Anti-Platelet Aggregation of Panax Notoginseng Triol Saponins by regulating GP1BA for Ischemic Stroke Therapy

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Research

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Abstract

**Background:** Panax notoginseng triol saponins (PTS) has been used clinically for ischemic stroke therapy (IST) by potential anti-platelet aggregation and neuro-protective in China for more than sixteen years, but its mechanisms are still unclear. In this study, anti-platelet aggregation related protein analysis and computer simulations of drug-protein binding interactions were performed for exploring mechanism of PTS against ischemic stroke by ischemia reperfusion model.

**Methods:** Three doses of PTS were administered orally in middle cerebral artery occlusion (MCAO) model rats; Panax notoginseng total saponins (PNS) and the combination of PTS and aspirin were chosen as comparison. The cerebral infarct size and water content in brain tissue, histomorphological observation, related factors and platelet receptor expression in serum, as well as binding affinity of PTS and platelet adhesion receptor were detected to evaluate therapeutic effect and explore possible mechanisms of anti-platelet aggregation.

**Results:** Compared with PNS, PTS showed stronger and more extensive anti-platelet aggregation effect on MCAO model rats. The combination of PTS and aspirin might reduce the gastrointestinal adverse reactions by regulating TXA\(_2\)/PGI\(_2\) ratio. However, PTS could reduce the chance of VWF-mediated platelet adhesion to damaged vascular endothelium, and thus enhance the probability of anti-platelet aggregation and anti-thrombosis under pathological conditions.

**Conclusion:** Our results showed that GP1BA was closely related with anti-platelet aggregation action of PTS, which provided new scientific evidences for its clinical application.

**Background**

Epidemiological studies showed that stroke was leading cause of death after heart disease, with clinical features such as high morbidity, high recurrence rate and high disability rate [1]. China has the highest incidence of ischemic stroke and the number of new stroke patients is increasing by 1.5 to 2 million per year, which has become a major health threat to residents and a serious economic and psychological burden on patients and their families [2, 3]. Therefore, the prevention and improvement of ischemic stroke has become an urgent problem in clinical medicine. The formation of intravascular thrombosis, which leads to vascular stenosis, and the further formation of cerebral artery obstruction are the main causes of ischemic stroke, while platelets play a key role in the pathogenesis of ischemic stroke, and their activation degree is a key factor determining the formation and development of thrombosis to a certain extent [4, 5].

Modern pharmacological studies have shown that arachidonic acid pathway, adenosine diphosphate pathway and platelet activation factor pathway are three main pathways of platelet activation aggregation through different mechanisms. Among them, arachidonic acid is metabolized by cyclooxygenase into thromboxane A2 (TXA\(_2\)), which induces platelet aggregation, while adenosine diphosphate and platelet activating factor activate phospholipase C through specific receptors acting on the membrane surface to promote the release of Ca\(^{2+}\) in the calcium reservoir, which induces platelet and
leukocyte adhesion, aggregation and release responses [6]. Currently, as representative anti-platelet agents, aspirin, clopidogrel, dipyridamole, etc. have been widely used in the prevention and treatment of stroke, and the clinical efficacy has been widely confirmed. As a cyclooxygenase inhibitor, aspirin can irreversibly inhibit cyclooxygenase, and promote the inability of arachidonic acid in platelets to be converted into intracyclic peroxide at the same time, thereby inhibiting the formation of TXA$_2$, effectively dilating blood vessels, and further exerting antiplatelet aggregation and antithrombotic effects [7]. However, according to clinical statistics, adverse response of aspirin was appeared in 5–45% of patients, which is an independent risk factor for gastrointestinal damage and poor prognosis of coronary heart disease, as well as the main reason for poor medication compliance and even withdrawal [8]. Therefore, the application of aspirin in the treatment of ischemic stroke was extremely limited by the potential clinical risks.

