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RESEARCH ARTICLE



# The antithrombosis effect of dehydroandrographolide succinate: *in vitro* and *in vivo* studies

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**ABSTRACT**

**Context:** Dehydroandrographolide succinate (DAS) is mainly used in the clinical treatment of various infectious diseases. Its potential effects on platelet aggregation and blood coagulation systems have not been reported systematically.

**Objective:** To explore whether DAS exerts an antithrombotic effect and its internal mechanism.

**Materials and methods:** Human blood samples and Sprague-Dawley (SD) rats divided into control, aspirin (30 mg/kg), and DAS groups (200, 400 and 600 mg/kg) were used to measure the platelet aggregation rate, coagulation function, coagulation factor activity, and contents of thromboxane $B_2$ ($TXB_2$) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$). The histopathology of the SD rat gastric mucosa was also observed. All rats were administered intragastric or intraperitoneal injections once a day for 3 consecutive days.

**Results:** Compared to control group, DAS significantly inhibited the platelet aggregation rate ($ED_{50}$ = 386.9 mg/kg) by decreasing $TXB_2$ levels (1531.95 ± 649.90 pg/mL to 511.08 ± 411.82 pg/mL) and activating antithrombin III (AT-III) (103.22 ± 16.22% to 146.46 ± 8.96%) ($p < 0.05$). In addition, DAS significantly enhanced the coagulation factors FV (304.12 ± 79.65% to 443.44 ± 75.04%), FVII (324.19 ± 48.03% to 790.66 ± 225.56%), FVIII (524.79 ± 115.47% to 679.92 ± 143.34%), FX (34.90 ± 7.40% to 102.76 ± 29.41%) and FXI (38.12 ± 10.33% to 65.47 ± 34.08%), increased the content of Fg (2.18 ± 0.39 to 3.61 ± 0.37 g/L), shorten the PT (10.42 ± 0.44 to 9.22 ± 0.21 s), APTT (16.43 ± 1.4 to 14.07 ± 0.75 s) and TT time (37.04 ± 2.13 to 32.68 ± 1.29 s) ($p < 0.05$), while the aspirin group showed no such effect on these items but showed reduced activity of FII (89.21 ± 21.72% to 61.83 ± 8.95%) and FVIII (524.79 ± 115.47% to 306.60 ± 29.96%) ($p < 0.05$). Histopathological changes showed aspirin-induced gastric mucosa haemorrhage and the protective effect of DAS in the gastric mucosa.

**Conclusions:** DAS is more suitable than aspirin in thromboprophylaxis treatment, which provides a reliable theoretical and experimental basis for its clinical application.

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Platelet aggregation; thromboxane; aspirin; haemorrhage; andrographolide

## Introduction

The formation of thrombi is the main step leading to heart, brain, and peripheral vascular events and is the direct cause of death or disability. The formation of thrombi is mainly composed of two steps: primary hemostasis resulting from platelet activation, and secondary hemostasis that activates the process of fibrin formation and thrombus reinforcement. White thrombus is the main product of primary hemostasis; its formation depends on various factors (such as thrombin, platelet activating factor, and thromboxane $A_2$ [$TXA_2$]) to activate platelets. Afterwards, with the cooperation of the coagulation factor cascade, platelets are induced to form a stable thrombus by secondary hemostasis (Bye et al. 2016). Since the white thrombosis process is reversible, prevention and intervention in primary hemostasis may avoid the occurrence of secondary hemostasis and prevent the damage caused by subsequent thrombotic events.

In current clinical treatment, certain side effects have been observed during the use of commonly employed antiplatelet agents for targeted primary hemostasis, such as thrombin inhibitors, ADP receptor inhibitors, and cyclooxygenase (COX) inhibitors. For example, because of the non-selective inhibition of COX by aspirin, low reactivity, serious side effects, gastrointestinal haemorrhage, and other problems may occur during treatment (Group et al. 2018). The thromboxane receptor inhibitor terutroban exerts a secondary preventive effect on patients with thrombotic cerebral ischaemia, but the haemorrhagic side effects of terutroban are still slightly higher than those of aspirin (Bousser et al. 2011; Lee and Ovbiagele 2011). Clopidogrel also

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has the problem of low reactivity and bioavailability in some patients. Aspirin combined with clopidogrel is often used for antiplatelet aggregation therapy; however, the risk of haemorrhage has not been effectively improved (Yusuf et al. 2001; Bates et al. 2011; Katsanos et al. 2015), and in some patients, this treatment also causes problems such as decreases in the numbers of neutrophils and platelets and aplastic anaemia (Maurício et al. 2014). Therefore, demand for antiplatelet aggregation drugs with high efficiency, low toxicity, a low haemorrhage risk, and low prices in clinical practice is still great.

