Effects of Preoperative Glycyrrhizin Infusion for the Prevention of Venous Thrombosis on the Tissue Expression of Antithrombin in a Rat Model

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Objective: Using a thrombus model prepared by ligation of the inferior vena cava (IVC), the influences of the glycoside, glycyrrhizin, on plasma antithrombin levels and antithrombin mRNA expression levels in the liver and IVC with the inhibition of venous thrombosis were investigated.

Materials and Methods: The rat IVC was exposed and ligated for 24 h immediately after the intravenous administration of 300 mg/kg glycyrrhizin. Among antithrombotic drugs, the Xa inhibitor, fondaparinux sodium, was used as a control drug.

Results: The mean thrombus weight was significantly smaller in the glycyrrhizin-treated group (18.3 mg) than in the saline-treated group (34.3 mg). In contrast, the inhibition of thrombosis was not observed in the fondaparinux-treated group. Antithrombin mRNA expression levels in the liver were significantly lower in the ligated groups than in the baseline control group. The mean plasma antithrombin level was significantly lower in the glycyrrhizin group (96.6%) than in the saline group (114.4%), but was not significantly different from that in the baseline control group (102.4%).

Conclusion: The pretreatment with glycyrrhizin inhibited venous thrombosis, and antithrombin mRNA expression levels in the liver and IVC as well as plasma antithrombin levels were significantly lower than those in the saline group.

Keywords: glycyrrhizin, antithrombin, DVT

Introduction

Antithrombin is a 58-kD protease inhibitor that is mainly synthesized in the liver and circulates as a plasma protein. It regulates blood coagulation by directly inhibiting the serine proteases of the clotting cascade, with the most important targets being thrombin, factor Xa, and factor IXa. A sequence-specific pentasaccharide present in only a fraction of heparin molecules mediates the high-affinity binding and anticoagulant activation of antithrombin by this polysaccharide. Antithrombin has lysine binding sites to which heparin binds at a molar ratio of 1:1. The half-life of thrombin is reduced to 20 ms in the presence of a high concentration of heparin, which is an approximately 2000-fold acceleration of this reaction. Griffith previously identified thrombin-heparin binding as the most important factor for efficient thrombin inhibition by antithrombin.

P- and E-selectins have been known to mediate the linkage of endothelial cells to neutrophils through the binding to sialyl-Lewis X glycoproteins, which are expressed on the surface of neutrophils. Neutrophils adherent to the endothelium also undergo transendothelial migration, leading to endothelial cell sloughing and exposure of the underlying basement membrane to the accelerated formation of deep vein thrombosis (DVT).

Glycyrrhizin, which is a natural triterpenoid saponin with a molecular mass of 840 Daltons, has been approved for useful drug for the treatment of allergic disorders and chronic hepatitis in Japan. We previously showed that glycyrrhizin was effective on the prevention of the tissue damage caused by ischemia-reperfusion in the rabbit hind limb.

Mendes-Silva et al. were the first to demonstrate that glycyrrhizin exhibits antithrombotic activity in vivo and, it has, thus, been characterized as a potential thrombin inhibitor. Assafim et al. showed that glycyrrhizin was effective in preventing venom-induced thrombus formation through the generation of thrombin by prothrombin activators and platelet-activating components. Glycyrrhizin was previously demonstrated to bind to thrombin exosite I and block the effects of the enzyme on fibrinogen and platelets.

Glycyrrhizin, an agent with a chemical structure analogous to that of sialyl-Lewis X and the ability to bind P- and L-selectins, may be useful for blocking the P-selectin-mediated thrombotic cascade due to its competitive binding to sialyl-Lewis X oligosaccharides on neutrophils and subsequent blocking of neutrophil adhesion to the vascular endothelium.
Fondaparinux sodium\(^{12,13}\) (fondaparinux) is an anticoagulant with a chemically synthesized antithrombin binding site of unfractionated heparin that binds to antithrombin and inhibits activated factor X (F Xa). It has been approved for use in the prophylaxis of venous thromboembolism following orthopedic surgery. In the present study, we compared the effects of the preoperative administration of glycyrrhizin on antithrombin levels in plasma and antithrombin mRNA expression levels in the liver and inferior vena cava (IVC) with the inhibition of venous thrombosis with those of a fondaparinux treatment.

