

Inhibitory Effect of Protocatechuic Acid on Platelet Aggregation Induced by High Shear Stress

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Research

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Abstract

Background Platelet aggregation helps stop bleeding and thrombosis and blocks atherosclerosis, a cause of heart and cerebrovascular disease. Presently, bleeding and drug resistance are the most common side effects of clinically used antiplatelet drugs, thus, it is particularly important to identify new antiplatelet drugs that can avoid or reduce these side effects.

Method In this study, we used microfluidic chips in vitro to simulate the highly narrow bionic blood vessels of atherosclerosis to investigate the platelet aggregation on shear force-induced drug inhibition. Use flow cytometry to detect platelet activation markers (CD62P). And thromboelasmograph and automatic coagulation analyzer were used to detect the coagulation function of blood after the action of protocatechuic acid.

Results we found that platelets strongly aggregated after passing through the highly narrow microfluidic channel, and the aggregation can be inhibited by protocatechuic acid and CD42b but not by aspirin, tirofiban and other drugs. and protocatechuic acid inhibits platelet aggregation mainly by inhibiting interactions between platelet glycoprotein 1b (GPIb) and von Willebrand factor (vWF) but does not activate platelets. It did not increase the expression of CD62P, a platelet activation marker. In addition, protocatechuic acid did not significantly inhibit platelet aggregation induced by other endogenous agonists, such as collagen and ADP. It was found that the inhibitory effect of protocatechuic acid on platelet aggregation did not affect blood production, coagulation time, or coagulation function, reducing the drug resistance and frequent bleeding caused by aspirin.

Conclusion Protocatechuic acid could selectively inhibit high shear force-induced platelet aggregation without affecting blood clotting function. However, the effect of the combination of the Protocatechuic acid and aspirin or other drugs remains to be studied. Therefore, we will conduct further studies on protocatechuic acid for new antithrombotic drugs, which can prevent thrombosis without affecting clotting time.

Introduction

Thrombosis is the leading cause of mortality and morbidity in industrialized countries, accounting for approximately 50% of all non-accidental deaths in the United States every year¹. Most patients affected by these diseases suffer from atherosclerosis, which narrows blood vessels, reduces blood flow, and causes thrombosis². Moreover, atherosclerosis is primarily hemorrhage and leads to thrombosis, which causes fibrous tissue proliferation and calcification and the gradual degeneration and calcification of the

middle layer of the artery, resulting in the thickening of the arterial wall and narrowing of the vascular lumen. Stenosis and post-stenosis low-flow blood flow rates are fast, the blood flow pattern is complex, and the rapid flow produces high shear stress, which has been proven in vitro to activate and aggregate platelets. Shear-induced platelet aggregation has been demonstrated³ by the interaction of the von Willebrand factor (VWF) with the platelet GP Ib-IX-V complex and platelet cross-linking via integrin GPIIb/IIIa¹. The adhesion and homotypic aggregation of platelets at vascular injury sites are the basis for normal hemostasis and pathological thrombosis through the exposure of subendothelial matrix proteins (vascular factors), blood biochemical activators (thrombin, ADP, and TXA₂), and blood flow dependence synergistic signaling by mechanical factors (hemodynamics) occurs with platelet activation and associated aggregation. However, in pathological conditions, once there is parameter imbalance, especially when an atherosclerotic plaque ruptures or high shear force is formed at its pathological stenosis, platelet activation, adhesion, and aggregation occur, which directly leads to pathological thrombosis and the blockage of blood vessels. Moreover, this leads to tissue and organ blood supply disorders, leading to the occurrence of cardiovascular and cerebrovascular diseases and atherosclerotic lesions^{4,5}. At present, the commonly used antiplatelet drugs, such as aspirin and clopidogrel, have been effective in inhibiting thrombosis. However, not all patients benefit from these drugs. Clinical observations have shown that 10% of patients with cardiovascular diseases experience recurring thromboembolism events after long-term use of aspirin⁶. Additionally, an increasing number of clinical reports have stated that an excessive dosage of drugs leads to increased bleeding, such as intracranial hemorrhages and gastrointestinal bleeding. Clinically, aspirin and clopidogrel dosages should be monitored at all times to avoid negative outcomes. Therefore, studies regarding new antiplatelet drugs are urgently needed. The interactions between the platelet membrane glycoprotein Ib (GPIb) receptor and vascular hemophilia factor (VWF) play key roles in platelet adhesion, especially under high shear stress (e.g., shear rate > 1200s⁻¹). Therefore, the GPIb receptor is considered a potential anti-thrombotic target⁷. The study of GPIb receptor antagonists is of great significance for exploring new anti-platelet drugs.

High shear-induced platelet aggregation primarily exists in abnormalities of the blood vessel wall, and normal blood flow does not produce high shear stress on the blood vessel walls. Normal blood flow produces shear stress, usually below 1000s⁻¹, which generally does not cause platelet aggregation. Shear stress induces platelet aggregation in a gradient-dependent manner. The higher the degree of stenosis, the greater the shear stress and the stronger the ability to induce platelet aggregation. When the shear rate exceeds 1000s⁻¹ in normal circulation, the initial platelet count depends on the binding of glycoprotein (GP) Ib to immobilized VWF, even if the extracellular matrix⁸ or vascular structure⁹ exhibit multiple reaction components. Because the development of a thrombus narrows the vascular lumen and increases the shear stress rate of blood flow locally, the continued recruitment of platelets also depends on VWF-GPIb. Hemostasis and arterial thrombosis occur in different hemodynamic environments. The shear stress rate of 20,000–40,000 S⁻¹ in or just upstream of coronary artery stenosis may be 100 times higher than in the absence of an obstruction^{10–12}, and 10 times higher than in microvessels¹³.

In our study, protocatechuic acid was found to have anti-platelet aggregation, especially for platelet aggregation induced by high shear stress due to vascular stenosis. Protocatechuic acid is a natural phenolic acid found in many vegetables and fruits and is an effective active ingredient in many traditional Chinese medicines, such as *Salvia miltiorrhiza*, *Hibiscus*, *Acanthopanax sessiliflora*, and bitter *Phyllanthus*. In the in vitro microfluidic model, protocatechuic acid had obvious anti-platelet aggregation effects induced by high shear stress. Studies have shown that protocatechuic acid inhibits platelet aggregation primarily by inhibiting the binding of the platelet glycoprotein Ib and von Willebrand factor (vWF), and the inhibition is stronger than other anti-platelet drugs at present. Under the same conditions, existing antiplatelet drugs, such as aspirin and Tirofiban hydrochloride, prolonged coagulation time to varying degrees, while protocatechuic acid did not. Therefore, we believe that protocatechuic acid has a better therapeutic potential in anti-platelet aggregation.

