

## Basic and clinical study on the antithrombotic mechanism of glycosaminoglycan extracted from sea cucumber

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**Objective** To investigate the antithrombotic mechanism of glycosaminoglycan ( GAG ) extracted from sea cucumber.

**Methods** We studied the effects of GAG on the coagulant pathway by measuring clotting time. The antithrombin mechanism of GAG was checked by assaying its effects on the thrombin activity in normal human pooled plasma, purified human heparin cofactor II system and antithrombin III system. The effects of GAG on the assembly, dispersion, and structure of fibrin gels as well as on the activity of plasmin were studied by means of turbidimetry, electron microscopy, and chromogenic substrate assay. We studied the effect of GAG on the expression and transcription of tissue factor ( TF ) and thrombomodulin ( TM ) in LPS ( lipopolysaccharide )-stimulated human umbilical vein endothelial cells ( HUVECs ), and used heparin as a control. HUVECs were treated with different concentrations of GAG ( 1 µg/ml, 5 µg/ml, and 10 µg/ml respectively ) and 5 µg/ml heparin as a control together with LPS ( 1 µg/ml ). After incubation for 6 hours, TF and TM were investigated by ELISA and the mRNA study was carried out by RT-PCR. In a clinical trail, a series of variables were observed before and after treatment with GAG in patients recovering from cerebral ischemic stroke or suffering from ischemic heart disease.

**Results** The TT and APTT were significantly prolonged by GAG ( 0.1 µg/ml ). GAG inhibited thrombin activity in the presence of HCII with a second order rate constant of  $1.14 \times 10^7 \text{m}^{-1} \cdot \text{min}^{-1}$ , which was 4.6 times higher than that of ATIII. GAG significantly inhibited the polymerization of fibrin monomer and enhanced the activity of plasmin in a concentration dependent manner. GAG could impair TF mRNA expression and up-regulate TM mRNA expression. The result of clinical trail showed that the fat metabolism was enhanced in addition to the anticoagulant and the blood viscosity reducing effects. No side-effect was found.

**Conclusions** GAG mainly affected on the intrinsic pathway of blood coagulation. GAG was similar to dermatan sulfate both in the efficiency and in the mechanism of antithrombin. The acceleration of clot lysis by GAG depended on its ability to increase the activity of plasmin, to inhibit the polymerizing of fibrin monomer, and consequently, to alter the architecture of the fibrin net work. This effect on HUVECs appears to be at a transcriptional level and might be relevant for the antithrombotic action of GAG. GAG possess anticoagulant activity in vivo and it is a promising drug for antithrombotic therapy.

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Holothurian glycosaminoglycan ( GAG ), a glycosaminoglycan extracted from the body wall of the sea cucumber, is primarily composed of sulfated L-fucose. Previous studies demonstrated that GAG has antithrombotic and anticoagulant activities, but the mechanism of which is not yet completely understood. To further investigate the antithrombotic mechanism of GAG, we studied the effects of GAG on coagulant pathway, thrombin activity, clot lysis, the activity of plasmin, the expressions and transcriptions of TF and TM in endothelial cells in vitro. In a clinical study, a series of variables were observed before and after oral administration GAG in patients recovering from cerebral ischemic stroke or suffering from ischemic heart disease.

## METHODS

### Materials

The following materials were purchased from the companies listed: chromozymTH ( Boehringer Mannheim,

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Department of Hematology, the Second Affiliated Hospital, Hunan Medical University, Changsha 410011, China ( Zhang GS )

Germany), Factor IIa (Haematologic Technology) Dermatan sulfate (DS), heparin, fibrinogen, aprotinin, thrombin, lipopolysaccharide (LPS) and standard TF were purchased from Sigma Chemical Co (St Louis, USA). Urokinase (UK, Baoli Co., China), China agkistrodon actulase venom (CAAV) (Guangdong Institute of Snake Venom, China), plasmin activity Kit (Gangci, Shanghai, China), TF ELISA Kit (America Diagnostic Immubind), TM ELISA Kit (Diagnostica Stago, France), TRIzol and SuperScript II Amplification Kit (Gibco-BRL). Human antithrombin-III (AT-III) and heparin cofactor II were purified as described by Griffith.<sup>1</sup> Plasminogen was purified from normal plasma in our lab.