**Materials and Methods**

**Animals**
The experimental protocols used conformed to the Institutional Committee for Animal Care and Experiments in Osaka City University, Graduate School of Medicine and were approved by the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

Male Sprague-Dawley rats (8–9 w) were purchased from SLC, Inc. (Shizuoka, Japan) and fed in separate cages in an air-conditioned room with free access to food and water. Venous thrombosis was induced in the IVC by its ligation as described by Reyers et al.\(^13\) with slight modifications. In brief, animals were anesthetized with 0.7 ml of a mixture of 3 ml of xylazine hydrochloride (20 mg/ml) and 12 ml of ketamine hydrochloride (50 mg/ml) by an intraperitoneal injection, and underwent midline laparotomy. The IVC was directly approached by careful blunt dissection and ligated at the level of the IVC just below the bifurcation level of the left renal vein. Rats were administered either an intravenous injection of glycyrrhizin (300 mg/kg body weight) (Minophagen Pharmaceutical, Tokyo, Japan) just before IVC ligation through the IVC proximal to the ligated suture after 24 h of ligation and were centrifuged at 3000 rpm for 15 min in order to obtain supernatant plasma fluid. Plasma antithrombin and TAT were assessed using commercially available kits (Testzim S AT III: Chromogenix, Tokyo, Japan; and TAT[S]: TBF, Tokyo, Japan, respectively).

**Study 1: Measurement of thrombus wet weights**

After 24 h of IVC ligation, the thrombus within the IVC was collected through longitudinal dissection and its wet weight was measured.

**Study 2: Measurement of antithrombin and the thrombin-antithrombin complex (TAT) in rat plasma**

Citrated blood samples from rats were collected from the IVC proximal to the ligated suture after 24 h of ligation and were centrifuged at 3000 rpm for 15 min in order to obtain supernatant plasma fluid. Plasma antithrombin and TAT were assessed using commercially available kits (Testzim S AT III: Chromogenix, Tokyo, Japan; and TAT[S]: TBF, Tokyo, Japan, respectively).

**Studies 3 and 4: Quantitative RT-PCR analysis of antithrombin mRNA expression in the IVC and liver**

The IVC and liver, which were harvested after 24 h of IVC ligation, were used in an RT-PCR analysis to confirm the effects of glycyrrhizin or fondaparinux on antithrombin gene expression levels in these samples. Each tissue was kept in RNA Later Solution at 4°C overnight. After 1 day, the tissues were removed and stored at –80°C until used. Total RNA was extracted from approximately 10 mg of tissue using an RNAsesy Fibrous Tissue Mini Kit (QIAGEN Co. Ltd., Germany) and concentrations were measured with a spectrophotometer. Total RNA was reverse transcribed into cDNA using a High capacity cDNA reverse transcription kit (ThermoFisher Scientific MA, Waltham, USA). The Taqman Gene Expression Assay was used for relative quantitative measurements of the mRNA expression levels of antithrombin (Rn01483153_m1) and antithrombin (Rn01527840_m1) was used for normalization in the data analysis.

PCR reactions used 10 ng of the cDNA template in a 20-µl reaction volume, and were run on the 7500 Fast Real-Time PCR System (ThermoFisher Scientific MA, Waltham, USA). Data analyses were performed using 7500 software V2.0.6 on the Windows XP OS. The results obtained from glycyrrhizin or fondaparinux-treated and baseline control rats were expressed relatively, with expression in the targets being compared with those of saline-treated rats that were calibrated with the expression of an internal control gene, hypoxanthine phosphoribosyltransferase 1 (Hprt1).\(^14\)

**Statistical analysis**

All values were expressed as the mean ± standard deviation (SD). The Mann-Whitney U-test was used to evaluate the significance of differences. P <0.05 was considered significant.
The tissue expression of antithrombin

Results

Study 1: Thrombus wet weights

Thrombi were consistently recognized in all IVC of the rats treated with or without glycyrrhizin and fondaparinux. The mean weight of the thrombus was significantly lower in the glycyrrhizin-treated group (18.3 ± 17.7 mg (n = 7)) than in the saline-treated group (34.3 ± 12.8 mg (n = 7)) (P = 0.035). However, no significant differences were noted between the fondaparinux (22.6 ± 8.29 mg (n = 7)) and saline (33.3 ± 24.45 mg (n = 6)) groups (P = 0.89).