Panax notoginseng, as the representative herbs for promoting blood circulation, is widely used in treating ischemic stroke. Saponins are the recognized active constituents and Q-marker of panax notoginseng, which are mainly divided into diol and triol saponins [9]. Panax notoginseng triol saponins (PTS) is the active pharmaceutical ingredients of Sanqi Tongshu Capsule which has marketed in China from 2003 for ischemic stroke therapy. Ginsenoside Rg$_1$, ginsenoside Re and notoginsenoside R$_1$ are main parts of PTS with total content more than 67%, rests are small amount of other saponins, flavonoid, polysaccharides, and amino acids. It has obvious cerebral protective effects on experimental cerebral ischemia and cerebral infarction animals, and strong activities in reducing platelet aggregation and platelet adhesion, as well as good therapeutic effect in improving outcomes after ischemic stroke as a safety complementary medicine to aspirin [10, 11]. Among them, Rg$_1$ exhibits the strongest anti-platelet activity, which can achieve anti-thrombosis by inhibiting platelet aggregation, reducing blood viscosity, enhancing fibrinolytic system activity and promoting the release of vascular endothelial NO; R$_1$ can inhibit the production of peroxides and leukocyte adhesion; Re enhances adenylate cyclase activity, Ca$^{2+}$ levels of platelet cell and inhibits platelet activation [12, 13]. Multiple years of clinical application had demonstrated curative effect of PTS; however, the treatment mechanism of PTS is not sufficient, especially in the systematic and in-depth interpretation of targets and pathways of anti-platelet aggregation, which affects the current clinical value of the drug.

Platelets can adhere to collagen on the vascular wall, and thus cause the release of inducers and activate the aggregation to form thrombus, which involves a variety of endogenous substances such as proteins and signal factors in the body. Studies have shown that during the initial adhesion of platelets, as an important kind of platelet membrane glycoproteins, the platelet receptor Glycoprotein Ib-α (GP1BA) can effectively combine with VWF and fibrinogen to form glycoprotein Ib/Ⅷ/Ⅲ complex and participate in platelet aggregation, which is a key step of thrombosis [14, 15]. Platelet membrane glycoprotein receptor can increase the rate of platelet aggregation, induce thrombus formation, and eventually cause ischemic stroke disease [16]. It is reasonable to believe that GP1BA maybe play a crucial role in the process of thrombosis.
Based on the above researches and discussions, three doses of PTS (PTS-H, PTS-M and PTS-L) were administered orally in middle cerebral artery occlusion (MCAO) model rats [17], and panax notoginseng total saponins (PNS) and the combination of PTS and aspirin were chose as comparison to evaluate therapeutic effect. The cerebral infarct size and water content in brain tissue, histomorphological observation, related factors and platelet receptor expression in serum, as well as binding affinity of PTS were detected to further investigate the mechanism of PTS against platelet aggregation for ischemic stroke therapy, especially, the effect between PTS and aspirin in arachidonic acid metabolism and influence of PTS on binding of glycoprotein Ib/II/IIIa complex and VWF was detailedly discussed. Meanwhile, the molecular mechanism between PTS and specific protein by surface plasmon resonance (SPR) [18] and molecular docking simulation [19] were conducted in this study.

Materials And Methods

Materials

PTS (Lot No.:20190318) was provided by Chengdu Huasun Technology Group (Chengdu, China). Aspirin Enteric-coated Tablets (Lot No.:BJ45216) was purchased from Bayer Health Care (Milano, Italy). Xuesaitong Soft Capsule (Lot No.:20190322) with 75% content of PNS (saponins which were extracted from notoginseng, including Ginsenoside Rg₁, Ginsenoside Rb₁, Ginsenoside Re, Notoginsenoside R₁, and Ginsenoside Rd) was purchased from Kunming Shenghuo Pharmaceutical (Kunming, China). Reference Standards of Ginsenoside Rg₁, Ginsenoside Re and Notoginsenoside R₁ were purchased from National Institutes for Food and Drug Control (Beijing, China). Toluidine Blue O was purchased from Scientan (Beijing, China). TTC, and other chemical reagents were purchased from Chengdu Chron Chemicals (Chengdu, China). All chemicals were of analytic grade and used as received. The kits for determining cAMP, TXA₂, TXB₂, PGI₂, 6-keto-PGF1α was purchased from Elabscience Biotechnology (Wuhan, China). The platelet protein extraction kit was obtained from Bestbio Biotechnology (Shanghai, Chinese). The kits for determining NO and NOS were purchased from Nanjing KeyGen Biotech (Nanjing, China). The peripheral blood platelet separation kit was obtained from Haoyang Biological Manufacture (Tianjin, China). PAF was purchased from Abcam (Cambridge, USA). GP1BA was purchased from Proteintech (Rosemont, USA). CM5 Chip, Peptide Coupling Kit, GST Capture Kit and other buffers used in SPR test were purchased from GE Healthcare Life Sciences.