### Coagulation assays

Activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT) were carried out using commercially available reagents in blood coagulation analyzer (ST4 Diagnostica Stago). Different concentrations of GAG (1  $\mu\text{g}/\text{ml}$ , 5  $\mu\text{g}/\text{ml}$ , and 10  $\mu\text{g}/\text{ml}$ ) were added to normal plasma. After a 3 min-incubation at 37°C, APTT, TT and PT reagents were added respectively and assays were carried out on the coagulation analyzer.

### Thrombin activity assay

DS (2 mg/ml), heparin (8.375 IU/ml), GAG (0.25 mg/ml) were added to normal plasma (1 – 2  $\mu\text{l}$ ), AT-III (final concentration 28 nmol/L) and HCII (142 nmol/L), then mixed with factor IIa (24.5 nmol/L). Tris/PEG buffer was added up to total volume 100  $\mu\text{l}$ . After 1 minute incubation at room temperature, 500  $\mu\text{l}$  of chromozym TH (100  $\mu\text{mol}/\text{L}$ ) was added. Absorbance at 405 nm was measured continuously from 0 to 120 seconds. The second order rate constant was determined according to the equation.  $K_2 = \text{Ln}([P]_0/[P]) / t \cdot a / [\text{HCII}]$ , where t is reaction time;  $[P]_0$  and  $[P]$  are the remaining thrombin activity at time t and initial thrombin activity, respectively; and  $[\text{HCII}]$  is initial HCII concentration.

### Fibrin monomer polymerization assay

Plasminogen-rich or plasminogen-free human fibrinogen (2.0 mg/ml) solution (0.05 mol/L Tris-HCl, pH 7.45) was mixed with various amounts of GAG (5, 10, 20  $\mu\text{g}/\text{ml}$ ). Thrombin solution (0.5 U/ml) was added to the mixture with or without aprotinin (10 IU/ml) and the  $A_{340}$  was measured continuously for 10 minutes.

Plasminogen-free human fibrinogen solution was mixed with various concentrations of GAG (5, 10, 20  $\mu\text{g}/\text{ml}$ ). After incubation at room temperature for 10 minutes, the assay was carried out following the method previously described.<sup>2</sup>

### Effect on the mass-length ratio ( $\mu$ ) of fibrin

Purified fibrinogen solution (1.5 mg/ml in HBS, pH 7.35) was mixed with various amounts of GAG (5, 10, 20  $\mu\text{g}/\text{ml}$ ) and aprotinin (10 IU/ml). After incubation at room temperature for 10 minutes, thrombin (0.5 U) was added to the mixture. Effect of GAG on the mass-length ratio of fibrin was performed as previously described.<sup>3</sup>

### Effect of GAG on fibrin fibre diameter

The samples were incubated overnight at 37°C. Fibrin fibre diameter was observed under electron microscopy as Gruber described.<sup>4</sup>

### Effect of GAG on the dissolution of fibrin gel

Fibrin degradation product (FDP) release and measurement of clot lysis using a turbidity technique were performed as Gruber<sup>4</sup> and McDonagh<sup>5</sup> described.

### Effect of GAG on plasmin activity

500 U/ml of UK was added to a solution of plasminogen (0.1 mg/ml) to completely convert plasminogen to plasmin. Various amounts of GAG were added to the mixture. Determination of plasmin was carried out following the procedure provided in the plasmin activity assay kit.

### Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured according to the method of Jaffe et al.<sup>6</sup> Cells were used after reaching confluency. HUVECs were divided into 6 groups: (1) control group: without any treatment; (2) LPS stimulated group: cells were treated by LPS (1  $\mu\text{g}/\text{ml}$ ); (3) heparin group: cells were incubated simultaneously with LPS (1  $\mu\text{g}/\text{ml}$ ) and heparin (0.625 IU/ml); (4) LPS plus 1  $\mu\text{g}/\text{ml}$  GAG group: cells were incubated simultaneously with LPS (1  $\mu\text{g}/\text{ml}$ ) and GAG (1  $\mu\text{g}/\text{ml}$ ); (5) LPS plus 5  $\mu\text{g}/\text{ml}$  GAG group: cells were incubated simultaneously with LPS (1  $\mu\text{g}/\text{ml}$ ) and GAG (5  $\mu\text{g}/\text{ml}$ ); (6) LPS plus 10  $\mu\text{g}/\text{ml}$  GAG group: cells were incubated simultaneously with LPS (1  $\mu\text{g}/\text{ml}$ ) and GAG (10  $\mu\text{g}/\text{ml}$ ). After another 6 hours in culture, cells were used in assays.