Materials And Methods

Materials: protocatechuic acid, PDMS-glass chip, CalceiAm fluorescent dye for sodium citrate vein blood vacuum shearer tube, RSP01-CS two-way push-pull precision injection pump, IXT1 inverted fluorescence microscope, plasma cleaner, CD42b antibody, dimethyl sulfoxide, acetylsalicylic acid, tirofiban, adenosinediphosphate, collagen, thromboelastograph, Kaolin activator, coagulation factor detection kit, Sysmex CA7000 automatic coagulation analyzer and its supporting reagents JAPAN, and FITC-CD62p.

Fabrication of microfluidic chips

The microfluidic chip for platelet adhesion and aggregation analysis consists of narrow microchannels and sample pools on both sides and outlets (Fig. 1). The working principle is that the blood samples are loaded into the sample pool, and the negative pressure generated by the outlet controls the blood sample flow through the microchannels at a set shear rate, and platelet adhesion and aggregation behavior are observed in the microchannels. Microfluidic chips were fabricated by dry film soft lithography¹⁴. The chip mask pattern was designed by Coreldraw 12.0 software and printed on transparent film using an inkjet printer. The two-layer photosensitive dry film (single layer thickness 35 μm) was laminated on a glass plate using a film-covering machine. The photosensitive dry film was irradiated by ultraviolet light through the mask for 50 s, and was developed with 1% sodium carbonate to form the chip mask. The prepolymer mixed at a 10:1 weight was poured onto the dye of the chip, and the bubbles were removed by vacuum evacuation. The prepolymer was cured for 3 h at 60°C. The solidified PDMS substrate was peeled from the photosensitive dry film anode, and the sample pool and outlet were formed by punching with a flat-end puncher (7 and 1.5 mm in diameter). The sample pool and outlet were irreversibly bonded with the clean glass carrier after being treated by an oxygen plasma cleaner (30 W, 1 min) to form a PDMS-glass microfluidic chip, A schematic diagram of the experimental device are shown in Fig. 1A,B.

Blood sample collection

Blood samples were collected from 20 healthy volunteers recruited from the Physical Examination center of Yongchuan Hospital affiliated to Chongqing Medical University from March to June 2019, and 10

volunteer patients with atherosclerosis recruited from the Cardiovascular Medicine department. Selection criteria: Healthy volunteers: no history of medication, operations, or alcoholism within one month, and hematocrit, platelet count, coagulation function (PT, APTT) and thromboelastogram (R, K, Angle, MA, CI) were within the normal reference range. Volunteer patients with atherosclerosis: the patient was not treated surgically at first admission. This study was approved by the Ethics Committee of Yongchuan Hospital affiliated with Chongqing Medical University (No. 2018035), and all subjects provided written informed consent. Venous blood samples were collected using a vacuum, and were anticoagulated with 1:9 (v/v) 3.2% sodium citrate and used as soon as possible. To test the inhibition of platelet adhesion and aggregation, tirofiban hydrochloride of different concentrations was prepared with 10 ml 0.9% sodium chloride solution added to 1 ml blood sample as the drug treatment group, and 10 ml 0.9% sodium chloride solution only was added as the control group. Finally, 1 mmol/L calcein AM fluorescent dyes were added to blood samples at a concentration of 1:500 (v/v). The samples were shaken gently and incubated at 37 °C in an incubator for 15 min. Calcein AM, as a living cell fluorescent dye, penetrates cell membranes and enters the cell. Calcein emits strong green fluorescence after being sheared by intracellular esterase. Therefore, Calcein AM was used to fluorescently label platelets in blood samples.

Detection of platelet aggregation

Microfluidic chips were treated with plasma cleaner (30 W) for 2 min to increase their hydrophilicity. The modified microfluidic chips were placed on the carrier of the inverted fluorescence microscope. The chip outlet was connected to the injection pump with a polytetrafluoroethylene tube (inner diameter 1.0, outer diameter 1.5 mm). The flow shear rate of the blood samples in the microchannel was controlled by the pullback mode. The flow rates were 10, 50, and 100 $\mu\text{l}/\text{min}$, respectively. The relationship between the fluid shear rate and injection pump flow rate in the microchannel was calculated according to Poiseuille's law, i.e., $v=6Q/a^2b$. Among them, V (s^{-1}) represents shear rate, Q ($\mu\text{l}/\text{s}$) represents flow rate, a (mm) represents depth of microchannel, and B (mm) represents width of microchannel. When blood began to flow into the microchannels, Streampix 5.0 software was used to control the camera to record fluorescence-labeled platelet adhesion and aggregation on the collagen surface at a frame rate of 1 frame/s (objective, *20), and 180 fluorescent images were recorded for 3 min. A schematic diagram and photos of the experimental device are shown in Fig. 1.

Detection of platelet activation markers

The AM fluorescent dye-labeled sodium citrate anticoagulated whole blood sample was treated with 2 μl dimethyl sulfoxide, ADP, ADP+PAC, Ristomycin, Ristomycin+PAC, and PAC, and was sequentially collected via a microfluidic chip. The specimen at the exit was fixed in paraformaldehyde solution. The machine was tested within 24 h to detect the expression of platelet CD62p.

Detection of platelet aggregation induced by gradient shear force

AM fluorescein-labeled sodium citrate anticoagulated whole blood samples were divided into three groups, and dimethyl sulfoxide, CD42b, and tirofiban hydrochloride were added, respectively. Then, the

mixtures were incubated at 37°C in darkness for 10 min, and the blood flow rate was adjusted (100 µl/min). When the blood began to flow into the microchannel, Streampix 5.0 software was used to control the camera to record the images of fluorescence-labeled platelet adhesion and aggregation on the collagen surface at a frame rate of 1 frame/s (objective lens, ×20), and a total of 300 frames were recorded for a 5 min sequence of fluorescent images. The degree of Platelet aggregation was then measured in a microfluidic chip device.

Detection of platelet aggregation after inhibitor action

Prepare a certain concentration of inhibitor and 1 ml sodium citrate anticoagulated whole blood samples were treated with aspirin, tirofiban hydrochloride, CD42b, and procatechin acid with AM fluorescent dye, respectively, and were incubated at 37°C for 10 min in darkness. Then, The degree of platelet aggregation was measured in a microfluidic chip device.