*Andrographis paniculata* (Burm. f.) Wall. ex Nees (Acanthaceae) (AP), is a traditional medicine that is mainly distributed in South China and Southeast Asia (Okhuarobo et al. 2014; Kumar et al. 2021). The plant contains the highest level of andrographolide, a diterpene lactone and major component used for treatment (Sareer et al. 2014; Hossain et al. 2021). Current research has found that andrographolide is a clinical drug with an α,β-unsaturated lactone group that inhibits nuclear factor-kappa B (NF-κB) DNA binding, exhibits potent anti-inflammatory properties and prevents acute lung injury (Zhu et al. 2013; Nguyen et al. 2015). It has also been shown to increase chemotherapeutic sensitivity in colorectal cancer and to exert an antitumor effect by inhibiting the Wnt/β-catenin signalling pathway. According to a few previous studies, AP extract produces antiplatelet effects *in vitro*. One study found that two diterpene lactones, including andrographolide, extracted from AP may inhibit thrombin-induced platelet aggregation (Thisoda et al. 2006). In another study, andrographolide and AP inhibited platelet-activating factor (PAF)-induced human blood platelet aggregation (Amroyan et al. 1999). Although, in recent years, AP has been reported to inhibit platelet aggregation potentially, it has not been observed to affect the mRNA expression of COX or the production of $TXB_2$ and P-selectin in clinical study (Sirikarin et al. 2018). In conclusion, a clear consensus on the specific mechanism underlying these phenomena is currently unavailable.

Moreover, andrographolide is insoluble in water and not easily absorbed, leading to a decrease in its bioavailability. DAS, a derivative of andrographolide that is refined by esterification, dehydration and salification of andrographolide and finally refined into potassium sodium dehydroandroandrographolide succinate, has a much higher absorption efficiency than andrographolide. Compared with andrographolide, this derivative is less likely to cause cytotoxicity and is safer to use. At present, DAS and its similar diterpene lactones are mainly used in the clinical treatment of respiratory tract infection and virus-induced fever (Reddy et al. 2005; Wiart et al. 2005; Wintachai et al. 2015; Peng et al. 2016; Tan et al. 2017), as a vasorelaxant (Yoopan et al. 2007) and to treat other diseases. Therefore, in this paper, by testing human blood *in vitro* and rats *in vivo*, we analysed the effect of DAS on primary and secondary hemostasis of the coagulation system and its underlying mechanism to provide theoretical support for its clinical application.

## Materials and methods

### Experimental drugs

DAS (0.2 g/bottle) was obtained from Hidragon Pharma Co., Ltd., Hubei, China, batch number: 1901083. Aspirin was obtained from Bayer Pharma Co., Ltd., Beijing, China, batch number: BJ50050.

### Human blood

All human blood studies were approved by the Medical Ethics Committee at Jiangxi Provincial People's Hospital (Ethics Committee protocol number: 2019 − 017). Volunteers were recruited after explaining all details of the experiments and were allotted time to ask any questions and consider participation. Afterwards, volunteers signed a consent form. Fifty healthy volunteers aged 50–60 years old were selected, with equal numbers of males and females, and blood was drawn in two tubes (2.0 mL per tube) with a 3.8% sodium citrate solution for anticoagulation (1:9).

### Animals

The animal study was approved by the Medical Ethics Committee of Jiangxi Provincial People's Hospital (Ethics Committee protocol number: 2019 − 017). Male rats, SD strain, body weight 250–270 g, purchased from Hunan Slack Jingda Experimental Animal Co., Ltd., Hunan, China, licence number SCK (Xiangmu) 2019 − 0004. Based on changes in the platelet aggregation rate, previous experiments and reference values, the minimum number of animals in each group calculated using PASS software (PASS 15.0, NCSS, Kaysville, Utah, USA) is $n = 8$ when $\alpha = 0.05$, $1-\beta = 0.90$, and $SD = 7.20$, 19.04, 18.46, and 21.01. When $\alpha = 0.05$, $1-\beta = 0.95$, and $SD = 7.97$, 6.67, 13.86, and 16.52, $n = 7$ human blood samples are needed. Two and three spare sample were added to each animal and human group to avoid missing data or loss to follow-up and to increase the power of the test. Finally, the total number of samples in each group *in vitro* and *in vivo* was confirmed to be $n = 10$ for a total of 50 (Noordzij et al. 2011).

### Reagents

Arachidonic acid was purchased from Helena, USA, production batch number: 21985364. The hemagglutination reagent was a Japanese original kit provided by Sysmex Co., Ltd. The Thromboxane $B_2$ ELISA kit, ab133022, was purchased from Abcam Co., Ltd., USA, batch number GR3261335–1; the 6-keto-$PGF_{1\alpha}$ ELISA kit, ab133023, was purchased from Abcam Co., Ltd., USA, batch number GR3251905–2.