### Procoagulant activity (PCA) assay

After three washes using cold PBS buffer (pH 7.4), cells were scraped from the dish and suspended in 500  $\mu\text{l}$  of cold PBS. After centrifugation at 12 000 g for 20 minutes at 4°C, cells were suspended in 50  $\mu\text{l}$  cold PBS and PCA was measured by a one-stage clotting assay. Cells were incubated with 50  $\mu\text{l}$  of plasma for 180 second, then 50  $\mu\text{l}$  of 0.05 mol/L  $\text{CaCl}_2$  were added and the clotting time was monitored on a blood coagulant analyzer (ST4 Diagnostica Stago). PCA was expressed as units of activity per well,

using calibration curves prepared with dilutions of a standard human TF preparation , which clotted human plasma in 45 seconds as a unit.

### TF antigen ( TF: Ag ) and TM antigen ( TM: Ag ) assay

After cell culture , cells were washed three times using cold PBS and 250  $\mu$ l 1% Triton X-100/TBS was added to each well. After incubation at 4°C for 2 hours , the lysate was centrifuged at 12 000 g for 20 minutes at 4°C . Total cell protein was determined spectrophotometer at 280 nm. TF :Ag and TM: Ag were measured by using TF ELISA Kit and TM ELISA Kit. The units of TF:Ag and TM: Ag were expressed as pg per mg cell protein and  $\mu$ g per mg cell protein.

### RNA isolation and reverse transcription polymerase chain reaction ( RT-PCR )

Total RNA was extracted by a single-step method. The RNA was quantified by optical density at 260 nm and analyzed by formaldehyde agarose electrophoresis. First strand cDNA was synthesized by the SuperScript II Amplification System. TF forward primer , 5 'GGATGTGAAGCAGACGT-ACT3 ' ; TF reverse primer , 5 'GTGTAGAGATATAGCC-AGGA3 ' . The TF primers produce an expected product of 535 bp. TM forward primer , 5 'GCTGCCGATGTCATTT-CCTT3 ' ; TM reverse primer , 5 'TAGTTAGGGTAGCAGTG-GCA3 ' . TM primers produce an expected product of 866 bp. To normalize the PCR amplification in different cDNA preparations , a second set of PCR oligonucleotide primers against a control protein , glyceraldehyde-3-phosphate dehydrogenase ( G3PDH ) , was synthesized : G3PDH forward primer , 5 ' ACCACAGTCCATGCCATCAC3 ' ; G3PDH reverse primer , 5 'TCCACCACCCTGTGCTGTA3 ' . G3PDH primers produce an expected product of 452 bp. PCR was performed using the reverse transcription products. After 5 minutes denaturation at 95°C , 35 cycles were performed at 95°C for 1 minute , 58°C for 1 minute , 72°C for 1 minute. 10  $\mu$ l of PCR amplification products were checked on a 2% agarose gel using ethidium bromide staining.

### Clinical trial of GAG

Subjects consisted of 84 patients recovering from cerebral ischemic stroke , 67 patients with ischemic heart disease , and 53 normal middle-aged volunteers , each of which were divided into two groups according to the GAG dosage administered( 60mg/d and 120mg/d ). Before and after oral administration of GAG , the following parameters were measured : APTT , PT , TT and fibrinogen were assayed with a Stago ST4 coagulation analyzer ; blood viscosity( 10s<sup>-1</sup> , 60s<sup>-1</sup> , 120s<sup>-1</sup> ) and plasma viscosity were measured by LBY-N6 rheometer ; triglyceride ( TG ) , cholesterol ( Ch ) , apolipoprotein A ( Apo-A ) and apolipoprotein B ( Apo-B ) were measured by a Beckman CX7 biochemical analyzer.