Detection of platelet aggregation induced by inducers

Prepare a certain concentration of Inducer and 1 ml sodium citrate anticoagulated whole blood samples were treated with collagen (4µg/ml), ADP (16M), ristomycin (0.4mg/ml) then incubated at 37°C for 10 min in darkness. Then, The degree of platelet aggregation was measured in a microfluidic chip device.

Detection of blood coagulation function after drug action

The protocatechuic acid solution (8 µmol/L) of 2 µl was added into 1 ml whole blood solution and incubated at 37°C for 10 min. The coagulation function was detected by thromboelastograph. And then add 1ml of whole blood into the kaolin activator tube and mix well, take 340µl of whole blood into the thromboelastometer for detection, record the detection time and graph. Secondly, the blood was centrifuged at 1782g for 5 minutes, and the prothrombin time (PT) and activated partial thromboplastin time (APTT) of the plasma were collected and recorded in detail.

Statistical analysis

The recorded data were input using Excel software, and SPSS 19.0 was used for data analysis. The count data were expressed as mean ± calibration difference ($\bar{x} \pm s$). Analysis of variance using completely randomized design data was statistically significant at $P < 0.05$.

Results

Computational fluid dynamics (CFD) model of the chip and the change in shear force inside the model

In order to study the changes of platelets after passing through the narrowed area, we used a microfluidic chip to simulate the narrowed area of atherosclerosis. Studies have shown that platelet aggregation occurs in areas of blood flow disturbance after vascular injury. To study the effect of rheological disorders on platelet aggregation kinetics in vivo, we used microfluidic chips to simulate blood flow in

blood vessels in vitro, and the effect of stenosis on platelet aggregation. We use a fixed flow rate syringe to control the flow rate in the tube. When local stenosis was greater than 80%, the speed and degree of platelet aggregation were significantly accelerated. Without the effects of stenosis, there would be no platelet aggregation. To better understand the relationship between local flow changes and platelet aggregation, we performed CFD modeling of stenotic vessel geometry. The stenosis of the model was fixed at 80% (Fig. 2). Vascular inner wall shear force distribution caused by different stenosis of the blood vessel wall was not the same. The shear force histogram displays red as high and blue as low shear force distribution. In the stenosis model, vascular stenosis and the high-shear region of the proximal stenosis vessel (Fig. 2A) can be seen. The normal low WSS region still appears in the distal stenosis vessel, and there is a fixed shear force near the stenosis. In the gradient change area, WSS showed a gradual decline (Fig. 2). In addition, in the color histogram, red indicates high and blue indicates low blood flow velocity. The cross-section blood flow velocity maps before and after the stenosis and the stenosis itself show that blood flow velocity increases rapidly near the stenosis and stenosis area, and the blood flow velocity at both ends near the stenotic blood vessel shows a gradient decrease. Platelets also gradually aggregate into stable platelet aggregates in areas where shear forces show a gradient.

Gradient shear forces can induce platelet aggregation, and does not activate platelets

To study the effects of changes in shear forces on stenosis vascular on platelet aggregation, we set up blood flow through the narrow and straight channels at a particular speed. The blood flow velocity in the microfluidic chip was controlled to 10, 50, and 100 $\mu\text{l}/\text{min}$, respectively, so that the shear force in the stenosis reached 1000, 5000, and 10000 s^{-1} . The results show (Fig. 3C-D) that when the shear rate is 1000 s^{-1} , there is a small fluorescence intensity scattered in the upstream and downstream of the narrow vessels and in the narrow channels, and less platelet aggregation occurs. When the shear rate increased to 5000 s^{-1} , the stenosis platelet aggregation increased slightly downstream of the channel, and was primarily distributed downstream of the narrow channel, where the shear force gradient changed. When the shear rate increased to 10000 s^{-1} , platelet aggregation downstream of the narrow channel increased significantly, forming a high-brightness aggregate. In the straight channel, regardless of the flow rate, there was no obvious aggregation of the platelets. The results show in Fig. 3A-B. Therefore, we believe that the high shear force caused by vascular stenosis reached a particular level, and the shear force gradient caused platelet aggregation.

To study the effect of shear platelets on platelets themselves, we investigated the activation status of platelets after aggregation. Flow cytometry was used to detect the activation of CD62p before and after platelets passed through the narrow channel, and it was found that the expression of CD62p did not increase significantly after platelet aggregation occurred (Fig. 4). At the same time, the activator ADP significantly increased CD62p content after platelet aggregation (Table 1). The treatment of platelets with protocatechuic acid inhibited platelet aggregation and did not enhance platelet CD62p expression. This shear-induced platelet aggregation did not activate platelets.

Table 1
(Single-factor ANOVA analysis) (n = 10, x ± s)

Different treatment factors	(CD62p%)	<i>p</i>
Control	7.24 ± 2.35	-
ADP	50.23 ± 4.72	0.651
Ristomycin	10.82 ± 2.49	0.537
protocatechuic acid	11.09 ± 2.01	0.000

Protocatechuic acid can inhibit platelet aggregation induced by high shear force, and primarily inhibits the interaction between GP1b- α and vWF

To study the effect of protocatechuic acid on platelet aggregation, we formulated protocatechuic acid to a certain concentration to treat blood, respectively, 1 $\mu\text{g/ml}$, 2 $\mu\text{g/ml}$, 4 $\mu\text{g/ml}$, 8 $\mu\text{g/ml}$, 16 $\mu\text{g/ml}$. Then through the narrow channel it was found that when procatechin concentration was 8 $\mu\text{g/ml}$, the degree of platelet aggregation has been significantly weakened, and it is believed that the lowest concentration of the optimal platelet aggregation concentration was reached, it was shown in Fig. 5A. At the same time, we combined protocatechuic acid with different inhibitors and found that in the group with protocatechuic drugs, Platelet aggregation was significantly reduced, and similar results were obtained with the addition of CD42b. When combined with CD42b, the effect of inhibiting platelet aggregation was improved; therefore, we considered the additive effect of the two drugs (Fig. 5B). To study the mechanism by which protocatechuic acid inhibits platelet aggregation, we investigated the effects of protocatechuic acid on agonists ADP, collagen, and ristomycin-induced platelet aggregation through and in the narrow channels, respectively. It was found that protocatechuic acid did not inhibit platelet aggregation induced by ADP but could inhibit platelet aggregation induced by ristomycin (Fig. 5B). Ristomycin primarily induces the combination of GP1b- α and vWF to cause platelet aggregation. Protocatechuic acid can inhibit this mechanism. We believe that protocatechuic acid also inhibits platelet aggregation by inhibiting this pathway.

