Research Paper

Impact of Xuefu Zhuyu Decoction on Reducing Hyporesponsiveness of Aspirin in Acute Coronary Syndrome

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The main objective of this study is to evaluate the effect of Xuefu Zhuyu decoction on aspirin resistance in acute coronary syndrome patients. We enrolled 45 patients with aspirin resistance and divided them randomly into control group (group A) and treatment group (group B). We applied Xuefu Zhuyu decoction to the group B for 4 w. Before treatment and after 4 w of treatment, we evaluated the platelet reactivity, and performed biochemical and molecular biology analyses from both subgroups. Through logistic regression analysis, we assessed the association between improvement of platelet function and confounding factors. Lastly, clinical follow-ups for 1 mo and 12 mo were performed to analysis the therapeutic effects. We found the aspirin reaction unit was clearly on decline 602.35±26.49 *vs.* 542.48±43.09 (before treatment *vs.* after treatment; p<0.001) in group B. Through quantitative reverse transcription-polymerase chain reaction and radioimmunoassay, we assessed the expression of cyclooxygenase-1 and thromboxane A2, and the results indicated that after treatment, the thromboxane A2 decreased 503.4±12.0 *vs.* 447.8±48.5 (before treatment *vs.* after treatment; p<0.001). In group B, hemorheology improved significantly, and plasma viscosity, whole blood viscosity along with fibrinogen decreased significantly (all p<0.01). Meanwhile, in group B, the glucose was correlated with aspirin reaction unit and the expression of thromboxane A2 was a protective factor of aspirin reaction unit, and aspirin resistance patients would suffer less major adverse cardiac events (p=0.024). Xuefu Zhuyu decoction might reduce hyporesponsiveness of aspirin by inhibiting thromboxane A2 and improve the hemorheological index, which are of great significance for the prevention in cardiovascular events.

Key words: Aspirin, platelet function, Xuefu Zhuyu decoction, coronary heart disease, cardiovascular events

Many researchers believe that thrombosis caused by platelet activation and aggregation after rupture and bleeding of atherosclerotic plaque is the main mechanism in atherosclerotic disease, especially in Acute Coronary Syndrome (ACS). Antiplatelet therapy is the main treatment in Coronary Artery Disease (CAD), especially after Percutaneous Coronary Intervention (PCI). It can prevent platelet aggregation and thrombosis, and was used for the primary and secondary prevention of cardiovascular disease[1].

Aspirin can prevent the conversion of arachidonic acid to Thromboxane A2 (TXA2), which is a main metabolite of prostaglandin synthesis by inhibiting Cyclooxygenase (COX)[2]. Taking aspirin (75-150) mg every day can inhibit the biosynthesis of thromboxane, inhibit platelet aggregation and reduce the risk of cardiovascular disease. However, there are differences in individual responses to antiplatelet therapy. Aspirin cannot always prevent the formation of TXA2 or completely inhibit the activation of COX receptor on platelets. Therefore, some patients undergoing antiplatelet therapy will still experience thrombotic events[3], which we refer as Aspirin Resistance (AR) or aspirin hyporesponsiveness. AR refers to the failure of aspirin to achieve the expected pharmacological effect, for instance, inhibition of platelet activity or prevention of ischemic events[4,5].

Currently for the clinical prevention of AR, modern medicine often applies other drugs or doubles the dose of aspirin, but there is a great risk

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of bleeding, and changing to other drugs also has the possibility of drug resistance. Since the early 1970s, activating blood circulation to dissipate blood stasis has become the most widely used and deeply studied Traditional Chinese Medicine (TCM) treatment principle for the prevention and treatment of CAD. Wang Qingren, a famous doctor in the Qing Dynasty, created 5 prescriptions for promoting blood circulation and removing blood stasis, among which Xuefu Zhuyu decoction is the classic agent. Our latest TCM study published abroad found that Xuefu Zhuyu decoction may effectively improve clopidogrel resistance[6]. Therefore, we wonder that Xuefu Zhuyu decoction may also improve the low response of aspirin. Hence, in present research, our group attempted to assess the effect of Xuefu Zhuyu decoction on AR as well as the effect of TCM on coronary heart disease.

