues, inhibition of adenosine receptor and thromboxane synthase have been suggested [24, 25, 26]. As well known, an increased intracellular cAMP level leads to inhibition of platelet activation, adhesion, and release of granule

contents. In addition, arachidonate metabolism regulates platelet function and thrombosis. TXA<sub>2</sub> and PGI<sub>2</sub> are major metabolites of cyclooxygenase activation in platelets and in endothelial cells, respectively [27]. TXA<sub>2</sub> is a powerful platelet aggregating and vasoconstricting agent, whereas PGI<sub>2</sub> is a potent anti-platelet and vasodilator substance. Rhee et al. [28] have been demonstrated the intracellular PGI<sub>2</sub> increase led to many inhibitory effects in platelet aggregation, ATP release, thromboxane formation and calcium efflux upon stimulation. To realize the PSS-induced calcium mobilization suppression, we further measured the platelet cAMP level, TXB<sub>2</sub> release, 6-keto-PGF<sub>1α</sub> contents in platelets and found that PSS increased intracellular cAMP. PSS at high concentration reduced the production of TXB<sub>2</sub> and elevated the production of PGI<sub>2</sub>. However, PSS decreased the ratio of TXA<sub>2</sub> to PGI<sub>2</sub> in platelets in a concentration-dependent manner.

NO which is generated in platelets activates guanylate cyclase and elevates the platelet cGMP level. It is well known that NO attenuates platelet activation and inhibit platelet aggregation *in vitro* and *ex vivo*. We investigated the effects of PSS on the cyclic nucleotides production. PSS was able to enhance cAMP level in ADP-induced platelet aggregation and at higher concentration to enhance cGMP production in non-stimulated platelets.

We also examined the effect of PSS on coagulation in normal rats by classical coagulant assays PT, TT, APTT and fibrinogen content. The *ex vivo* study demonstrated that PSS prolonged APTT and TT. This prompted that PSS inhibited the coagulation function in several species mainly by effecting prothrombin, fibrinogen and blood coagulation factor V, VII, X in plasma. However, ASA had no significant effect on any parameter of coagulation function, suggesting that the mechanism of PSS on antithrombotic activity was different from ASA.

Our results suggested that PSS inhibited ADP-induced platelet aggregation in a dose-dependent manner. This inhibitory effect by PSS seems to be due to lowering of [Ca<sup>2+</sup>]<sub>i</sub> and the up-regulation of intracellular levels of cAMP and cGMP in ADP-stimulated or non-stimulated platelets. In addition, the mechanisms of PSS anti-platelet were related to its antioxidation properties. These results also suggest, therefore, that PSS may be effective in inhibiting thrombosis.

#### Conflict of interest statement

The authors reported no potential conflicts of interest.

#### Corresponding author:

Dr. Gao Yuan,  
Department of Pharmacology, School of Medicine,  
Zhengzhou University,  
Zhengzhou, Henan450052, China  
E-mail: [manman2002521@hotmail.com](mailto:manman2002521@hotmail.com)

#### References

1. Puri RN. ADP-induced platelet aggregation and inhibition of adenylyl cyclase activity stimulated by prostaglandins[J]. *Biochemical Pharmacology*, 1999, 57:851-859.
2. Cho HJ, Cho JY, Rhee MH, Park HJ. Cordycepin (3'-deoxyadenosine) inhibits human platelet aggregation in a cyclic AMP- and cyclic GMP-dependent manner[J]. *Eur J of Pharmacology*, 2007, 558:43-51.
3. Liu FC, Liao CH, Chang Y, et al. A new insight of anti-platelet effects of sirtinol in platelets aggregation via cyclic AMP phosphodiesterase[J]. *Biochemical Pharmacology*, 2009, 77:1364-1377.
4. Higashi Y, Noma K, Yoshizumi M, Kihara Y. Endothelial function and oxidative stress in cardiovascular diseases[J]. *Circ J*, 2009, 73:411-418.
5. Zhao HW, Li XY. Ginkgolide A,B, and huperzine A inhibit nitric oxide-induced neurotoxicity[J]. *International Immunopharmacology*, 2002, 2:1551-1556.
6. Gadi D, Bnouham M, Aziz M, et al.. Parsley extract inhibits *in vitro* and *ex vivo* platelet aggregation and prolongs bleeding time in rats [J]. *Journal of Ethnopharmacology*, 2009, 125:170-174.
7. Dogne JM, Leval XD, Benoit P, et al. Recent advances in antiplatelet agent[J]. *Current Medicinal Chemistry*, 2002, 9:577-589.
8. Koetzner L, Grover G, Boulet J, Jacoby HI. Plant-derived polysaccharide supplements inhibit dextran sulfate sodium-induced colitis in the rat [J]. *Dig Dis Sci*, 2010, 55(5):1278-1285.
9. Barahona T, Encinas MV, Mansilla A, et al. A sulfated galactan with antioxidant capacity from the green variant of tetrasporic *Gigartina Skottsbergii* (Gigartinales, Rhodophyta). *Carbohydr Res*, 2012, 347(1):114-120.
10. Yang Y, Hu SJ, Li L, Chen GP. Cardioprotection by polysaccharide sulfate against ischemia/reperfusion injury in isolated rat hearts [J]. *Acta Pharmacol Sci*, 2009, 30(1):54-60.
11. Li H, Liu X, Li Y, et al. Effects of the polysaccharide from *pholiota nameko* on human cytokine network in serum[J]. *Int J Bio Macromol*, 2012, 50(1):164-170.
12. Yoon SJ, Yu MA, Pyun YR, et al. The nontoxic mushroom *Auricularia auricula* contains a