To study the mechanism of high-shear-induced platelet aggregation, we treated platelets with different inhibitors and observed platelet aggregation. We utilized GP1b antibody CD42b, GPIIb/IIIa receptor antagonists tirofiban, and aspirin, respectively, to treat whole blood, and used microfluidic chips to detect platelet aggregation after passing through the narrow channels. This study found that platelet aggregation significantly reduced after blood passed through the narrow channel after treatment with CD42b, while platelets still aggregated after tirofiban- and aspirin-treated blood passed through the narrow channel. Aspirin and tirofiban were used in combination with CD42b, showed an inhibitory effect similar to CD42b. Platelets without CD42b-added inhibitors still aggregated. It was found that CD42b can inhibit platelet aggregation induced by high shear force, but tirofiban cannot. CD42b mainly blocks GP1b- α , thereby inhibiting the binding of GP1b- α to vWF and preventing the first phase response of platelet

aggregation. In this study, the addition of CD42b inhibited platelet aggregation, indicating that this high shear-induced platelet aggregation, for the same aggregation mechanism, that the change of shear force induces the combination of GP1b- α and vWF to induce platelet aggregation. The pharmacological effects of Tirofiban competitively inhibits the binding of fibrinogen and platelet GPIIb/IIIa receptors after platelet activation, thereby inhibiting platelet aggregation (Fig. 4). However, in this study, platelet aggregation was not inhibited, which may also prove that platelets did not activate during the process of aggregation and release the corresponding receptors.

Protocatechuic acid has no effect on the hemagglutination function

In order to study whether protocatechin has an effect on blood coagulation function, we tested the blood coagulation function after drug action. First prepare a certain concentration of protocatechuic acid solution, 1 $\mu\text{g/ml}$, 2 $\mu\text{g/ml}$, 4 $\mu\text{g/ml}$, 8 $\mu\text{g/ml}$, 16 $\mu\text{g/ml}$, then added 2 μl into whole blood for thromboelastography test and coagulation function test. It was found that different concentrations of protocatechuic acid solution had no effect on the functions of thromboelastography results, nor did it affect the platelet aggregation function; Similarly, the thrombin time PT and APTT measured in plasma taken after centrifugation were not significantly abnormal. Therefore, we believed that the use of protocatechuic acid did not affect the blood clotting function.

Table 2
The effects of different concentrations on the coagulation function of samples

The concentration of PCA	PT	APTT	<i>P</i>
0	12 \pm 1.04	36 \pm 5.36	0.378
1	11 \pm 0.87	38 \pm 6.48	0.521
2	12 \pm 1.41	34 \pm 4.98	0.286
4	10 \pm 0.97	35 \pm 6.59	0.378
8	11 \pm 1.51	35 \pm 6.21	0.495
16	12 \pm 0.86	37 \pm 4.20	0.542

Discussion

Pathological atherosclerotic arterial thrombosis is mediated by platelets and depends on the local fluid environment¹⁵⁻¹⁸. On this basis, platelet thrombosis is an important cause of cardiovascular disease, and platelets play an important role in the formation of atherosclerotic thrombosis. Platelet aggregation plays a key role in the normal coagulation mechanism. Platelets participate in thrombogenesis through adhesion, aggregation, and release. Therefore, anti-platelet aggregation drugs play an important role in the treatment of thrombotic diseases. When shear stress is high, such as thrombosis in stenotic arteries, the platelet glycoprotein (GP) Ib-IX-V complex binds to the vascular von Willebrand factor (vWF) fixed on

exposed subendothelial membranes of vessels damaged by angioplasty or atherosclerotic plaque rupture^{16,19-21}. The integrin GP IIb-IIIa complex plays a role in platelet adhesion to the subendothelial matrix²². The mural thrombosis is primarily formed under the exposed artery endothelium through two steps of platelet adhesion and aggregation. The high shear stress found in stenosed arteries was mediated by the von Willebrand factor (vWF). Through the glycoprotein (GP) Ib-IX-V complex, platelets initially adhere to the vWF of the subendothelial matrix. At present, the most commonly used anti-platelet drugs are primarily aimed at antagonizing each link in the formation of platelet thrombosis and exerting the function of inhibiting platelet aggregation. For example, aspirin inhibits the synthesis of thromboxane A₂ by inhibiting cyclooxygenase (COX-1), thereby inhibiting platelet adhesion activity; clopidogrel and others play an anti-platelet aggregation role by inhibiting platelet membrane ADP receptor; and tirofiban and others inhibit platelet aggregation by antagonizing the platelet membrane GPIIb/IIA receptor. Some traditional antiplatelet drugs play an important role in anti-thrombosis. However, the problems of drug resistance and bleeding events reported in recent years (Yende and Wunderink, 2001; Chen, 2004) are also increasing. GP IIb/IIIa receptor inhibitors have strong anti-platelet activity and relatively small effects on the vascular system, thus, they have received special attention. However, the risk of bleeding appears to be proportional to the effect of anti-platelet activity (Lefkovits et al, 1995; Scarborough et al., 1999). Therefore, the traditional antiplatelet drugs still cannot fully meet clinical needs, and the search for new, safe, effective drugs that can avoid adverse reactions to a certain extent continues. Compared to these traditional antiplatelet drugs, protocatechuic acid inhibits platelet aggregation by antagonizing the interaction between platelet membrane glycoprotein GPIb receptor and VWF. Platelet GPIb-IX-V complex induces pathological arterial thrombosis by binding vWF fixed on the intima of bare blood vessels. Previous studies have shown that this initial step of blocking platelet adhesion reduces thrombosis and the long-term restenosis of injured stenosed vessels^{23,24}. In this study, protocatechuic acid is derived from a phenolic acid in many vegetables and fruits and is an active component in many traditional Chinese medicines. It has prominent anti-condyloma acuminatum and anti-thrombosis activity by selectively inhibiting shear-induced platelet activation and aggregation. In human whole blood platelets, protocatechuic acid reduces platelet activation induced by high shear stress by blocking the interaction between vWF and platelet receptor GP Ib. Antithrombotic activity was demonstrated in the stenosed vessels simulated by microfluidic chips in vitro. Compared to the traditional antiplatelet drugs aspirin and tirofiban, the blood coagulation function was not significantly prolonged. This study suggests that protocatechuic acid inhibits platelet aggregation by inhibiting the adhesion of the glycoprotein (GP) Ib-IX-V complex to vWF. However, the mechanism that is adopted remains to be further studied.