MATERIALS AND METHODS

Study population:

In this study, the used samples represent a clinical cohort subgroup[7]. In brief, a set of patients with AR, seeking medical advice at The First Affiliated Hospital of Ningbo University, were enrolled from 2013 to 2018. Of these 258 patients, our group randomly chose 45 AR patients and randomly divided them into group A (control group, 22 patients) and group B (treatment group, 23 patients). The clinical phenotype data related to the above patients, including coexisting diseases, age, sex, smoking and alcohol abuse were collected. In both groups, the AR patients went on with conventional Dual Antiplatelet Treatment (DAPT) with aspirin (Bayer Medical and Health Care Co., Ltd, specification: 100 mg) and clopidogrel (Sanofi Co., Ltd, specification: 75 mg) daily. We administered group B with Xuefu Zhuyu decoction (twice daily) lasting for 4 w. During this period, the included samples did not take anticoagulant medicine (such as low-molecular weight heparins or rivaroxaban) or any other herbs, which might help in activating blood circulation to eliminate blood stasis.

After aspirin treatment for 1 mo, we performed platelet function measurements at that timepoint because of platelet activity stability. Before and after 4 w of treatment with Xuefu Zhuyu decoction, we evaluated the platelet reactivity and performed biochemical and molecular biology analyses by collecting peripheral venous blood in these 2 subgroups. The clinical follow-ups for 1 mo and 12 mo were performed to analyse the therapeutic effects of Xuefu Zhuyu decoction in AR patients.

Preparation of Xuefu Zhuyu decoction:

The Xuefu Zhuyu decoction (also called Xuefu Zhuyu Tang) is composed of many Chinese herbs or ingredients, including Honghua (*Carthami flos*) 6 g, Taoren (*Semen persicae*) 10 g, Chuanxiong (Rhizoma *Ligusticum chuanxiong*) 9 g, Danggui (Radix *Angelica sinensis*) 9 g, Sheng di huang (Radix *Rehmannia*) 10 g, Chaihu (Radix *Bupleurum*) 6 g, Zhike (*Fructus aurantii*) 6 g, Niuxi (Radix *Achyranthes bidentata*) 9 g, Chishao (Radix *Paeoniae rubra*) 9 g, Jiegeng (Radix *Platycodon*) 5 g, and Gancao (Radix *Glycyrrhiza*) 5 g. These mentioned medicinal ingredients and herbs are purchased from Ningbo Mingbei Chinese Medicine Co., Ltd and decocted (twice daily, 45 min every time) in about 550 ml water for oral application[8].

Platelet function measurements:

Dual antiplatelet therapy (aspirin+clopidogrel) was conducted for 1 mo. The VerifyNow® P2Y12 system was operated to detect the platelet function of patients and analyze the different reactivities of aspirin. The VerifyNow® P2Y12 analyzer is a whole blood bedside detection system. Through the expression of optical signals, a VerifyNow® test value >550 Aspirin Reaction Units (ARU) was defined as AR[9].

Hemorheological analysis:

Patients were instructed to avoid strenuous exercise and labour before blood collection. Generally, patients were required to rest for 15 min before blood collection. 5 ml of pre-elbow venous blood (heparin anticoagulant tube) was collected on an empty stomach. The clinical hemorheological examination items mainly include whole blood viscosity; plasma viscosity and erythrocyte deformability are observed.

Expressions of Cyclooxygenase-1 (COX-1) and TXA2:

We evaluated COX-1 messenger Ribonucleic Acid (mRNA) expression through quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), which is a key molecule of aspirin's

antiplatelet effect. Since the peripheral blood samples were collected before and after treatment, Peripheral Blood Mononuclear Cells (PBMC) was isolated and the total RNA was extracted using the RNeasy Plus Universal Kit (Qiagen). We synthesize complementary Deoxyribonucleic Acid (cDNA) with PrimeScript™ RT reagent kit and genomic DNA (gDNA) Eraser (TaKaRa Bio, Kusatsu, Japan). Then 1 µg RNA was applied and template cDNAs were diluted by 1:4. Lastly, we quantify the relative expression of COX-1 through Applied Biosystems (ABI) 7500 qRT-PCR System (Applied Biosystems, Foster City, California). Here, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was chosen for normalization. The primers for qRT-PCR amplification were designed by Premier 5 software (Table 1). After measuring the samples in triplicate, we calculated the average value of COX-1 mRNA expression by the relative quantification method.

Since TXA2 was unstable *in vivo* and difficult to determine directly, the concentrations of Thromboxane B2 (TXB2, which is a stable metabolite of TXA2 in plasma) were analyzed by radioimmunoassay. The concentrations of TXB2 were used as indicators to judge the contents of TXA2, and the kit was provided by Beijing North Institute of Biotechnology.