### Statistical analysis

Data are expressed as mean  $\pm$  SD. Statistical analysis was performed using Student test.

## RESULTS

### Effects of GAG on TT , APTT and PT

The effect of GAG and heparin on the thrombin clotting time of normal plasma is shown in Table 1. TT was markedly prolonged by GAG ( 0.5  $\mu$ g/ml ) and heparin ( 0.1  $\mu$ g/ml or 0.0125 IU/ml ). Similarly , the APTT was significantly prolonged by GAG ( 0.1  $\mu$ g/ml ) and heparin ( 0.0125 IU/ml ). On the contrary , GAG and heparin had no effect on PT prolongation until they were at high concentrations ( 10  $\mu$ g/ml and 5  $\mu$ g/ml , respectively ).

### Effects of GAG on the activity of thrombin

In the presence of AT-III , GAG inhibited the activity of thrombin ; the remaining activity of thrombin at 60 and 120 seconds was 50% and 84% , respectively. Compared with heparin , GAG only inhibited 12.3% and 16% of thrombin activity at 60th and 120th minute , respectively ( Fig. 1 ).

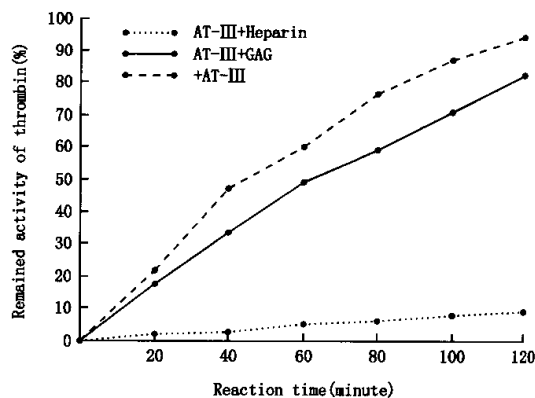


Fig. 1. Inhibitory effect of GAG on thrombin in the presence of AT-III ( 28 nmol/L ).

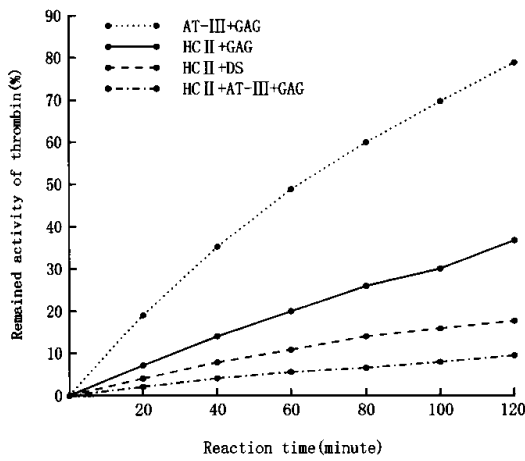
In the presence of HCII , GAG inhibited thrombin activity and the activity of thrombin was 19.8% and 36.8% at 60 and 120 seconds , respectively. Figure 2 shows that the effect of GAG in the presence of HCII is greater than that of GAG in the presence of AT-III .

In the presence of HCII and AT-III together , the activity of thrombin was significantly inhibited by GAG. The inhibitory effect is greater than the summation of each effect ( Tables 1 and 2 ) , implying a synergistic effect.

**Table 1.** Effects of GAG and heparin on TT , APTT and PT ( n = 2 )

Concentration (μg/ml)	TT (second)		APTT (second)		PT (second)	
	GAG	Heparin	GAG	Heparin	GAG	Heparin
0	20.47±0.42	20.47±0.42	37.65±0.07	37.6±0.07	14.1±0.21	14.13±0.21
0.1	21.50±0.14	22.85±0.21*	57.95±0.21**	45.1±0.35**	13.7±0.28	13.30±0.14
0.5	22.95±0.64*	33.65±2.33**	70.4±1.20**	79.4±1.91**	13.9±0.78	13.35±0.07
1.0	31.35±0.35**	115.6±0.85	120.6±7.50**	>251	13.0±0.14	12.85±0.07
5.0	64.05±3.32**	>251	187.95±5.73**	>251	13.5±0.28	18.80±0.14**
10.0	>251	>251	>251	>251	23.5±0.14*	133.05±4.45**

\* P < 0.05 , \*\* P < 0.01 .