Division of groups and middle cerebral artery occlusion model

All animals in our research were provided by the Experimental Animal Center of Chengdu University of Traditional Chinese Medicine, and raised in SPF-class housing of laboratory with a controlled condition (20-22℃, relative humidity of 50-60%, and 12 h light-dark cycles). They were fed with water and commercial rat pellet diet. All animal experiments were permitted by the Sichuan Provincial Committee
for Experimental Animal Management. After acclimation, the adult male SD rats (300±20 g) were given the preventive drug at 10 mL/kg by gavage, once a day for 6 consecutive days. And then the animals were divided into 7 groups with different forms of administration: sham surgery and model control (distilled water), positive control (PNS at 28 mg/kg), high-, medium- and low-dose PTS (100, 50 and 25 mg/kg, respectively) as well as combined treatment (PTS at 50 mg/kg and aspirin at 21 mg/kg) groups. Specifically, the MCAO model of rats was established at 30 min after gavage on Day 6, and the animals were irradiated using incandescent light to maintain their anal temperature at (37±1) °C until recovery of activity. The rats underwent fasting for a period of 12 hours prior to the experimental procedures with water available ad libitum, and were anesthetized. The intraoperative and postoperative room temperature was maintained at about 25°C, reperfusion was performed after 2 h of completely blocking the blood supply from the middle cerebral artery. Determination of a successful model: (1) positive Horner's syndrome on the right side; (2) hemiplegia on the left side mainly manifesting as forelimb movement abnormality. In the sham surgery group, all the procedures were performed except no insertion of a fishing line. The animals were dosed twice in process of model establishment, at 2 hours after ischemia before reperfusion and 6 hours after reperfusion, respectively, and then the bloods and brains were collected after reperfusion for 22 h.

**Determination of cerebral infarct size and water content in brain tissue**

After reperfusion for 22 h, the rats were decapitated and their rhinencephalon, lower brain stem and cerebellum were removed. After weighing, coronal sections with a thickness of about 2 mm were taken from brain tissues, and each side of the ischemic brain tissue was cut into 5 pieces. Then, the pieces of brain were immediately placed in 2% TTC solution and incubated for 30 min at 37 °C in the dark. After staining, the normal brain tissue was rosy, while the cerebral infarct area was white. The total area and infarcted area of ischemic brain tissue in each slice were calculated by Nodus DanioScope Version 1.0.109. The percentage of the brain in infarct area to the total brain was taken as the infarct size (%). The stained brain tissue was weighed before and after dried in an oven at 105 °C for 48 h to a constant weight, respectively, and the water content in brain tissue was calculated according to the following formula:

\[
\text{water content in brain tissue (\%)} = 100\%.
\]

**Histomorphological observation**

Sections were taken from the same part of brain tissue for rats in each group. The tissue samples were fixed with 4% paraformaldehyde, embed with paraffin, and then cut into pieces (4 µm) using a Leica RM2235 microtome. Subsequently, H&E and Nissl's staining were implemented for observing
histopathological changes using the microscopic imaging system, and the Nissl body numbers were quantitatively analyzed by Image Pro Plus 6.0 software.