Statistical analysis:

We adopted Statistical Package for Social Sciences (SPSS), version 20.0 software for complete series of statistical analysis. Continuous variables were expressed as mean±standard deviation and t-test was used for inter-group comparison. Categorical variables were expressed as percentages and Fisher's exact test or Chi-square (χ^2) test were used for comparison. Logistic regression was used to test the interaction of confounding variables on the effect of Xuefu Zhuyu decoction. A two-sided $p<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

From 2013 to 2018, through platelet function evaluation by VerifyNow® P2Y12, 63 ACS patients whose ARU were >550 were defined as the existence of AR. Among these patients, 45 persons who met the requirements[10] were recruited for present investigation. These patients were divided randomly into control group (group A, 22 patients) and treatment group (group B, 23 patients). As shown in Table 2, the patients' baseline characteristics like coexisting diseases, age, sex, smoking and alcohol abuse, etc. were summarized. All demographic and clinical data were well-matched.

TABLE 1: PRIMERS OF qRT-PCR AMPLIFICATION FOR COX-1 mRNA ANALYSIS

Name	Group	Base sequence (5'→3')
COX-1	Forward primer	CTCCCAGGAGTACAGCTACGA
	Reverse primer	CCAGCAATCTGGCGAGAGA
GAPDH	Forward primer	GGACCTGACCTGCCGTCTA
	Reverse primer	AGGAGTGGGTGTCGCTGT

TABLE 2: BASELINE CHARACTERISTICS OF PATIENTS

Characteristics	Group A (n=22)	Group B (n=23)	t/χ^2	p
Male, n (%)	13 (59.1)	14 (60.9)	0.015	0.903
Hypertension, n (%)	14 (63.6)	12 (48.0)	1.158	0.282
Diabetes mellitus, n (%)	7 (31.8)	5 (21.7)	0.584	0.445
Dyslipidemia, n (%)	9 (40.8)	13 (56.5)	1.097	0.295
Current smoking, n (%)	7 (31.8)	6 (26.1)	0.18	0.672
Alcohol abuse, n (%)	6 (27.3)	5 (21.7)	0.186	0.666
Age (years)	62.36±9.27	61.83±6.55	0.226	0.823
Body mass index (kg/m^2)	24.53±2.13	24.58±1.83	-0.086	0.932
ARU	599.27±34.24	602.35±26.49	-0.187	0.853
COX-1	0.65±0.02	0.65±0.02	-0.323	0.748

TXA2 (ng/l)	503.7±11.7	503.4±12.0	-1.139	0.261
Plasma viscosity (mPa·s)	2.04±0.10	2.02±0.14	0.519	0.606
Whole blood viscosity (mPa·s)	5.09±0.22	5.07±0.15	0.305	0.762
Fibrinogen (g/l)	3.89±0.21	3.95±0.21	-0.96	0.342
Hematocrit (%)	50.95±3.17	50.27±2.90	0.744	0.461
Vascular lesions (n)				
Lymphatic malformation	3	2	0.003	0.958
Left Anterior Descending (LAD) artery	13	11	0.573	0.449
Left Circumflex (LCx) coronary artery	11	8	0.884	0.361
Right Axis Deviation (RAD)	8	9	0.037	0.848
Stent, n				
1	6	8	-0.296	0.587
2	12	10	-0.551	0.458
>3	4	5	0.089	0.766

As described in Table 3, 20 patients in group A retained AR under conventional Western-medicine treatment, whereas 52.2 % of patients in group B retained AR after combination therapy with Xuefu Zhuyu decoction (p=0.002).

Furthermore, our group discovered that after patients had received Xuefu Zhuyu decoction for 4 w, the value of ARU was remarkably downgrading 602.35±26.49 *vs.* 542.48±43.09 (before treatment *vs.* after treatment, p<0.001) (Table 3 and fig. 1). These were illustrated that Xuefu Zhuyu decoction ameliorated platelet function under aspirin treatment.

Through qRT-PCR and radioimmunoassay, we assessed the expression of COX-1 and TXA2 to determine whether Xuefu Zhuyu decoction would influence aspirin's antiplatelet effect. After treatment with Xuefu Zhuyu decoction, the results indicated that TXA2 decreased 503.4±12.0 *vs.* 447.8±48.5 (before treatment *vs.* after treatment; p<0.001), but the change in COX-1 mRNA expression was insignificant 0.65±0.02 *vs.* 0.65±0.03 (before treatment *vs.* after treatment; p=0.734) (fig. 2). In group A, the changes in COX-1 and TXA2 were also insignificant (Table 3).