Study 2: Measurement of antithrombin and TAT levels in rat plasma

The mean plasma antithrombin values for the baseline control, saline-treated, and glycyrrhizin-treated rats were 102.4 ± 11.2, 114.4 ± 10.6, and 96.6% ± 9.4%, respectively (Fig. 1A). The plasma concentration of antithrombin was significantly lower in the glycyrrhizin-treated group than in the saline-treated group (P = 0.01). The mean plasma antithrombin levels in the saline-treated and fondaparinux-treated groups were 128.0 ± 12.6 and 118.4% ± 9.4%, respectively (Fig. 1B), which were significantly higher levels than that in the baseline control group (102.4% ± 11.2%). No significant difference was observed between the fondaparinux-treated and saline-treated groups (P = 0.086). Furthermore, no significant difference was noted in TAT levels between the saline-treated (n = 7) and glycyrrhizin-treated (n = 7) groups or between the saline-treated (n = 6) and fondaparinux-treated (n = 7) groups (P = 0.61 and 0.39, respectively).

Study 3: Expression of antithrombin mRNA in the liver

Figure 2 shows the relative ratios of the mRNA expression of antithrombin in the liver from baseline control,
saline-treated, and glycyrrhizin-treated rats after 24 h of ligation. The relative ratios of the baseline control, saline-treated, and glycyrrhizin-treated livers for antithrombin were 1 ± 0.104, 2.385 ± 0.133, and 1.889 ± 0.246, respectively. A significant increase was observed in the expression of antithrombin mRNA in saline-treated and glycyrrhizin-treated livers (P = 0.0062, P = 0.0045, respectively), while induced mRNA levels were significantly lower in the glycyrrhizin-treated group than in the saline-treated group (P = 0.0027). Antithrombin mRNA expression ratios in the liver in the baseline control, saline-treated, and fondaparinux-treated groups were 1.0 ± 0.1, 1.83 ± 0.21, and 1.75 ± 0.29, respectively (Fig. 3), and were significantly higher in the saline-treated and fondaparinux-treated groups than in the baseline control group (P = 0.011 and P = 0.008, respectively). However, no significant differences were observed between the fondaparinux-treated and saline-treated groups.

**Study 4: Expression of antithrombin mRNA in the IVC**

Figure 4 shows the relative ratios of the mRNA expression of antithrombin in the IVC from baseline control, saline-treated, and glycyrrhizin-treated rats after 24 h of ligation. The means ± SD of the relative ratios of the baseline control, saline-treated, and glycyrrhizin-treated IVC were 1.0 ± 0.831, 2.405 ± 0.991, and 1.358 ± 0.342, respectively. The expression of antithrombin mRNA was significantly weaker in the glycyrrhizin-treated group than in the saline-treated group (P = 0.032); however, no significant difference was observed in induced mRNA levels between the baseline control and glycyrrhizin-treated groups (P = 0.16).

The antithrombin mRNA expression ratios in the IVC were 1.0 ± 0.18, 1.8 ± 0.36, and 2.16 ± 0.55 in the baseline control, saline-treated, and fondaparinux-treated groups, respectively (Fig. 5), and were significantly higher in the saline-treated and fondaparinux-treated groups than in the baseline control group (P = 0.011 and P = 0.008, respectively). However, in contrast to that in the glycyrrhizin group, the antithrombin mRNA expression ratio was not lower in the fondaparinux-treated group than in the saline group.

The antithrombin mRNA expression ratio in the liver was 18947.1-fold that in the IVC in the baseline control group, suggesting that antithrombin is mainly produced in the liver.

**Discussion**

In the process of the inhibition of venous thrombosis by the preoperative administration of glycyrrhizin, antithrombin production in the liver, its level in the plasma, and its mRNA expression level in the IVC were significantly lower than those in the saline-treated group.
The Tissue Expression of Antithrombin

After being produced in the liver, circulating antithrombin is bound by the endothelial heparin sulfate proteoglycan and is thereby anchored to the endothelial plasma membrane, on which it is strategically situated to bind and inactivate thrombin.\(^{15}\)

A thrombus adheres to the endothelium, causing compression, hypoxia, and inflammation, which ultimately induce endothelial cell damage.\(^{16}\) A histological analysis by light microscopy previously revealed an injured endothelium with prominent inflammatory cells infiltration after 24 h of ligation of the IVC.\(^{17}\) Mo et al. also reported that, after 24 h of IVC clipping, endothelial injury was clearly observed under a light microscope.\(^{18}\)

A scanning electron microscope analysis of veins subjected to 24 h of stasis showed an injured or sloughing endothelium with an exposed basement membrane and large thrombus, and adherent or emigrated leukocytes and platelets were detected in the region around the thrombus.\(^{19}\) Zhang et al. demonstrated that, after IVC ligation for 24 h, the histological findings of the IVC under a transmission electron microscope were the adhesion of inflammatory cells and a thrombus to endothelial cells, causing the loss of the endothelium.\(^{19}\)