**Fig. 2.** Inhibitory effect of GAG on thrombin in the presence of HCII ( 142 nmol/L ) or AT-III ( 28 nmol/L ) .

In the presence of HCII , the second order rate constant for GAG is  $1.14 \times 10^7 \text{ m}^{-1} \cdot \text{min}^{-1}$  . In the presence of AT-III ,  $K_2$  for GAG is  $2.48 \times 10^6 \text{ m}^{-1} \cdot \text{min}^{-1}$  . The  $K_2$  for GAG in the presence of HCII is as 4.6 times as the  $K_2$  for GAG in the presence of AT-III .

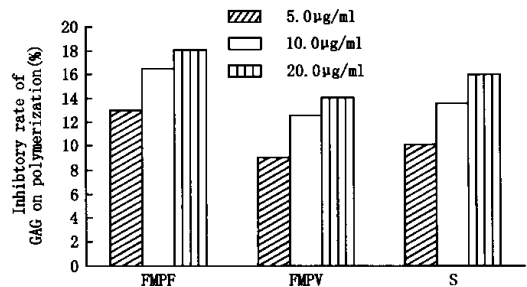
**Table 2.** Effects of GAG on the activity of thrombin in the presence of heparin cofactors

Heparin cofactor + activators	Activity of thrombin ( % )	
	60th second	120th second
AT-III		
+ heparin	5.7	12.3
+ GAG	50.0	84.0
HCII		
+ DS	10.9	20.0
+ GAG	19.8	36.8
HCII + AT-III		
+ GAG	5.2	10.8
GAG only	88.2	100

**Effect of GAG on fibrin monomer polymerization**

The polymerization of fibrin monomer was significantly inhibited by 5 μg/ml GAG. As GAG concentrations increased , the polymerization of fibrin monomer was accelerated. Polymerization was impaired when the GAG concentration was over 100 μg/ml. When either aprotinin or

plasminogen-free fibrinogen were added to the reaction mixture , even 100 μg/ml GAG did not inhibit polymerization , but increase the polymerization of the fibrin monomer. GAG was able to inhibit the polymerization of fibrin monomer ( Fig. 3 ) .



**Fig. 3.** After incubate GAG and Fg solution containing 2.97 mg/ml Fg for 10 minutes , the inhibitory rate of GAG on polymerization was measured. FMPF : faction of fibrin monomer polymerization ; FMPV : CAAV induced polymerization velocity of fibrin monomer ; S : concentration of fibrin monomer that could polymerise .

**Effect on the structure of fibrin gels**

The mass-length ratio of fibrin fibres formed in the presence of GAG ( 5 , 20 , 40 μg/ml ) increased by 1.49 , 1.73 and 2.28 times , respectively. Electron microscopy showed that the average diameter of fibrin fibres formed in the presence of GAG at 20 μg/ml was  $2.598 \pm 0.250 \text{ nm}$  ( n = 20 ) and the control was  $1.611 \pm 0.288 \text{ nm}$  ( P < 0.001 ) ( data not show ) .

**Effect of GAG on the dissolubility of fibrin gels**

The thrombin induced clot containing GAG was more easily lysed , and the dissolution of the clot was faster than the control ( P < 0.005 ) .

**Effect of GAG on plasmin activity**

Plasmin activity was enhanced by 27.98% , 34.88% and 37.21% in the presence of GAG at 15 , 30 and 60 μg/ml , respectively. Degradation of the clot by plasmin was enhanced by GAG .

**Effect of GAG on the Expression of TF and TM in Stimulated Endothelial Cells**

LPS ( 1 μg/ml ) significantly enhanced PCA , TF : Ag levels , and decreased the TM : Ag level expression in HUVECs compared to controls ( absence of LPS ) . PCA enhanced by LPS was significantly decreased by 5 μg/ml GAG and 0.625 IU/ml heparin ( Table 3 ) . The increased TF:Ag level was significantly reduced by 0.625 IU/ml heparin , 1 μg/ml GAG , 5 μg/ml GAG and 10 μg/ml GAG ( P < 0.001 , P < 0.05 , P < 0.001 , P < 0.001 , respectively ) , in which 5 μg/ml GAG was more significant