Before treatment, there was no clear difference in hemorheological indices like plasma viscosity, whole blood viscosity, fibrinogen and hematocrit between the two groups (all p>0.05). However, in group B, hemorheological indices improved significantly, plasma viscosity, whole blood viscosity and fibrinogen decreased significantly (all p>0.05), while no remarkable difference was existed in haematocrit (t=1.645, p=0.144) (Table 3 and fig. 3).

In group A, after 4 w, the plasma viscosity was improved, but other indices (whole blood viscosity, fibrinogen and haematocrit) did not change significantly (all p>0.05) (Table 3).

While considering the genetic and nongenetic factors which influence the therapeutic effects of Xuefu Zhuyu decoction in AR, we implemented a logistic regression analysis to assess the association between improvement of platelet function and these confounding factors. After receiving the treatment of Xuefu Zhuyu decoction, the results revealed that value of glucose was correlated with the ARU, while the expression of TXA2 was protective factor of AR (Table 4).

For these 2 groups of patients, we performed clinical follow-ups for 1 mo and 12 mo. In the 1 mo follow-up, there are 2 stent thrombosis in group A and 1 bleeding in group B. In the 12 mo follow-up, there were 6 Major Adverse Cardiovascular Events (MACEs) in group A including 2 non-fatal myocardial infarctions, 1 non-fatal strokes, 2 stent thrombosis and 1 cardiovascular deaths, as well as 1 case of bleeding. Meanwhile, in group B, there are 3 patients with minor or major bleeding in 12 mo follow-up. After receiving the treatment of Xuefu Zhuyu decoction, the results indicated that the AR patients (group B) would suffer less MACEs (p=0.024) and the risk of bleeding were similar with group A (Table 5).

TABLE 3: CHANGE OF PLATELET FUNCTION, COX-2, TXA2 AND HEMORHEOLOGY INDICES BEFORE AND AFTER TREATMENT

Group	Parameters	Before treatment	After treatment	t/χ^2	p
A	AR, n	22	20	-	-
	ARU	599.27±34.24	588.68±39.20	1.812	0.084
	COX-1	0.65±0.02	0.63±0.04	1.356	0.189
	TXA2 (ng/l)	503.4±12.0	502.8±16.7	0.192	0.85
	Plasma viscosity, mPa·s	2.06±0.10	2.01±0.09	2.23	0.037
	Whole blood viscosity, mPa·s	5.09±0.22	5.02±0.23	2.02	0.056
	Fibrinogen (g/l)	3.89±0.21	3.85±0.21	1.904	0.071
	Hematocrit (%)	50.95±3.17	50.38±3.41	1.768	0.092
B	AR, n	23	12	-	-
	ARU	602.35±26.49	542.48±43.09	6.266	0
	COX-1	0.65±0.02	0.65±0.03	-0.344	0.734
	TXA2,ng/L	503.4±12.0	447.8±48.5	5.934	0
	Plasma viscosity, mPa·s	2.02±0.14	1.56±0.12	16.175	0
	Whole blood viscosity, mPa·s	5.07±0.15	3.45±0.15	48.234	0
	Fibrinogen (g/l)	3.95±0.21	3.17±0.26	12.658	0
	Hematocrit (%)	50.27±2.90	49.64±2.65	1.645	0.114