**Determination of related factors and platelet receptor expression**

Blood samples were collected and centrifuged at 3500 rpm for 10 min to obtain serum and plasma for investigating the following indexes: cAMP, Ca\(^{2+}\), NO, NOS, TXA\(_2\), TXB\(_2\), PGI\(_2\) and 6-keto-PGF1\(\alpha\). In which, cAMP, Ca\(^{2+}\), NO and NOS was determined using corresponding test kit, and the content of TXA\(_2\), TXB\(_2\), PGI\(_2\) and 6-keto-PGF1\(\alpha\) was determined using ELISA. Besides, platelets were collected with platelet isolation kits from the blood. Platelets were also collected with platelet isolation kits from the rats in each group. Besides, the total proteins were collected with a kit for the rest, and the expression level of GP1BA and PAF was determined by Western Blot.

**Methods of SPR Test**

After coupling captured molecule and pre-concentration of ligand, these results indicated that GP1BA could be optimally captured at pH 5.0. The relevant parameters were as follows: Rmax=analyte Mw/ligand Mw×RL× Sm. Anti-GST antibody: 26kDa, GP1BA: 56.7 kDa; Rmax: typically, 100 RU; Sm: stoichiometric ratio, typically 1 (actual captured > RL); GP1BA: pI=5.63, pH of sodium acetate buffer (3.5<pH<pI). GP1BA and samples (Rg1, Re, R1) with different concentrations were prepared and injected for test, the contact time and the flow rate were set according to system instructions. The obtained data were analyzed and fitted by PLEXERA SPR Date Analysis Module (DAM). Kinetic data such as fitting constant, dissociation constant and equilibrium dissociation constant was calculated based on the fitting curve to obtain the specificity of molecular binding and the binding process.

**Method of molecular docking**

The geometric structures of Ginsenoside Rg1, Re and Notoginsenoside R1 were optimized by using density functional method [20, 21] at the B3LYP level of theory [22, 23] with the 6-31G(d) basis set in Gaussian 16 package. The 3D crystal structure of human platelet receptor GP1BA was obtained from RCSB Protein Data Bank (PDB ID: 1P9A) [24]. All docking simulations were performed with the Lamarckian genetic algorithm in Autodock 4.2 software [25]. The docking images were generated by PyMOL.

**Statistical analysis**
The experimental data are subject to one-way ANOVA after being processed with SPSS 17.0 statistical package. Data presented as mean±standards deviation (n = 8). If P<0.05 or P<0.01, it indicates that there is significant difference.

Results

In vivo therapeutic effect of PTS on MCAO model

The chemical structure of main components of PTS was shown in Fig. 1a-c, the representative HPLC chromatograms of PTS and reference standard sample were presented in Fig. 1d, e. The chromatographic peak location of active ingredients in PTS (Ginsenoside Rg₁, Ginsenoside Re and Notoginsenoside R₁) was consistent with the standard sample. The animal experiments were carried out and the results were analyzed as shown in Fig. 2. Compared with the sham surgery group, the percentage of cerebral infarct size and water content in brain tissue of the model control group were significantly changed (P < 0.01). The cerebral infarction size of each treatment group decreased at different degrees, and the high-dose PTS group presented the lowest size, which was significantly different from that of the model group (P < 0.05). Compared with model group, the high-dose PTS group significantly reduced the brain water content (P < 0.01). These data indicated that the high-dose PTS could efficiently reduce the size of cerebral infarction and water content in brain tissue after ischemia-reperfusion. Compared with middle-dose PTS, there was no significant advantage between the combination of aspirin and PTS.

H&E staining was performed and the results observed by microscope was shown in Fig. 3a. In the model group, the necrosis and liquefaction of nerve tissue were observed, and formed sieve reticular lesion, which was clearly demarcated from the surrounding tissue; while a large number of nucleus fragments were observed in the infarcted area, and even caused nucleus condensation and fragmentation. After drugs treatment, the brain histopathology of each group was improved to some extent. Moreover, the Nissl staining was observed with microscope and presented in Fig. 3b, c, in which the Nissl bodies were highlighted with dark blue. The results revealed that a large number of Nissl bodies were destroyed in model group, while the number of Nissl bodies increased obviously after drugs treatment, especially in the high-dose PTS groups, the number of Nissl bodies was similar to that in sham group.