In this study, we observed in the in vitro blood vessels simulated by the microfluidic chip that the stenosis of the vessel wall has a great effect on the blood flow velocity in the vessel, and the shear force distribution of the inner wall of the vessel caused by different degrees of stenosis is not the same. Figure 2 shows that the blood flows into and out of the stenosis through a region of shear force with a gradient increase then decrease. In the stenosis, a fixed area of high shear force was present, but no large amounts of platelet aggregation were found. Therefore, in the absence of changes in the shear force of platelets, platelets do not aggregate, even under conditions of high shear forces. Moreover, the present

study found that platelet aggregation primarily occurs downstream of the narrow channel, and platelets gradually aggregate into stable platelet aggregates in this area. Figure 2 shows that there is a gradient-decreasing shear force variation zone downstream, and it is the decrease of this gradient that leads to platelet aggregation. In summary, this study shows that local shear changes, especially the shear deceleration stage, play a major role in enhancing platelet aggregation, which is also consistent with previous study results.

Protocatechuic acid is a water-soluble phenolic acid component²⁵⁻²⁸ that naturally exists in many foods and traditional Chinese medicines. It is also an active substance of many traditional Chinese medicines. It not only has pharmacological activities, such as anti-platelet agglutination, reducing myocardial oxygen consumption, improving myocardial oxygen tolerance, slowing heart rate, bacteriostasis, and analgesia²⁹⁻³¹, but also has antioxidant activities and tumor and neuroprotective effects^{32,33}. For example, some studies have found that protocatechuic acid has anti-inflammatory, bacteriostasis^{34,35}, anti-oxidation³⁶, and anti-tumor effects³⁷, and plays a role in the protection of cerebral hemorrhage³⁸. Therefore, protocatechuic acid is not only the active ingredient that exists in vegetables and fruits, but is also studied as a potential new drug, which could make a significant contribution to the future development of medicine. Our research regarding protocatechuic acid will continue. Protocatechuic acid, as a natural component of Chinese herbal medicine, is also one of the contents of this drug in terms of its metabolic mode, pathway, and mechanism in vivo. However, there have been few studies regarding protocatechuic acid metabolism in vivo³⁹. Chen Jiaquan et al.⁴⁰ studied the metabolic forms of protocatechuic acid in vivo. It was found that the main metabolic pathways of protocatechuic acid were the combination of hydroxyl groups with sulfuric acid and glucuronic acid, and the formation of O-methylation conjugates. There were some differences in metabolites detected in different parts of rats. The metabolites in plasma and urine were abundant and were not excreted in the original form of feces.

In this study, we found that only a certain degree of vascular stenosis can cause platelet aggregation, and the higher the degree of stenosis, the more obvious the platelet aggregation, while the straight channel did not cause platelet aggregation (Figure.A.B). Besides we found that protocatechuic acid has a highly selective inhibitory effect on platelet aggregation induced by vascular stenosis, but no obvious inhibitory effect on that induced by the platelet activator ADP(Fig. 5B, Fig. 6). The difference between shear- and agonist-induced platelet aggregation is currently unknown, as is the manner in which shear force induces platelet aggregation. Previous studies have shown that shear stress inducement differs from the mechanism of chemical inducers^{41,42}. Various inducers interact with the corresponding receptors on the platelet membrane, activate platelets through membrane transfer, activate another receptor, glycoprotein GP IIb/IIIa, on the membrane surface, and mediate platelet aggregation through fibrinogen bridging in plasma. Although this type of aggregation is also accomplished by stirring at a particular speed, the shear action is small and irregular, and some studies have shown that this type of aggregation cannot resist higher shear action, which is easy to dissociate under the action of shear force of blood flow, and cannot form a solid local thrombus. This means that in traditional aggregators, platelet aggregation may not exist in the arterial blood flow, especially in the case of high vascular stenosis or spasm, which often

results in abnormally high shear stress. Current studies have shown that shear-induced platelet aggregation primarily depends on the interaction between plasma vWF and GP1b/XI, a functional platelet receptor complex, rather than on fibrinogen in plasma. However, in the normal circulatory system, vWF does not spontaneously bind to the GP Ib-IX-V complex. Only when high shear stress occurs in vivo does the vWF interact with the exposed subendothelial matrix, which may be due to the activation of conformational changes in the vWF A1 region ⁴¹. In addition, the shear-dependent aggregation of platelets in the liquid phase may be initiated by the combination of the vWF and GP Ib-IX-V complex, which depends on the conformational changes of the vWF and GPIb-IX-V complexes ⁴². Configuration, therefore, GPIb/IX, as a vWF receptor on the platelet membrane first binds to GPIb/IX under the influence of high shear stress to promote platelet aggregation to form a preliminary thrombus. By blocking the binding of GP1b/IX to vWF by adding CD42b, we found that platelet aggregation significantly reduced, while adding tirofiban, a GP IIb/IIIa receptor inhibitor, did not affect platelet aggregation. Therefore, we speculate that the mechanism of protocatechuic acid on platelet aggregation induced by shear stress may be a combination of GP1b/IX or vWF, blocking the interaction between GP1b/IX and vWF, and, secondly, it may also be the protective effect of protocatechuic acid on platelet structure, resulting in platelet glycoprotein binding. The structure of the platelet GP Ib is more stable, which prevents the change to platelet GPIb structure caused by high shear stress. In addition, in recent years, an increasing number of protective effects of protocatechuic acid on the body, such as the effect of intracranial hemorrhage in mice ³⁸ and of the blood-spinal barrier hemorrhage in rats ⁴³, have been identified. However, how to inhibit this process remains to be studied.

In conclusion, in the present study, we used microfluidic chip technology to analyze and detect the effects of traditional antiplatelet drugs and protocatechuic acid on platelet adhesion and aggregation under physiological and pathological conditions in vitro. The results showed that traditional antiplatelet drugs had no obvious effect on platelet aggregation induced by high shear stress. Protocatechuic acid could selectively and effectively inhibit platelet aggregation induced by high shear stress by inhibiting vWF-GP Ib interaction and, thus, had an obvious antithrombotic effect. It does not interfere with normal blood coagulation function. Therefore, protocatechuic acid is a new research direction for antithrombotic drugs. We will use the microfluidic chip method to combine the original catechuic acid medication to attempt to utilize antithrombotic drugs and prevent complications of arterial thrombosis to achieve personalized analysis.