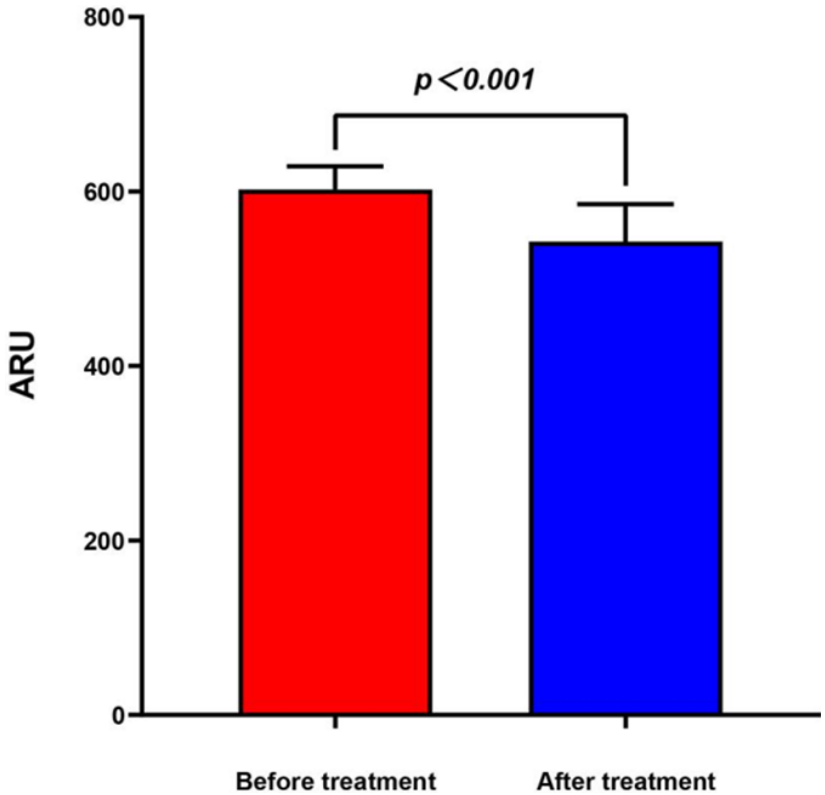

Fig. 1: Improved platelet function
Note: ( ): Before treatment and ( ): After treatment

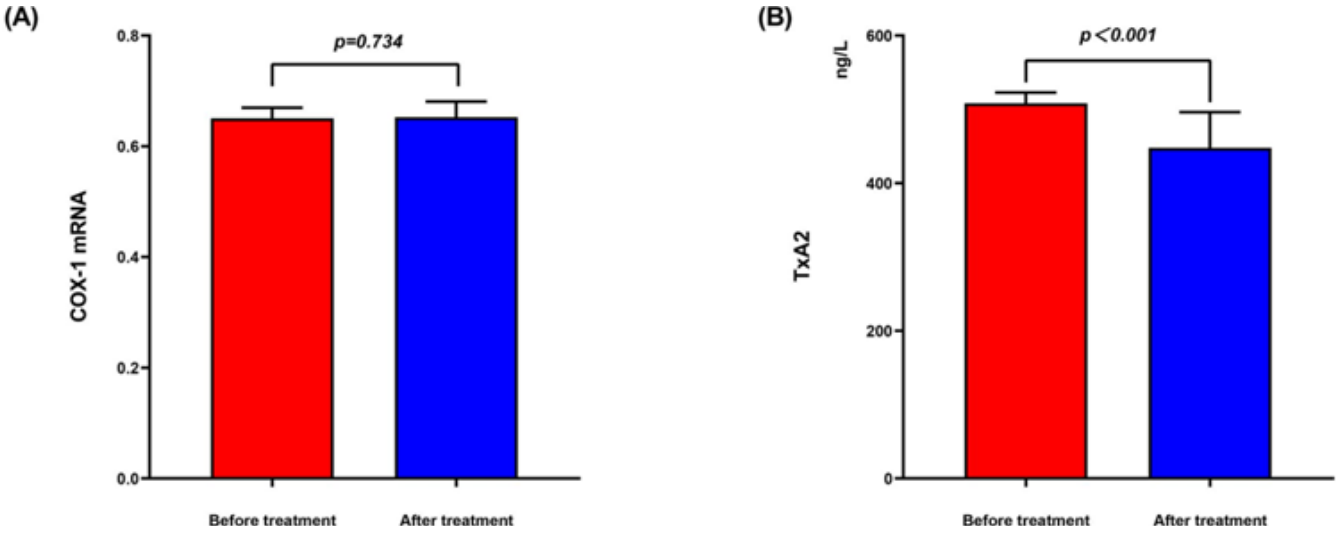

Fig. 2: Changes in COX-1 and TXA2, (A): COX-1 mRNA expression and (B): TXA2
Note: ( ): Before treatment and ( ): After treatment