PTS regulate platelet associated factors in serum of MCAO model

PGI₂ and TXA₂ were both in vivo metabolites of arachidonic acid, and TXB₂ and 6-keto-PGF₁α were hydrolyzed products of them. As shown in Fig. 4a-e, after ischemia, the balance of the ratio between PGI₂ and TXA₂ in blood, as an important indicator of AA-induced platelet aggregation, would be destroyed. In the animal experiment, it was found that PTS could regulate the ratio of TXA₂/PGI₂, which could maintain vascular tension and reduce the risk of gastrointestinal bleeding. When PTS was used alone, PGI₂
increased significantly compared with that of combination with aspirin. Similar results were also appeared in the inhibiting expression of TXA₂. The increased expression of TXB₂ and declined expression of 6-keto-PGF1α were also appeared in the group treated with the combination of PTS and aspirin, and the variation trend of the two metabolites was the same as their prototype substance.

On the other hand, both cAMP and calcium ions were intracellular second messengers, which displayed important effects on the formation and exacerbation of platelet aggregation (Fig. 5a, b). The MCAO model could reduce the concentration of cAMP and increase the content of free calcium ions in the serum of animal, which would cause the activation of platelets. The results revealed that PTS exhibited a dose-related effect on cAMP and calcium ions with slightly decreased the expression of calcium ions and significantly increased in cAMP. Besides, different concentrations of NO presented protective or toxic effects on blood vessels and nervous system. Maintaining the concentration of NO in normal range in the body was of positive significance for regulating the activation of platelets. In the acute stage of cerebrovascular disease, endothelial NOS and neuronal NOS were induced to express, and their activity would be up-regulated after cerebral ischemia, while the inducible nitric oxide synthase (iNOS) was activated to produce and release a large number of pathologic inflammatory factors, which played an important role in necrosis and apoptosis of brain cell. In this study, it was found that PTS with different doses could reverse the increasing trend of total nitric oxide synthase (tNOS), iNOS and NO with different degrees in rats after ischemia/reperfusion, especially for iNOS, which was much better than the positive control and the combination of aspirin and PTS groups (Fig. 5c-e).

**Regulating effect of PTS on expression of GP1BA and PAF in blood of MCAO model**

The expression of GP1BA was obviously decreased in model group, while the expression was increased in the medicated groups (Fig. 6a). The effect of PTS on the expression of GP1BA protein was stronger than that of PNS, especially in low-dose and medium-dose PTS groups. Meanwhile, compared with PTS alone, the combination of PTS and aspirin group showed no significant difference. On the other hand, the increased expression of PAF protein was found in model group (Fig. 6b). However, the expression of PAF in all the medicated groups was significantly declined (P < 0.05), and the combination of PTS and aspirin group showed the lowest expression of PAF, while the positive control group showed the highest. These results indicated that PTS could upregulate the expression of GP1BA protein and downregulate the expression of PAF protein. No previous studies reported that aspirin had effect on the expression of GP1BA and PAF, and the results in this study also indicated that no obvious enhancement effect was found by PTS combined with aspirin. Therefore, PTS and aspirin might have different targets in anti-platelet aggregation.

**PTS could be binded with GP1BA by SPR test and molecular docking**
The results of MCAO-model animal experiments suggested that the regulation of the expression of GP1BA protein might be an important mechanism for anti-platelet aggregation. However, how did PTS regulate the expression of this protein? SPR and molecular docking simulation technology could be used to accurately investigate the inter-molecular interactions and molecular recognition between the main monomeric compounds of PTS and GP1BA. Multi-cycle kinetics was applied in the experiment. The analytes (ginsenoside Rg$_1$, notoginsenoside R$_1$, and ginsenoside Re) were prepared by running buffer HBS-EP containing pH 4.5 sodium acetate. The concentration was set with 5 gradients, and blank control with only running buffer was set. The experimental data were fitted by affinity, and all the KD values of ginsenoside Rg$_1$, notoginsenoside R$_1$, and ginsenoside Re binding to GP1BA were stated in the µM grade (Fig. 7). All three monomer compounds exhibited great binding activity to platelet membrane glycoprotein GP1BA. However, the results showed that the KD value of ginsenoside Rg$_1$ was higher than that of other two compounds, preliminarily suggesting that the binding capacity of ginsenoside Rg$_1$ might be relatively low. In SPR test, it was required that the target protein was well coupled with the chip, and the coupling mode and condition needed to be explored. In this experiment, due to the low isoelectric point of the tested protein GP1BA, the amount of GP1BA protein captured on the CM5 chip was finally determined to be 987.1 RU by different pH assays. Although the value was not very high, the experiment displayed that the binding amount of different components of ginsenosides at different concentrations was different, and the results should be reliable.