Regarding vein wall disorders in the process of thrombogenesis, Downing et al. reported a significant increase in the number of neutrophils infiltrating the vein wall after 6 h in a rat IVC ligation model, suggesting functional damage to the vein wall and the accelerated formation of a thrombus.\(^{20}\) We also previously showed that a pretreatment for venous thrombosis with P-selectin blockade using glycyrrhizin, a carbohydrate analogous to the selectin ligand sialyl-Lewis X and glycomimetics, was beneficial for reducing neutrophil adhesion, thereby preventing the formation of venous thrombosis associated with IVC ligation for 6 h in rats.\(^{6}\)

Liver antithrombin mRNA expression levels were significantly higher in the ligated groups: the glycyrrhizin-treated, fondaparinux-treated, and saline-treated groups, than in the baseline control group. This result was attributed to a biological reaction to the formation of a thrombus induced by the stress of IVC ligation, and may serve as a stress marker of venous thrombosis; however, the underlying mechanisms have not yet been elucidated in detail. In contrast, Chan et al. demonstrated that 10 min of venous occlusion produced by the application of a sphygmomanometer cuff to the arm maintained midway between systolic and diastolic arterial blood pressure in normal female volunteers induced increases in plasma antithrombin III levels, suggesting the possible origin of antithrombin III as the vessel wall.\(^{21}\) The excessive production of coagulation factors such as thrombin has been reported with disseminated intravascular coagulation. Plasma antithrombin appears to be consumed by binding to a coagulation factor, thereby reducing its levels. However, Asakura et al. found that antithrombin levels were not decreased in most cases of acute promyelocytic leukemia with severe coagulopathy.\(^{22}\) However, measured values for antithrombin in plasma vary markedly in the acute phase of DVT, as many clinicians have experienced. Chan et al. assessed antithrombin III levels using a method involving a radioimmunoassay for the first time, and reported that patients with active DVT had significantly lower levels of antithrombin III than normal subjects.\(^{23}\) On the other hand, Kim et al. showed that serum antithrombin III levels did not significantly differ between DVT and non-DVT groups following total hip arthroplasty.\(^{24}\) In the present study, plasma antithrombin levels were significantly lower in the glycyrrhizin-treated group than in the saline-treated group, and this may have been due to significant reductions in antithrombin production in the liver. No significant differences were observed in plasma antithrombin levels between the glycyrrhizin-treated and baseline control groups. Therefore, the high plasma antithrombin levels detected in the present study may be a marker for the promotion of DVT.

Plasma TAT levels did not significantly differ between the glycyrrhizin-treated and saline-treated groups, suggesting the lack of thrombin consumption in the plasma. Therefore, heparin-bound antithrombin on the vascular endothelium may exhibit stronger anti-coagulation activity than free antithrombin in plasma, and, thus, have a more important role. Furthermore, antithrombin mRNA expression levels in the liver and IVC were significantly lower in the glycyrrhizin-treated group than in the saline-treated group, suggesting that these decreases were due to a significant decrease in thrombus formation, i.e., the grade of endothelial cell impairment.

The plasma concentration of intravenously injected glycyrrhizin was previously shown to peak during the 10 min following its injection and then declined, but was still detectable in the subsequent 10 h;\(^{25}\) therefore, glycyrrhizin may inhibit the adherence of neutrophils by binding to P-selectin within several hours of IVC ligation.

Myeloperoxidase (MPO) activity in the ischemia-reperfused muscles of rabbits was shown to increase in the absence of a glycyrrhizin infusion, but remained low in its presence, suggesting that an increase in oxidant production is secondary to enhanced endothelial permeability following ischemia-reperfusion and the infiltration of neutrophils into muscles, which may be prevented by a treatment with glycyrrhizin.\(^{7}\)

The clinical dose and administration method of fondaparinux are 5–7.5 mg/day and a subcutaneous injection, respectively, and its dose was 0.2 mg/kg (subcutaneous injection) in a study on intestinal ischemia-reperfusion in rats by Olanders et al.,\(^{26}\) suggesting that the dose (1.5 mg/kg) administered to rats in our study was comparatively high for animal experiments. 'Inflammation' and venous thrombosis