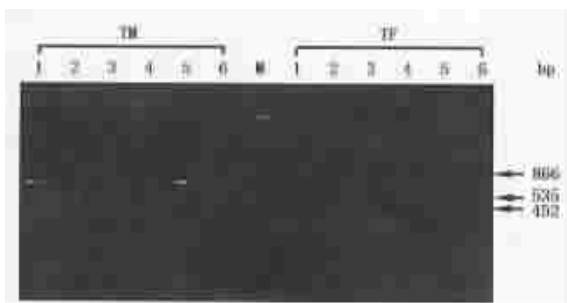
than 0.625 IU/ml heparin and 1 μg/ml GAG ( *P* < 0.005 ). At the same time , the decreased TM:Ag level was increased by GAG and heparin and 5 μg/ml GAG was more effective on TM:Ag levels than the other two groups. RT-PCR results showed that LPS could stimulate TF gene transcription and suppressed TM gene transcription , GAG could down-regulate TF mRNA expression and up-regulate TM mRNA expression , especially at a concentration of 5 μg/ml ( Table 3 , Fig. 4 ).

**Table 3.** Effects of GAG on the PCA, the expressions TF:Ag and TM:Ag in HUVECs

	n	PCA (U/well)	TF:Ag (pg/mg cell protein)	TM:Ag (ng/mg cell protein)
Control	2	1.19 ± 0.14	4033.31 ± 157.98	1631.70 ± 31.88
LPS induce	3	3.12 ± 0.41	14829.27 ± 754.47	669.41 ± 51.25
Heparin	3	1.87 ± 0.02*	8899.43 ± 233.72***	1254.20 ± 79.03***
LPS plus 1 μg/ml GAG	3	1.94 ± 0.33	12673.01 ± 668.84*	1161.74 ± 15.49**
LPS plus 5 μg/ml GAG	3	1.68 ± 0.05*	7418.59 ± 239.99***	1787.84 ± 82.62***
LPS plus 10 μg/ml GAG	3	2.38 ± 0.30	9021.37 ± 716.57***	1391.84 ± 25.53***

Compared with LPS stimulated group , \* *P* < 0.05 ; \*\* *P* < 0.005 ;

\*\*\* *P* < 0.001.



**Fig. 4.** Effects of GAG on the TF and TM transcription in HUVECs. 1. Control ; 2. LPS induced ; 3. Heparin ; 4. LPS plus 1 μg/ml GAG ; 5. LPS plus 5 μg/ml GAG ; 6. LPS plus 10 μg/ml GAG ; M : DNA Marker.

**Clinical trial of GAG**

In normal subjects , APTT , PT and TT were significantly prolonged and blood viscosity , plasma viscosity , TG and Ch were reduced after oral administration of GAG. Apo-A was enhanced and Apo-B was reduced in the 120 mg/d GAG group ( Table 4 ). In cerebral ischemic stroke patients , APTT and TT were prolonged and blood viscosity , Ch , TG and Apo-B were significantly decreased after oral administration of GAG. Plasma viscosity and Apo-A were decreased only in the 120 mg/d GAG group. PT and Fg showed no significant difference before and after treatment with GAG ( Table 5 ). In the ischemic heart disease group , APTT and TT were prolonged and blood viscosity was decreased ( Table 6 ).