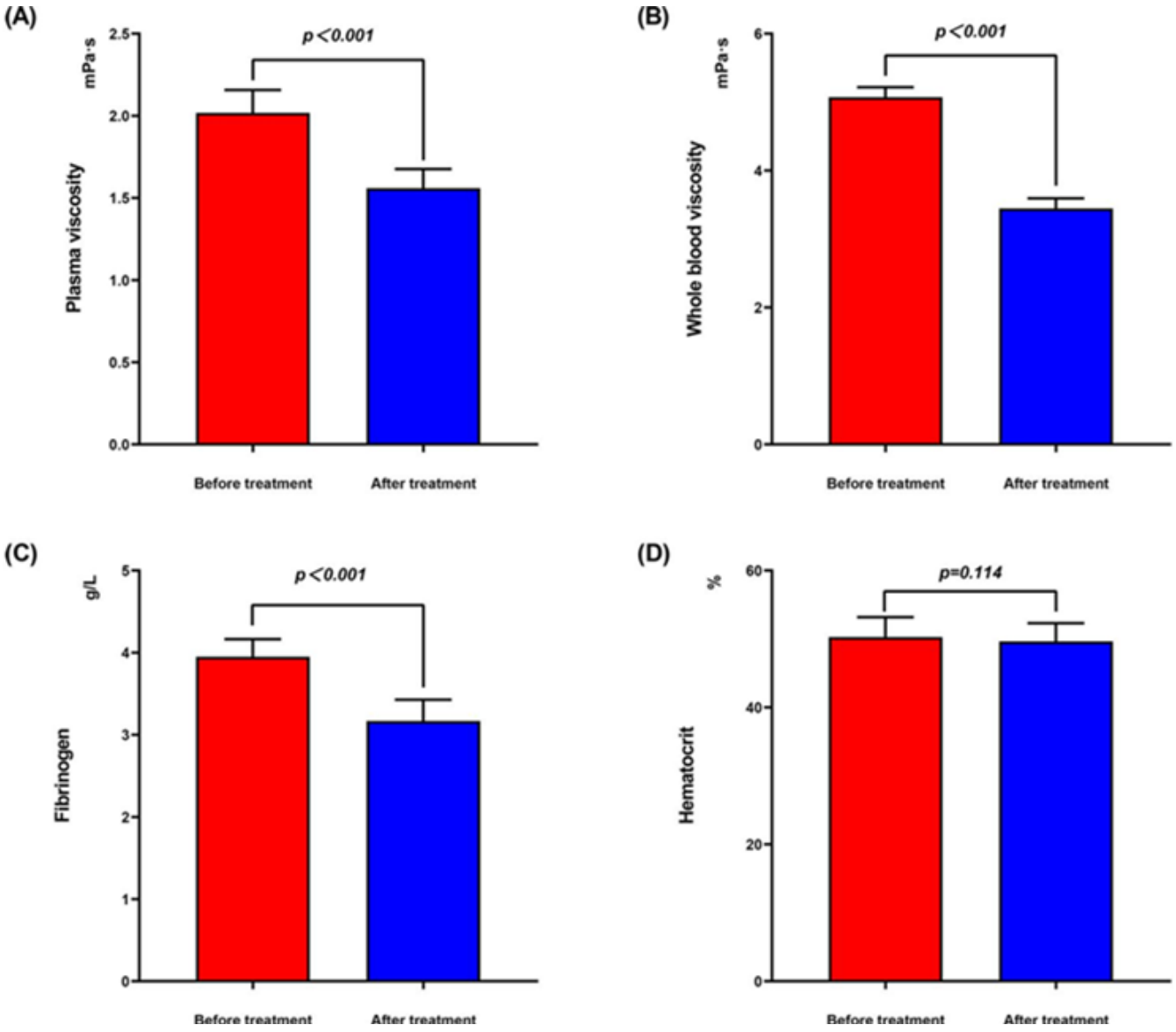

Fig. 3: Improvement in hemorheological indices, (A): Plasma viscosity; (B): Whole blood viscosity; (C): Fibrinogen and (D): Haematocrit
Note: ( ): Before treatment and ( ): After treatment

TABLE 4: LOGISTIC REGRESSION ANALYSIS OF THE THERAPEUTIC EFFECTS OF XUEFU ZHUYU DECOCTION IN AR PATIENTS

Effect	B	Standard error	Wald	p	Exponential value of B
Cox-1	0.072	0.041	3.674	0.061	1.152
TXA2	-1.247	0.573	4.763	0.031	2.357
Age	-0.066	0.031	1.942	0.176	1.262
Body mass index	0.165	0.013	1.875	0.144	1.534
Glucose	1.235	0.215	2.523	0.026	0.784
Constant	4.5612	5.2367	0.752	0.422	4.675

TABLE 5: OCCURRENCE OF CLINICAL EVENTS AFTER THE TREATMENT OF XUEFU ZHUYU DECOCTION IN AR PATIENTS

Events	Follow-up	Group A (22)	Group B (23)	χ^2	p
MACEs	1 mo	2	0	0.571	0.45
		1	3	0.326	0.568
Bleeding	12 mo	6	0	5.07	0.024
		2	4	0.145	0.704

The biological steps of platelet aggregation occurs in the following sequences as initiation, extension and stabilization[11]. By inhibiting both COX-1 and COX-2, TXA2 production is reduced for the lifetime of platelets (approximately 10 d). With a short biological half-life, TXA2 could be rapidly converted to TXB2, which play a role in promoting vasoconstriction and platelet aggregation. In human platelets, aspirin reduces TXA2 synthesis, resulting in limiting of platelet aggregation[12]. However, AR is a clinical phenomenon refers to the failure to fully activate platelets[13]. Modern medicine often applies other drugs or doubles the dose of aspirin to prevent AR, but this poses a great risk of bleeding.