Result of molecular docking analysis displayed that ginsenoside Rg$_1$ could form 10 hydrogen bond binding sites with N-terminal amino acid residues of protein, and form a stable structure of hydrophobic pocket with its surrounding residues. The hydroxyl in Ginsenoside Rg$_1$ was involved in H-bonding with Leu$^{214}$, Lys$^{189}$, Cys$^{211}$, Glu$^{212}$, Tyr$^{215}$, Arg$^{217}$ and Thr$^{266}$ residues in amino- (N-) terminal of GP1BA protein. Furthermore, Ginsenoside Rg$_1$ also formed hydrophobic contacts with the residues Gly$^{190}$, Arg$^{218}$, Gln$^{221}$, Cys$^{264}$ and Pro$^{265}$ in GP1BA protein, which strengthened the stability of bonding (Fig. 8). The binding free energy (The protein – ligand interaction energy) was − 25.8 kJ/mol, and Inhibition Constant, Ki was 30.0 µM. The molecular docking conformation of Ginsenoside Rg$_1$ and GP1BA proteins could be simulated by computer. The results of affinity and docking energy between the protein and the ligand also demonstrated that stable binding could be formed.

According to the previous studies [15], one side of the VWF A1 domain was wrapped by the amino N-terminal structure of GP1BA, forming two binding regions linked by interaction between solvated electrons; the two binding sites of α-thrombin were binded successively to two sites (25 amino acid residues) of the amino N-terminal structure of GP1BA, respectively, and formed a variety of protein-ligand complexes. This docking study revealed that ginsenoside Rg$_1$ also acted at the amino N-terminal of GP1BA, of which ARG-218 was also the common binding site of ginsenoside Rg$_1$ and α-thrombin, indicating that ginsenoside Rg$_1$ might prevent GP1BA from binding to VWF and thrombin by occupying the amino N-terminal of GP1BA, and inhibit the activity of GP1BA protein.
Discussion

As a major cerebrovascular disease, ischemic stroke becomes one of the main threats to human-beings’ health, and now is the second global cause of death [26, 27]. PTS is a quantified dry extract produced from the root of Panax Notoginseng (Burk.) F. H. Chen. It has a stable quality standard, which is included in Chinese Pharmacopoeia. Its preparation Sanqi Tongshu Capsule has been used clinically for IST by potential anti-platelet aggregation and neuro-protective in China since 2003 to now, but its mechanisms are still unclear. Herein, anti-platelet aggregation mechanism of PTS and its efficacy in IST were further investigated in this study.

Exposed in the pathological model animal, PTS showed a good therapeutic effect on decrease of cerebral infarction size, reduction of water content of brain tissue, pathological changes of brain tissue and recovery effect of nerve cell, as well as regulation of related factors and platelet receptor expression in serum. We also found PTS could exert its efficacy via the pharmacological functions of anti-platelet aggregation, vasodilation, anti-inflammation, and anti-oxidative stress etc. which were achieved by impacting the ratio of TXA₂ and PGI₂, activity of nitric oxide-related factor, release of second messenger of intracellular signaling cAMP and calcium ion. PTS could affect the expression of GP1BA and PAF, which are important proteins in platelet aggregation and thrombosis, proving that PTS may act on initial adhesion of platelet and platelet activation. Compared with PNS, PTS showed stronger and more extensive anti-platelet aggregation effect on MCAO model rats. The combination of PTS and aspirin might reduce the gastrointestinal adverse reactions by regulating TXA₂/PGI₂ ratio.