- polysaccharide with anticoagulant activity mediated by antithrombin [J]. *Thromb Res*, 2003, 112(3):151-158.
13. Han F, Yao W, Yang X, et al. Experimental study on anticoagulant and antiplatelet aggregation activity of a chemically sulfated marine polysaccharide YCP[J]. *Int J Biol Macromol*, 2005, 36(4):201-207.
  14. Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, 1962, 94: 927-929.
  15. Fan HY, Fu FH, Yang MY (2010). Anti-platelet and antithrombotic activities of salvianolic acid A[J]. *Thrombosis Research* 126: e17-e22.
  16. Jeng JH, Wu HL, Lin BR, et al. Anti-platelet effect of sanguinarine is correlated to calcium mobilization, thromboxane and cAMP production [J]. *Atherosclerosis*. 2007, 191:250-258.
  17. Jackson SP, Schoenwaelder SM. Anti-platelet therapy: in search of the magic bullet[J]. *Nat. Rev. Drug Discov*, 2003, 2:775-789.
  18. Shantsila E, Lip GY, Chong BH. Heparin-induced thrombocytopenia. A contemporary clinical approach to diagnosis and management [J]. *Chest*, 2009, 135(6):1651-1664.
  19. Lee GY, Chang TS, Lee KS, et al. Antiplatelet activity of BRX-018, (6*S*,*cis*)-Malonic acid3-acetoxy-6*a*9-bis-(2-methoxycarbonylacetoxy)-6,6*a*,7,11*b*-tetrahydro-indeno[2,1-*c*]chromen-10-yl ester methylester [J]. *Thrombosis Research*, 2005, 115:309-318.
  20. Scheinitz MD, Heidenreich PA. A cost-effectiveness analysis of combination antiplatelet therapy for high-risk acute coronary syndromes: clopidogrel plus aspirin versus aspirin alone [J]. *Ann Intern Med*, 2005,142:251-259.
  21. Saldeen T, Li DY, Mehta JL. Differential Effects of  $\alpha$ - and  $\gamma$ -Tocopherol on low-density Lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis[J]. *J American College of Cardiology*, 1999, 34:1208-1215.
  22. Xu R, Ye H, Sun Y, et al. Preparation, preliminary characterization, antioxidant, hepatoprotective and antitumor activities of polysaccharides from the flower of tea plant (*Camellia Sinensis*)[J]. *Food Chem Toxicol*, 2011, In Press.
  23. Weber C. Platelets and chemokines in atherosclerosis: partners in crime [J]. *Circ Res*, 2005, 96: 612-616.
  24. Rahman Kh, Billington D. Dietary supplementation with aged garlic extract inhibits ADP-induced platelet aggregation in humans [J]. *Human Nutrition and Metabolism Research Communication*, 2000,130:2662-2665.
  25. Rein D, Paglieroni TG, Pearson DA, Wun T, Schmitz HH, Gosselin R, Keen CL. Cocoa and wine polyphenols modulate platelet activation and function[J]. *Nutrition*, 2000, 130: 2120S-2126S.
  26. Ma FF, Gao Y, Qiao HL, Hu XJ, Chang JB. Antiplatelet activity of 3-butyl-6-bromo-1(3H)-isobenzofuranone on rat platelet aggregation[J]. *J Thromb Thrombolysis*, 2012, 33:64-73.
  27. Brito FCF, Kummerle AE, Lugnier C, Fraga CAM, Barreiro EJ, Miranda A LP. Novel thienylacylhydrazone derivatives inhibit platelet aggregation through cyclic nucleotides modulation and thromboxane A<sub>2</sub> synthesis inhibition[J]. *Eur. J. Pharmacol*, 2010, 638:5-12
  28. Rhee SG, Lee CW, Jhon DY. Phospholipase C isozymes and modulation by cAMP-dependent protein kinase[J]. *Adv Second Messenger Phosphoprotein Res*, 1993, 28:57-64.

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