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References

1. Mo et al. reported...
2. A thrombus adheres to the endothelium, causing...
3. A histological analysis by...
4. We also previously showed that a pretreatment for venous thrombosis with P-selectin blockade using glycyrrhizin, a carbohydrate analogous to the selectin ligand sialyl-Lewis X and glycomimetics, was beneficial for reducing neutrophil adhesion, thereby preventing the formation of venous thrombosis associated with IVC ligation for 6 h in rats.
5. Liver antithrombin mRNA expression levels were significantly higher in the ligated groups: the glycyrrhizin-treated, fondaparinux-treated, and saline-treated groups, than in the baseline control group.
6. However, Asakura et al. found that antithrombin levels were not decreased in most cases of acute promyelocytic leukemia with severe coagulopathy.
7. However, measured values for antithrombin in plasma vary markedly in the acute phase of DVT, as many clinicians have experienced.
8. Chan et al. assessed antithrombin III levels using a method involving a radioimmunoassay for the first time, and reported that patients with active DVT had significantly lower levels of antithrombin III than normal subjects.
9. On the other hand, Kim et al. showed that serum antithrombin III levels did not significantly differ between DVT and non-DVT groups following total hip arthroplasty.
10. In the present study, plasma antithrombin levels were significantly lower in the glycyrrhizin-treated group than in the saline-treated group, and this may have been due to significant reductions in antithrombin production in the liver.
11. No significant differences were observed in plasma antithrombin levels between the glycyrrhizin-treated and baseline control groups.
12. Therefore, the high plasma antithrombin levels detected in the present study may be a marker for the promotion of DVT.
13. Plasma TAT levels did not significantly differ between the glycyrrhizin-treated and saline-treated groups, suggesting the lack of thrombin consumption in the plasma.
14. Therefore, heparin-bound antithrombin on the vascular endothelium may exhibit stronger anti-coagulation activity than free antithrombin in plasma, and, thus, have a more important role.
15. Furthermore, antithrombin mRNA expression levels in the liver and IVC were significantly lower in the glycyrrhizin-treated group than in the saline-treated group, suggesting that these decreases were due to a significant decrease in thrombus formation, i.e., the grade of endothelial cell impairment.
16. The plasma concentration of intravenously injected glycyrrhizin was previously shown to peak during the 10 min following its injection and then declined, but was still detectable in the subsequent 10 h; therefore, glycyrrhizin may inhibit the adherence of neutrophils by binding to P-selectin within several hours of IVC ligation.
17. Myeloperoxidase (MPO) activity in the ischemia-reperfused muscles of rabbits was shown to increase in the absence of a glycyrrhizin infusion, but remained low in its presence, suggesting that an increase in oxidant production is secondary to enhanced endothelial permeability following ischemia-reperfusion and the infiltration of neutrophils into muscles, which may be prevented by a treatment with glycyrrhizin.
18. The clinical dose and administration method of fondaparinux are 5–7.5 mg/day and a subcutaneous injection, respectively, and its dose was 0.2 mg/kg (subcutaneous injection) in a study on intestinal ischemia-reperfusion in rats by Olanders et al., suggesting that the dose (1.5 mg/kg) administered to rats in our study was comparatively high for animal experiments.
are cross-linked. Fondaparinux did not inhibit venous thrombosis in the present study, and this may have been due to its weaker inhibitory effects on endothelial cell impairment, which subsequently occurs after ‘inflammation’ in a cascade initiated by the adhesion of neutrophils to the vascular endothelium at the local stressed site because of the absence of the inhibitory effects of fondaparinux on enhanced vascular endothelial permeability or MPO activity.26)

**Conclusion**

1. Plasma antithrombin levels were significantly lower in the glycyrrhizin-treated group than in the saline-treated group, but did not significantly differ from those in the baseline control group; therefore, high plasma antithrombin levels have potential as a marker of the promotion of DVT.

2. Antithrombin mRNA expression in the liver was significantly enhanced by ligation of the IVC, and this may serve as a stress marker of venous thrombosis.

3. In the glycyrrhizin-induced process of the inhibition of thrombosis, antithrombin mRNA expression levels in the liver and IVC were significantly lower than those in the saline-treated group, suggesting a relationship with the inhibition of thrombosis; however, the underlying mechanisms have not yet been elucidated in detail.

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**Disclosure Statement**

The authors declare no conflicts of interest.

**Author Contributions**

Study conception: NN
Data collection: NN, YK
Analysis: NN, YK
Investigation: NN, YK
Writing: NN
Critical review and revision: NN, YK
Final approval of article: NN, YK
Accountability for all aspects of the work: NN, YK.

**References**