In the molecular interaction test by SPR, we found ginsenoside Rg₁, ginsenoside Re, notoginsenoside R₁, all showed very good binding activities with GP1BA. All the three main components of PTS were dammarane-type tetracyclic triterpenoid saponins and owned the same parent nucleus structure. It could be concluded by organic structure analysis that the differences in the side chains of each compound had little impact on the docking with GP1BA. Panaxatriol-type saponins were interconverted under the influence of pH value, liver drug enzyme, and intestinal bacteria. For example, ginsenoside Re could be converted into ginsenoside Rg₁ in vivo [28, 29]. The human pharmacokinetic study demonstrated that the ginsenoside Rg₁ and notoginsenoside R₁ could be detected in healthy human blood after administration with Sanqi Tongshu Capsules via oral, especially for ginsenoside Rg₁, as the active ingredient with most content (greater than or equal to 50%) in PTS, its AUC₀–∞ and Cmax in human were much higher than those of notoginsenoside R₁ [30]. Therefore, in the study on molecular docking of PTS and GP1BA, the ginsenoside Rg₁, which has been confirmed to be fully distributed in the human blood system, was selected as the main study object. Taking the main component ginsenoside Rg₁ as the representative, molecular docking by computer simulation showed ginsenoside Rg₁ and GP1BA could form a stable structure of hydrophobic pocket. As mentioned previously for the structural similarity of the major components in PTS, it could also be speculated that other active components and metabolites in PTS might also bind to GP1BA protein in a similar manner. These studies proved that PTS can competitively inhibit the activity of GP1BA protein from the level of molecular interaction, showing that the PTS has
reduced the chance of VWF-mediated platelet adhesion to vascular endothelium at the site of vascular injury, and also reduced the binding of thrombin to platelets, thus reduced the probability of platelet aggregation and thrombosis under pathological conditions.

Combined with the results of previous studies [11, 31], it could be found that the anti-platelet aggregation action of PTS involved early-thrombogenesis by regulating GP1BA, platelet activation induced by ADP, PAF, AA, collagen and other factors, and activation signal transduction. The potential targets of PTS in the above action links were found to be GP1BA and PAF, and the efficacy as well as the safety for combination with aspirin were further clarified; meanwhile, GP1BA, as a new target protein, was related to the therapeutic effect of PTS against platelet aggregation. The above results provided new scientific evidences of PTS in clinical application of IS, and it would be helpful for the further mechanism research and clinical studies.

Conclusion

In this study, it was found that PTS exhibited a good overall therapeutic effect on ischemia-reperfusion rats after middle cerebral artery occlusion (MCAO) at the dose range of 25–100 mg/kg, which was manifested with significantly reducing the area of cerebral infarction and water content of brain tissue, as well as obvious histopathological improvement. By impacting TXA2/PGI2 ratio, activity of nitric oxide-related factor, and second messenger of intracellular signaling cAMP and calcium release in animals, PTS could exert its therapeutic effects via regulating relevant neural cytokines and the expression of GP1BA and PAF protein to anti-platelet aggregation. The equilibrium dissociation constants (KD) between all compounds (ginsenoside Rg1, ginsenoside Re, notoginsenoside R1) and GP1BA presented quite good binding activities by surface plasmon resonance (SPR) test. Furthermore, this study proved that PTS could competitively inhibit the activity of GP1BA protein from the level of molecular interaction, implied that PTS reduced the chance of VWF-mediated platelet adhesion to vascular endothelium at the site of vascular injury, and also reduced the binding of thrombin to platelets, thus reduced the probability of platelet aggregation and thrombosis under pathological conditions.

Abbreviations

GP1BA: Glycoprotein Ib-α; IST: ischemic stroke therapy; MCAO: middle cerebral artery occlusion; PGI2: prostaglandin I2; PNS: Panax notoginseng total saponins; PTS: Panax notoginseng triol saponins; SPR: surface plasmon resonance; TXA2: thromboxane A2; VWF: von Willebrand factor.