TCM, exhibiting great antiplatelet effects, are commonly used for post-PCI patients to activate blood circulation and thus remove blood stasis[8]. Xuefu Zhuyu decoction, applied to remove blood stasis in the chest of CAD, showed good effects on antiplatelet therapy in Chinese medicine practice[14]. Of this investigation, we discovered that Xuefu Zhuyu decoction could reduce the risk of AR. Here, the results showed that TXA2 decreased, which indicated that Xuefu Zhuyu decoction might reduce aspirin hyporesponsiveness by inhibiting TXA2 (fig. 4). In modern pharmacological experiments, the Xuefu Zhuyu decoction was proven that it could reduce biological process of platelet adhesion and aggregation, thereby inhibiting the clinical events of thrombosis. One Chinese study observed that Xuefu Zhuyu decoction combined with aspirin could greatly lower platelet aggregation level in patients with adenosine diphosphate and arachidonic acid as inducers which helps in improving the situation of AR[15]. Moreover, our results indicated that Xuefu Zhuyu decoction improved the hemorheological indices. This finding was similar to the former investigation in CAD patients due to blood-stasis syndrome, and it might be related to the Glycoprotein IIb (GPIIb) Human Platelet Antigen-3 (HPA-3) polymorphism[16]. One recent study suggested that Xuefu Zhuyu decoction for activating blood circulation to dissipate blood stasis would be an effective therapy to treat diabetic retinopathy through the Reactive Oxygen Species-Extracellular signal-Regulated Kinase 1/2 (ROS-ERK1/2) signaling pathway[17]. Therefore, Xuefu Zhuyu decoction plays important roles in anticoagulation along with improving platelet activity.

The whole decoction is composed of various Chinese herbs. Modern pharmacological studies have shown that Taoren, Honghua, Chishao and Chuanxiong can improve microcirculation, reduce blood viscosity, relieve red blood cell aggregation, expand coronary artery and peripheral vessels and reduce blood pressure. Danggui can reduce blood lipids and inhibit the formation of coronary atherosclerosis and Sheng di huang has a

cardiotonic effect. Niuxi and Zhike can relieve spasms, promote blood circulation and relieve pain. Throughout the whole prescription, there are many pharmacological effects, but the most prominent effects are anticoagulation, crown expansion and improvement of myocardial ischaemia[6]. A recent basic study identified a total of 210 chemical components of Xuefu Zhuyu decoction, screened out from the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database and analysis platform, docked with key targets, and proved that naringin, acteoside, ligustrazine and so on, were potential active components for activating blood, and the combination of *Fructus aurantii*, Chuanxiong, *Rehmannia*, *Glycyrrhiza*, and *Carthami flos* had antiplatelet aggregation activity[18].

To overcome AR, various Chinese traditional medicine researchers have performed many investigations. Antiplatelet therapy usually targets three aspects which includes decreased platelet adhesion, inhibition of platelet aggregation, and inhibition of platelet release[19]. First, Wang *et al.*[20] proved that combined with DAPT, *Panax notoginseng* saponins could raise the Phosphoinositide-3-Kinase/protein kinase B (PI3K/Akt) pathway of endothelial cells, and inhibit the platelet adhesion induced by endothelial injury. Secondly, another study found that the antiplatelet effect of Kyung-Ok-Ko (KOK) maybe involved in the inhibition of Adenosine Triphosphate (ATP) release and increasing the level of intracellular calcium[21]. Another study revealed that in rats model of acute myocardial infarction, *Panax quinquefolius* saponins enhanced the platelets inhibition of aspirin through the COX-1/TXA2 pathway, and activated the fibrinolytic system[22]. Last, Ge *et al.*[19] found that ligustrazine combined with DAPT may have synergistic antiplatelet effects *via* the inhibition of platelet release.

Although our research has yielded some meaningful conclusions, there are still some inherent limitations. First, our study was at the stage of observing intermediate indices. During platelet activation, whether there was an interaction in the signaling pathways still need to be further investigated. Moreover, large-scale, multicenter, clinical researches are needed to promote the pace of integration in TCM and for internationalization. Last, it was better to consider overall regulation and syndrome differentiation in TCM so as to enhance the antiplatelet effect and reduce adverse reactions.