**Table 4.** Effects of GAG on healthy subjects

	60 mg/d ( n = 25 )		120 mg/d ( n = 28 )	
	Before treatment with GAG	After treatment with GAG	Before treatment with GAG	After treatment with GAG
APTT ( second )	42.88 ± 4.37	47.48 ± 4.41#	43.28 ± 4.69	49.29 ± 5.23#
PT ( second )	12.16 ± 0.59	13.24 ± 1.03Δ	12.53 ± 0.84	14.94 ± 0.89#
TT ( second )	17.21 ± 0.55	29.62 ± 5.05#	17.79 ± 1.08	27.63 ± 3.05#
Fg ( g/L )	3.73 ± 0.79	3.99 ± 1.19	4.07 ± 1.19	3.88 ± 0.80
Blood viscosity ( mPa·s )				
10s <sup>-1</sup>	9.67 ± 2.47	8.26 ± 1.26*	10.40 ± 1.63	8.71 ± 1.38#
60s <sup>-1</sup>	5.67 ± 0.58	5.11 ± 0.51#	6.01 ± 0.62	5.41 ± 0.53#
120s <sup>-1</sup>	5.01 ± 0.40	4.56 ± 0.39#	5.24 ± 0.51	4.82 ± 0.37#
Plasma viscosity ( mPa·S )				
	1.76 ± 0.15	1.56 ± 0.16#	1.89 ± 0.13	1.77 ± 0.11*
TG ( mg/L )	165.38 ± 78.31	110.44 ± 59.29Δ	144.81 ± 41.64	110.38 ± 37.85#
Ch ( mg/L )	213.93 ± 34.25	155.88 ± 38.42#	203.31 ± 29.63	180.25 ± 25.66Δ
ApoA ( g/L )	1.29 ± 0.05	1.26 ± 0.12	1.23 ± 0.10	1.42 ± 1.08#
ApoB ( g/L )	0.81 ± 0.14	0.83 ± 0.27	0.91 ± 0.11	0.76 ± 0.10#

\* *P* < 0.05 ; Δ *P* < 0.01 ; # *P* < 0.001.

**Table 5.** Effects of GAG on the patients recovering from cerebral embolism

	60 mg/d ( n = 35 )		120 mg/d ( n = 32 )	
	Before treatment with GAG	After treatment with GAG	Before treatment with GAG	After treatment with GAG
APTT ( second )	45.50 ± 4.37	48.78 ± 6.81Δ	43.81 ± 3.66	48.17 ± 7.58Δ
PT ( second )	12.54 ± 0.92	12.78 ± 0.99	13.24 ± 1.03	13.50 ± 1.06
TT ( second )	16.80 ± 1.26	18.02 ± 1.62Δ	16.16 ± 1.36	17.15 ± 2.23Δ
Fg ( g/L )	4.21 ± 0.46	4.02 ± 0.71	3.99 ± 0.88	4.08 ± 0.9
Blood viscosity ( mPa·s )				
10s <sup>-1</sup>	10.28 ± 1.11	9.84 ± 1.15*	10.32 ± 1.79	9.47 ± 1.56*
60s <sup>-1</sup>	6.16 ± 0.24	5.78 ± 0.74*	6.12 ± 0.91	5.80 ± 0.79*
120s <sup>-1</sup>	5.21 ± 0.47	5.14 ± 0.65	5.78 ± 1.99	5.20 ± 0.52*
Plasma viscosity ( mPa·S )				
	1.85 ± 0.10	1.82 ± 0.16	1.80 ± 0.14	1.73 ± 0.16*
TG ( mg/L )	171.45 ± 90.69	133.75 ± 37.83*	176.75 ± 64.43	141.10 ± 48.17Δ
Ch ( mg/L )	215.95 ± 51.98	195.15 ± 27.27*	229.70 ± 85.03	184.13 ± 32.06Δ
ApoA ( g/L )	1.38 ± 0.15	1.33 ± 0.18	1.32 ± 0.08	1.42 ± 0.16Δ
ApoB ( g/L )	0.95 ± 0.16	0.86 ± 0.10Δ	0.93 ± 0.09	0.88 ± 0.10Δ

\* *P* < 0.05 ; Δ *P* < 0.01 ; # *P* < 0.001.