List Of Abbreviations

protocatechuic acidPAC

glycoprotein IbGP(1b)

von Willebrand factor VWF

adenosinediphosphate..... ADP

Declarations

There are couplers of results in this paper may be found interested by the general readers. 1. Computational fluid dynamics (CFD) model of the chip and the change in shear force inside the model; 2. Gradient shear forces can induce platelet aggregation, and does not activate platelets; 3. Protocatechuic acid can inhibit platelet aggregation induced by high shear force, and primarily inhibits the interaction between GP1b- α and vWF; 4. Protocatechuic acid has no effect on the hemagglutination function. They are closely related to the content.

Our manuscript has been Ethics approved and agreed, all data and materials in the article are truly usable, The authors have no conflict of interest. This work was funded by the project "Clinical Application Verification of Microfluidic Chips for Platelet Adhesion and Aggregation" in Yongchuan Hospital Affiliated to Chongqing Medical University.

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Figures

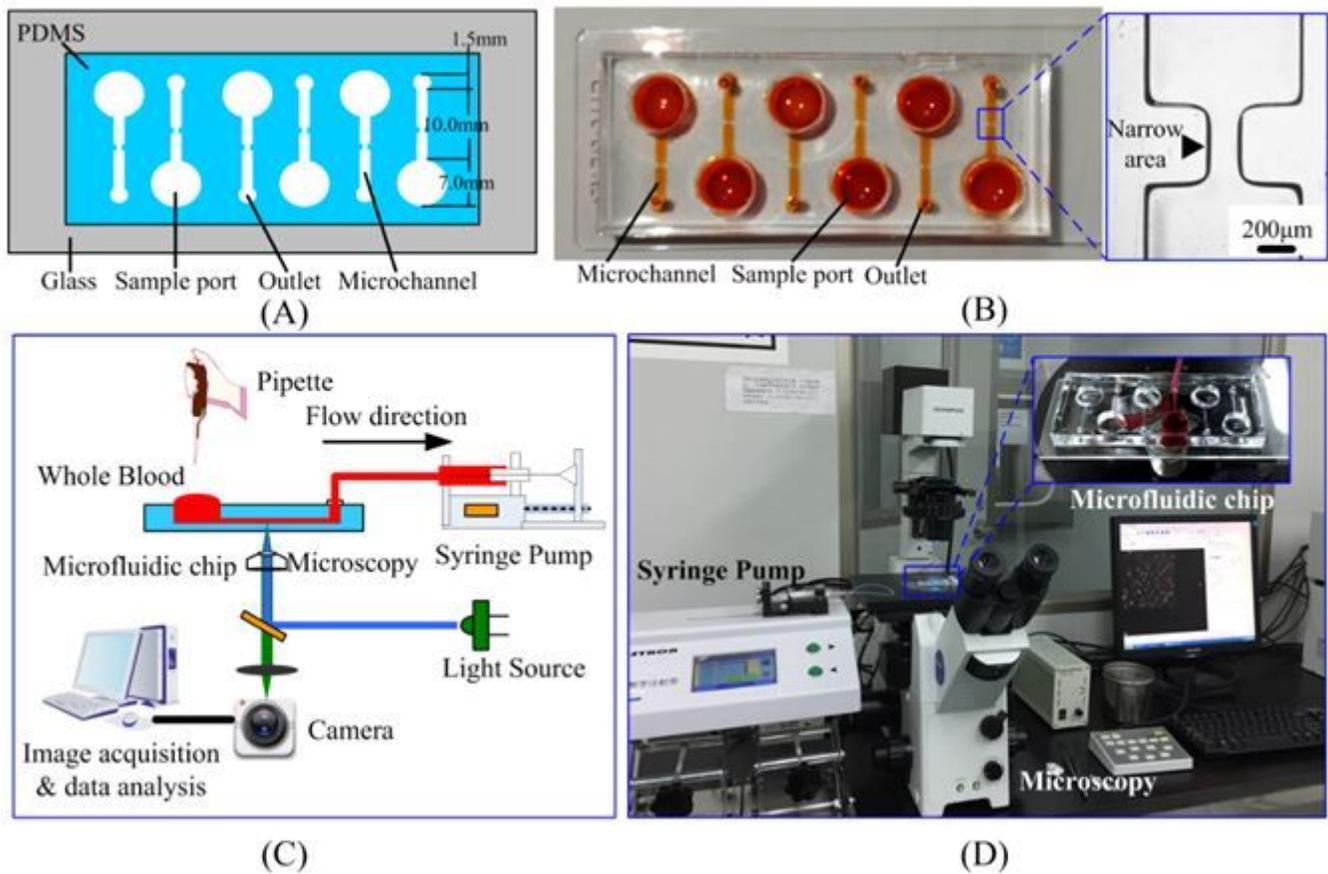


Figure 1

A, Microfluidic chip schematic; B, physical chart of the microfluidic chip; C, schematic diagram of the experimental device; D, photos of the experimental device.