Declarations

Acknowledgements

Not applicable.
Authors’ contributions

YJF and CP designed the study. ZYX, YX and JHS participated in animal experiments and related test. YT contributed to molecular docking analysis. DSL and XG provided sample extraction technical support and conducted the HPLC analysis. ZYX draft the manuscript. SY and XXF provided the technical support and advices for the study. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Ginsenoside Rg₁

Notoginsenoside R₁

Ginsenoside Re

Figure 1

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Chemical structures and representative HPLC chromatograms of PTS. 2D Chemical structure of (a) Ginsenoside Rg1, (b) Notoginsenoside R1 and (c) Ginsenoside Re. (d) HPLC chromatogram of Panaxtriol Saponins (Lot No.:20190318) and the content of Rg1, R1 and Re. (e) HPLC chromatogram of reference standard and the percentage of each reference. Difference in structures of the above three compounds were shown in blue or red. The determination method was based on the Chinese pharmacopoeia 2015 and the absorbance at 210 nm was monitored by HPLC.

**Figure 2**

(a) TTC staining images of each group after reperfusion for 22 h. Normal brain tissue appears red as shown by the blue arrow, and infarct tissue appears pale gray as shown by the red arrow. Quantitative analysis of the (b) cerebral infarct size and (c) water content in brain of each group. Comparing model control group with Sham surgery group, * P <0.05, ** P <0.01; comparing treatment groups with model control group, # P <0.05, ## P <0.01.
Figure 3

a) H&E staining (in 10× and 40×) and (b) Nissl staining (in 20×) analysis of brain tissue in each group after reperfusion for 22 h. In H&E staining sections, neuronal pyramidal cell and its dendrite could be observed obviously, and central Nissl bodies were dissolved in red neurons, and in Nissl staining, Nissl bodies were highlighted in dark blue. After modeling, neuronal cell degeneration and necrosis were observed and central Nissl bodies were dissolved, showing red neurons. (c) The number of Nissl bodies was analyzed by Image Pro Plus 6.0 software. Comparing model control group with Sham surgery group, * P <0.05; comparing treatment groups with model control group, # P <0.05
Figure 4

The level of (a) TXA2/PGI2 ratio, (b) TXA2, (c) PGI2, (d) TXB2, and (e) 6-keto-PGF1α in serum of each group were determined by ELISA after reperfusion for 22 h. Comparing model control group with Sham surgery group, * P <0.05, ** P <0.01; comparing treatment groups with model control group, # P <0.05, ## P <0.01; comparing treatment groups with positive control group, + P <0.05, ++ P <0.01; comparing PTS combined with aspirin group with PTS medium-dose group, ▲ P<0.05, ▲▲ P<0.01. (f) The difference effect of PTS and aspirin in arachidonic acid metabolism drew according to the above data and past researches.
Figure 5

The expression level of (a) Ca2+, (b) cAMP, (c) NO, (d) iNOS, and (e) tNOS in serum of each group were determined by ELISA after reperfusion for 22 h. Comparing model control group with Sham surgery group, * P <0.05, ** P <0.01; comparing treatment groups with model control group, # P <0.05, ## P <0.01; comparing treatment groups with positive control group, + P <0.05, ++ P <0.01; comparing PTS combined with aspirin group with PTS medium-dose group, ▲ P<0.05, ▲▲ P<0.01.

Figure 6

The expression ratio of (a) GP1BA and (b) PAF in each group compared with the sham group determined by western blot after reperfusion for 22 h. Comparing treatment groups with model control group, # P
Figure 7

Binding affinity results of PTS with GP1BA. Binding affinity curve of (a) Ginsenoside Rg1, (b) Notoginsenoside R1, and (c) Ginsenoside Re with GP1BA, respectively. This figure also showed the KD value of Ginsenoside Rg1, Notoginsenoside R1 and Ginsenoside Re binding with GP1BA.

The binding free energy = $-25.8$ kJ/mol
Figure 8

Schematic diagram of molecular docking simulated using PyMOL (The PyMOL Molecular Graphics System, Version 2.3 Schrödinger, LLC). Basic structure of GP1BA was represented by cartoon in light grey; protein residues in contact with the ligand were represented by wireframe; molecule structure of Ginsenoside Rg1 was represented by tube, and the blue dots represent hydrogen-bonding between the above two.

Supplementary Files

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