**Table 6.** Effects of GAG on the patients suffering from ischemic heart disease

	60 mg/d ( n = 33 )		120 mg/d ( n = 34 )	
	Before treatment with GAG	After treatment with GAG	Before treatment with GAG	After treatment with GAG
APTT ( second )	44.80 ± 6.08	49.13 ± 5.15Δ	43.74 ± 5.15	47.25 ± 7.46Δ
PT ( second )	13.00 ± 1.33	13.39 ± 1.94	13.18 ± 1.24	13.60 ± 1.70
TT ( second )	16.95 ± 1.35	18.23 ± 2.40Δ	16.99 ± 1.55	17.90 ± 1.80*
Fg ( g/L )	4.03 ± 0.68	3.81 ± 0.65	3.84 ± 0.61	3.62 ± 0.53
Blood viscosity ( mPa·s )				
10s <sup>-1</sup>	11.28 ± 3.14	9.91 ± 3.41*	11.62 ± 3.97	10.11 ± 3.50
60s <sup>-1</sup>	7.14 ± 2.68	6.37 ± 1.82	7.64 ± 2.03	6.45 ± 1.78*
120s <sup>-1</sup>	5.71 ± 1.80	4.62 ± 1.17	5.59 ± 1.43	4.96 ± 1.26*
Plasma viscosity ( mPa·S )				
	1.65 ± 0.25	1.59 ± 0.24	1.79 ± 0.26	1.64 ± 0.25Δ
TG ( mg/L )	166.50 ± 88.74	147.54 ± 45.03	159.64 ± 38.70	142.00 ± 37.50*
Ch ( mg/L )	211.25 ± 43.44	199.53 ± 38.50	219.50 ± 39.25	202.40 ± 37.60Δ
ApoA ( g/L )	1.29 ± 0.12	1.30 ± 0.14	1.31 ± 0.13	1.42 ± 0.15Δ
ApoB ( g/L )	0.93 ± 0.17	0.87 ± 0.12Δ	0.92 ± 0.13	0.80 ± 0.11Δ

\* *P* < 0.05 ; Δ *P* < 0.01.

## DISCUSSION

The results show that TT and APTT could be significantly prolonged by low concentrations of GAG, which could not prolong PT in vitro. We suppose that GAG mainly affects the intrinsic pathway of blood coagulation, which we confirmed by the thrombin generation assay. In a purified protein system, kinetic analysis of thrombin inhibition confirmed that GAG inhibited thrombin mainly in a HCII-dependent manner, and slightly in an AT-III-dependent manner. Nagase et al<sup>7</sup> showed that GAG was an anticoagulant with two different inhibitory activities, one being the HCII-dependent inhibition of thrombin and the other the ATIII- and HCII- independent inhibition of the activation of factor X by the factor IXa-factor VIIIa complex. In light of those findings, it is clear that further investigation of the effects of GAG on the components of intrinsic factor is required to elucidate the heparin cofactor-independent anticoagulant effect of GAG.

Our results shown that GAG could increase the mass-length ratio ( $\mu$ ) and decrease the polymerization of fibrin monomer. In the presence of GAG, the average diameter of fibrin fibres became thicken, and the fibrin clot was more easily degraded by plasmin. As  $\mu$  correlates positively with the absorbance of fibrin gel, so  $\mu$  increases when the absorbance increases. However, GAG can enhance the activity of plasmin and the effect of GAG was dose-dependent, so when the concentration of GAG reached a certain level (100  $\mu\text{g}/\text{ml}$ ), absorbance decreased. When aprotinin was added to the reaction mixture or plasminogen-free fibrinogen was used, even 100  $\mu\text{g}/\text{ml}$  GAG could not reverse the absorbance. This suggests that GAG not only inhibits the aggregation of fibrinogen and the polymerization rate of fibrin monomers, but also enhances the activity of plasmin. These preliminary results suggest that the acceleration of clot lysis by GAG depended on its ability to increase the activity of plasmin, to inhibit the polymerization of fibrin monomer, and consequently, to alter the architecture of the fibrin network.

These results show that GAG down-regulated both TF antigen and gene expression and up-regulated TM antigen and gene expression. This effect on HUVECs appears to be at a transcriptional level and might be related to the antithrombotic action of GAG. GAG's anticoagulant effect is

not dose dependent, which may due to its complex pharmacokinetics<sup>8</sup> and need further study.

The clinical trial indicated that GAG not only anticoagulates but also decreases blood viscosity. In normal subjects, blood and plasma viscosity were reduced after treatment by GAG, in both dose groups. This effect of GAG was also seen in the cerebral ischemic stroke patient and the ischemic heart disease patients groups.

We conclude that GAG, as an anticoagulant substance, mainly affect thrombin activity in an HCII-dependent manner. The acceleration of clot lysis by GAG depended on its ability to increase the activity of plasmin and to inhibit the polymerizing of fibrin monomer. GAG can down-regulate both TF antigen and gene expression and up-regulate both TM antigen and gene expression. GAG also decreases blood viscosity. From these data, GAG appears to be a promising antithrombotic agent.

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