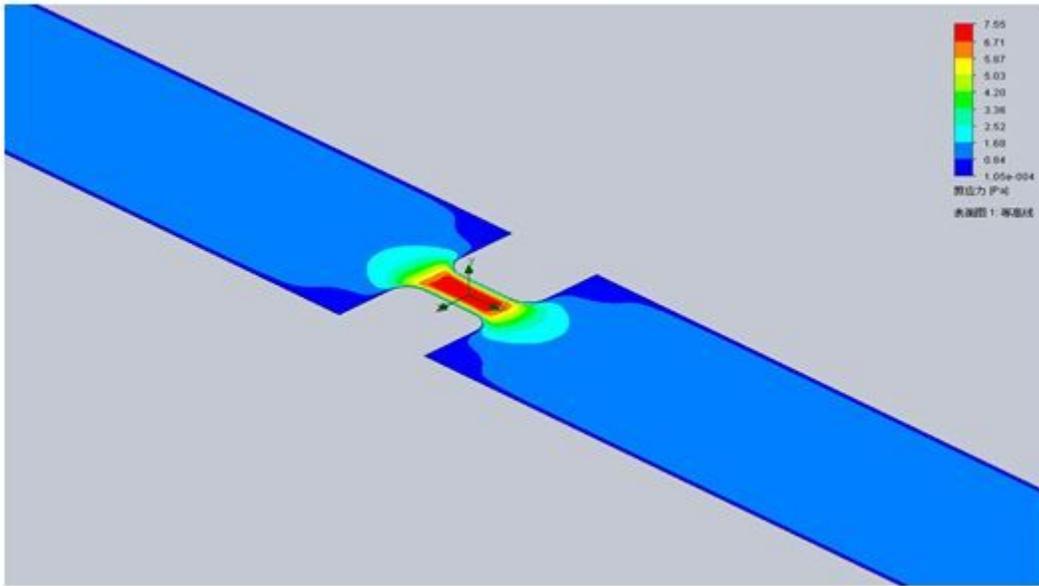


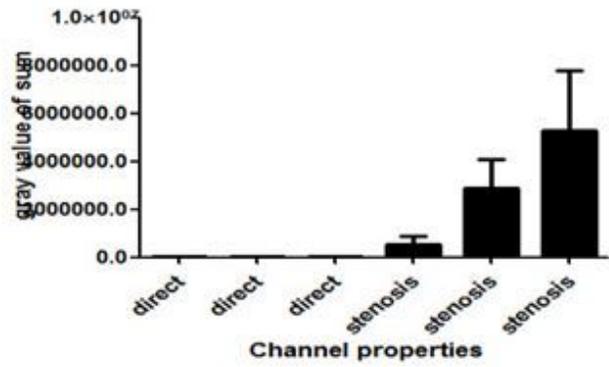
Figure 2

Simulation model of the narrow channel. Shear force distribution on the inner wall of the channel. An abnormally high shear force can be observed. Near the narrow section, the shear force presents a constant declining gradient.

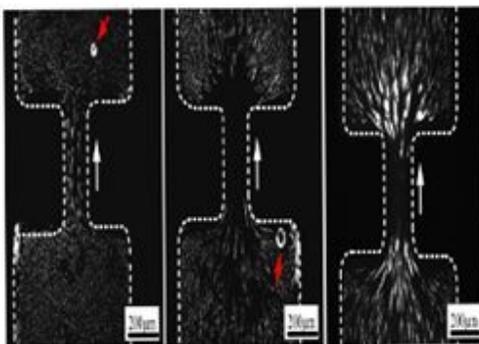


10 μL/min for 300s 50 μL/min for 300s 100 μL/min for 300s

A

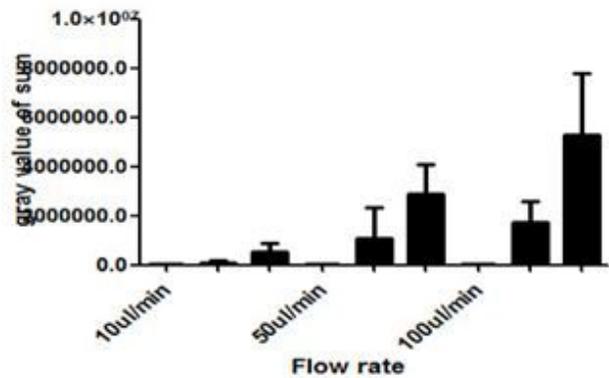


B



10 μL/min for 300s 50 μL/min for 300s 100 μL/min for 130s

C



D

Figure 3

A, Platelet aggregation through straight channel, no obvious platelet aggregation; microscopic images; B, platelet aggregation through straight channel; C, platelets at different flow rates through microfluidic bionic vessels, platelet aggregation, microscopic images; D, platelets at different flow rates through micro flow-controlled bionic blood vessels, platelet aggregation.

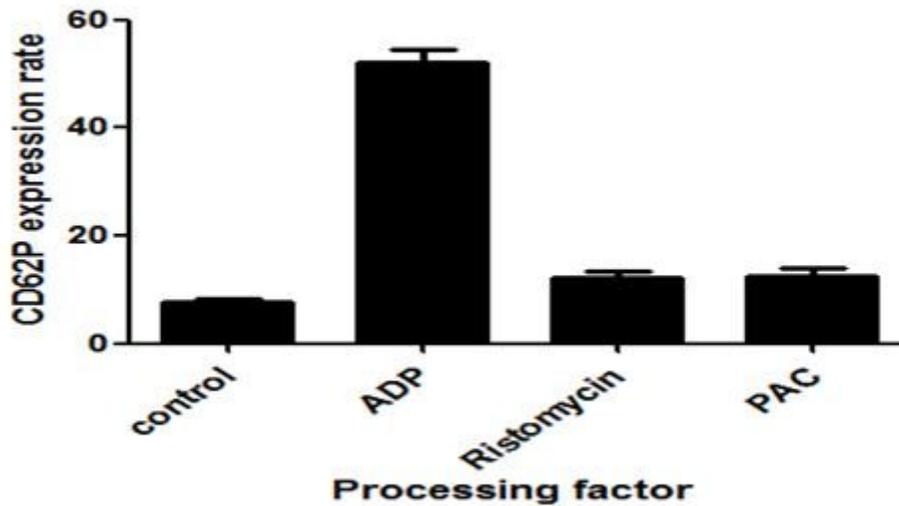


Figure 4

The expression of CD62p after platelets were treated by different factors; after ADP induction, platelets were significantly activated but not by high shearing force, and were treated with PCA and platelet activation was not enhanced.