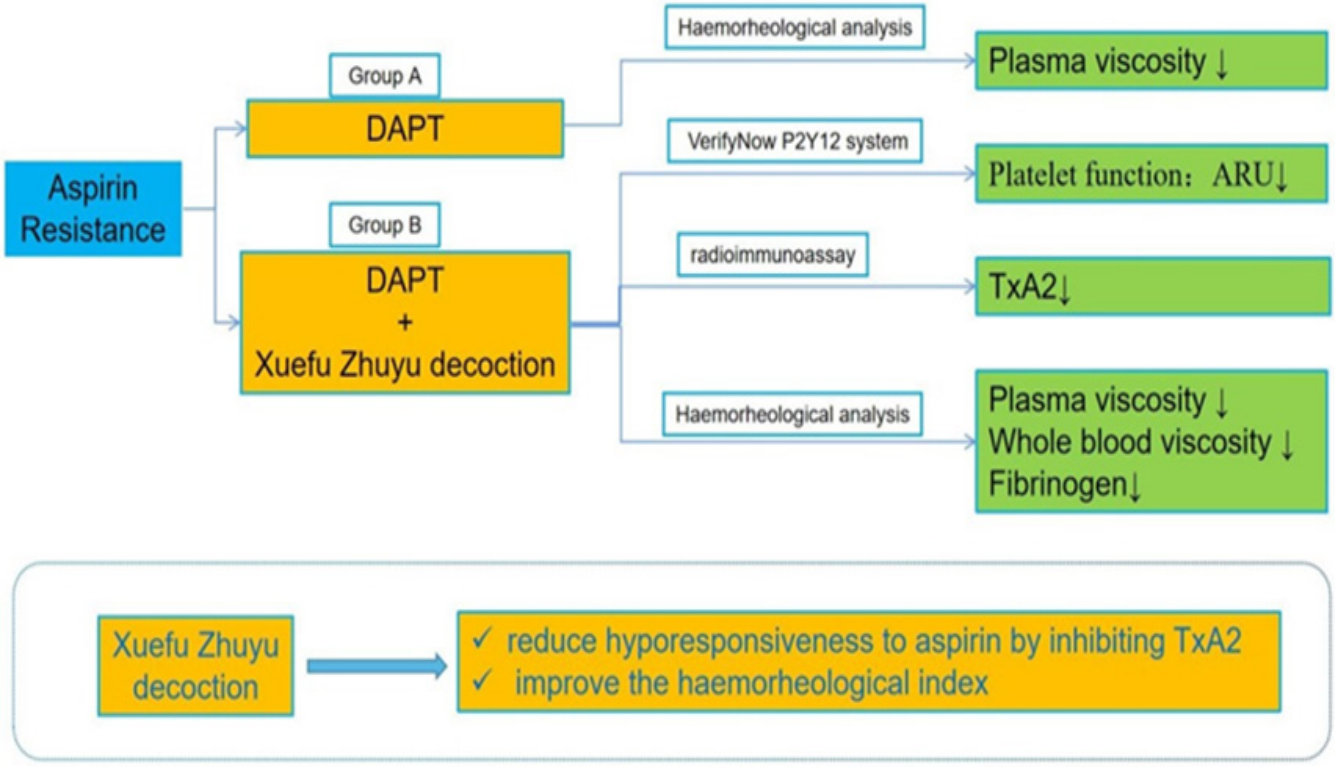

Fig. 4: Xuefu Zhuyu decoction reduce hyporesponsiveness to aspirin by inhibiting TXA2 and it may also improve the hemorheological index

In summary, our research revealed that Xuefu Zhuyu decoction might reduce hyporesponsiveness of aspirin by inhibiting TXA2 and this decoction may also improve the hemorheological index. Meanwhile the glucose was correlated with the ARU, while the expression of TXA2 was a protective factor of AR. And our findings are of great significance for the prevention of cardiovascular events. Large-scale, multicenter, clinical research and mechanistic research studies on signaling pathways would further support our present investigation.

Author contributions:

Resources and related data were collected by Yili Cai, Zhizhou Zhong and Zhen Fang. Yuanyuan Hu and Jin Yang have supported in using the software and the study was supervised by Xiaomin Chen and Jia Su. Qinglin Yu, Keqi Zhu and Jianhong Sun helped in writing the entire original draft, reviewing and editing.

Ethical approval:

The study was approved by the Ethics Committee of First Affiliated Hospital of Ningbo University on November 25, 2021 (Approval No. 2021-R111-YJ01), and all the participants enrolled in this study provided their written informed consent.

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Qinglin Yu, Keqi Zhu and Jianhong Sun are the equal contributors of this article and they are the first co-authors.

Conflict of interests:

The authors declared no conflict of interests.

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