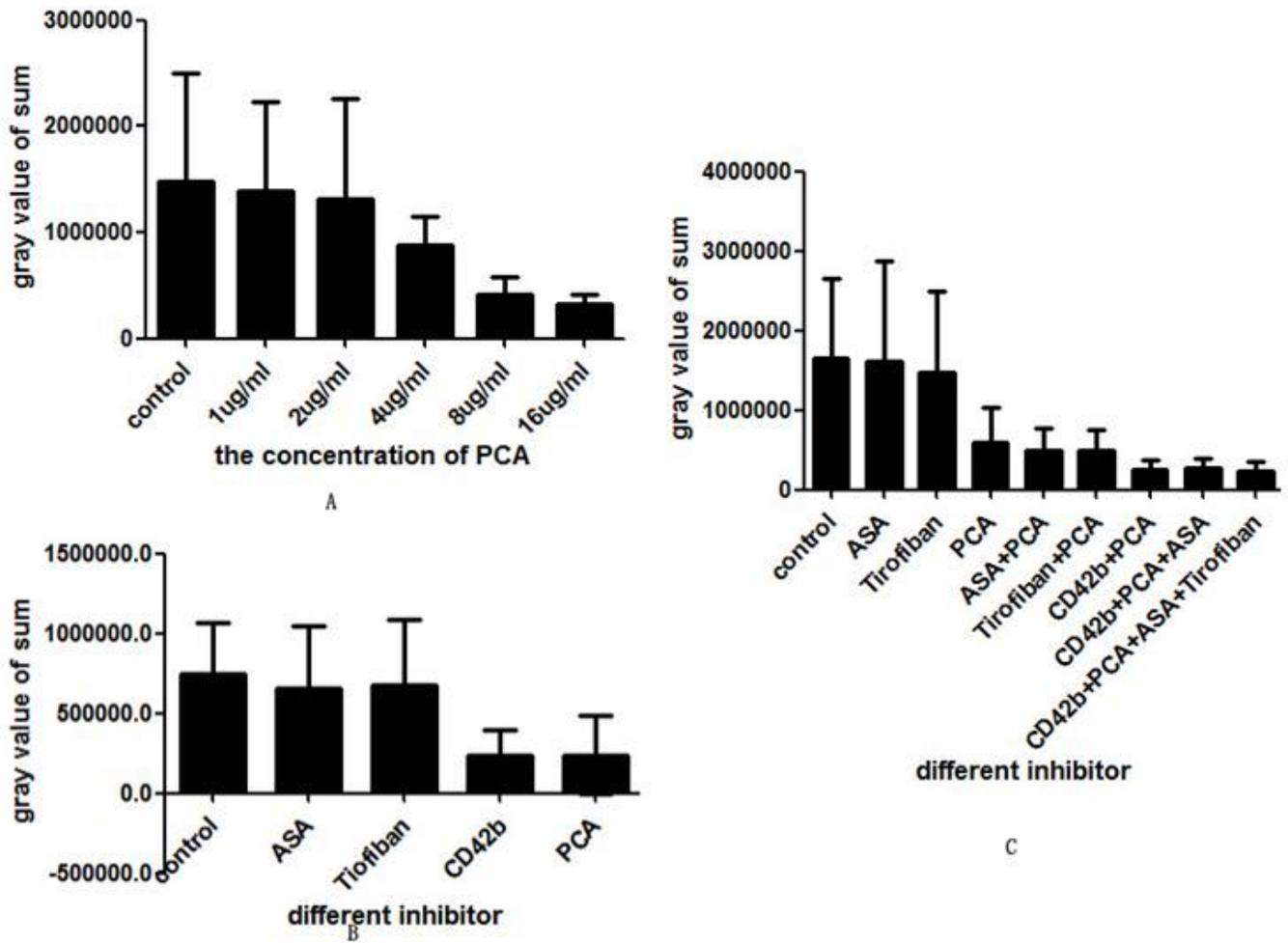


Figure 5

A, inhibits platelet aggregation at different concentrations of PCA. When the PCA concentration is at 8 ug/ml, significant inhibition has occurred. B. The effect of different inducers on the microfluidic bionic angiogenesis of the sample was found to have the same inhibitory effect on 8 ug/ml PCA and CD42b. C. Combination of different inhibitors to inhibit platelet aggregation.

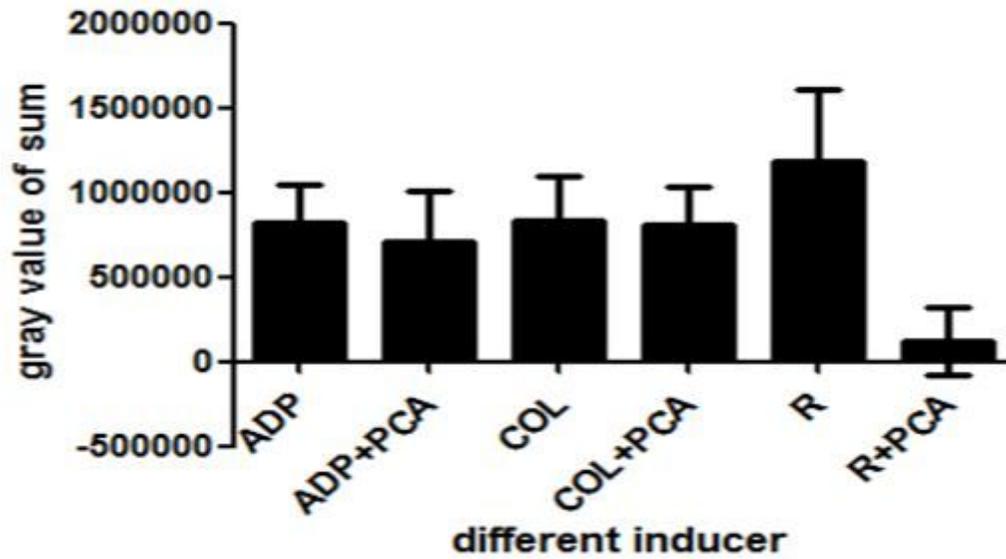


Figure 6

A. Different inducers induce platelet aggregation and PCA inhibits platelet aggregation induced by each inducer. PCA has a significant inhibitory effect on ristocetin-induced aggregation;

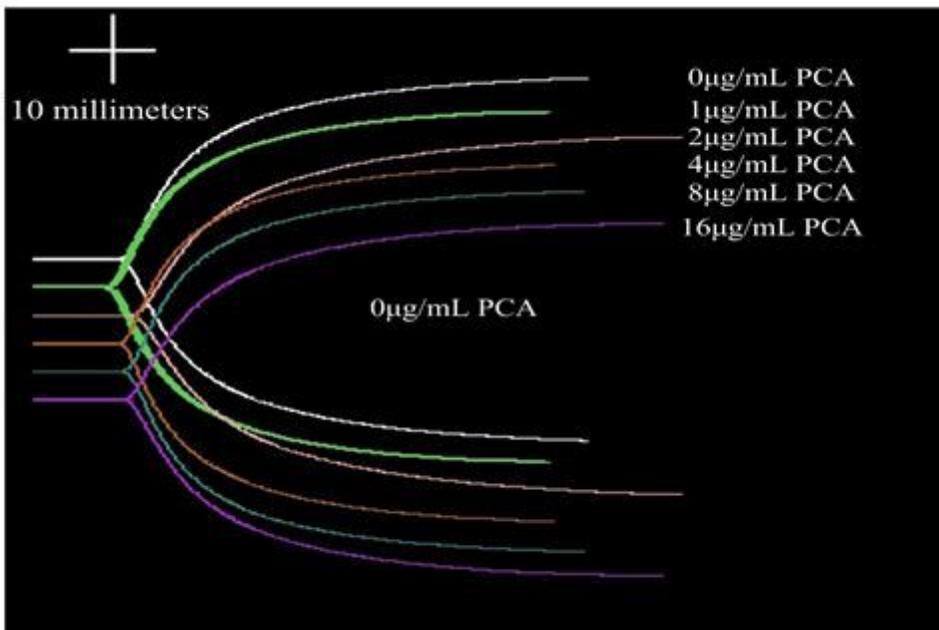


Figure 7

The effect of different concentrations on the results of the thrombus elasticity diagram of samples. There was no significant difference in the effects of various concentrations.