### Apparatus

The AggRAM aggregation remote analyser module, model BJF52001, was provided by Helena Laboratories Co., Ltd., USA. An automated blood coagulation analyser, model CA-8000, was purchased from Sysmex Medical Electronics Co., Ltd. (Shanghai). The microplate reader MULTISKAN Sky, model 1530, was purchased from Thermo Scientific Co., Ltd., USA. Centrifuges, continuously adjustable sample dispensers, and other equipment are commonly used devices in the laboratory.

### Platelet aggregation test with turbidimetry (PAgT) induced by arachidonic acid added to human blood in vitro

Anticoagulated blood was divided into five groups, namely, the control group (equal volume of normal saline), the aspirin group (aspirin 0.15 mg/mL), and the DAS low (0.5 mg/mL), medium (1.0 mg/mL), and high (1.5 mg/mL) dose groups. One tube from each group was incubated at 37 °C for 2 h and centrifuged at

room temperature to obtain platelet-rich plasma. Then, 225 µL of platelet-rich plasma and 25 µL of arachidonic acid (5 µmol/L) were added to the measuring tube, and the maximum platelet aggregation rate was measured with an AggRam platelet aggregation instrument. The other tube was used to detect coagulation factors and coagulation function after treatment.

### PAgT induced by arachidonic acid administered to SD rats

SPF-grade male SD rats were selected and randomly divided into 5 groups, each with 10 rats: a control group (equal volume of normal saline), an aspirin group (30 mg/kg), and DAS low (200 mg/kg)-, medium (400 mg/kg)-, and high (600 mg/kg)-dose groups. Aspirin was administered intragastrically once a day for 3 consecutive days. DAS was administered by intraperitoneal injection once a day for 3 consecutive days. On the night before the blood was collected, the rats were fasted without water deprivation. Twenty hours after the last treatment, the rats were anaesthetized in the heart with sodium pentobarbital (40 mg/kg) to draw blood, and two tubes (3.0 mL per tube) of blood were drawn and mixed with 3.8% sodium citrate anticoagulant (1:9). The blood was immediately centrifuged at room temperature to obtain platelet-rich plasma. Then, 225 µL of platelet-rich plasma and 25 µL of arachidonic acid (5 µmol/L) were added to the test tube, and the AggRam platelet aggregator was used to determine the maximum platelet aggregation rate. Another tube of anticoagulant-treated blood was used to determine coagulation function, coagulation factor activity, $TXB_2$ and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) levels.

### Detection of TXB2 and 6-keto-PGF1α

Since $PGI_2$ and $TXA_2$ have short half-lives and are unstable, this study detected the downstream stable end-products $TXB_2$ and 6-keto-$PGF_{1\alpha}$ to indirectly reflect the $PGI_2$ and $TXA_2$ contents. The blood was centrifuged for 10 min to obtain platelet-poor plasma, and ELISAs were performed to detect the contents of $TXB_2$ and 6-keto-$PGF_{1\alpha}$. The protocols were performed in accordance with the operating instructions. After colour development was complete, the colour was compared with a microplate reader at a main wavelength of 405 nm and a secondary wavelength of 580 nm.

### Coagulation function test

The anticoagulant-treated blood was centrifuged for 10 min, and the prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (Fg) content, thrombin time (TT) and antithrombin III (AT-III) activity were measured using an automated blood coagulation analyser and its supporting reagents.

### Detection of coagulation factor activity

The anticoagulant-treated blood was immediately used to measure the activity levels of the coagulation factors FII, FV, FVII, FVIII, FIX, FX, FXI, and FXII with an automated blood coagulation analyser and matching coagulation factor detection reagents.

### Observation of HE staining of the gastric mucosa

After the anticoagulated blood of each group of SD rats was obtained as described above, the gastric tissues of the rats were removed and immediately fixed with a formalin solution. Twelve hours later, dehydration, sectioning, HE staining, and optical microscopy observations were performed.

### Data analysis

The Shapiro-Wilk test was used to test the normality of each group. One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test were used to compare the results obtained from the four treatment groups with the control group. Paired *t*-tests were used to determine the significance of changes before and after the administration of the medication. All statistical analyses were performed using SPSS software (SPSS Statistics 26.0, IBM, Armonk, NY, USA), and the significance level was set to $p < 0.05$ ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

## Results

### DAS inhibits platelet aggregation

As shown in Figure 1(a), treatment with 200 mg/kg DAS induced a minimal decrease in the platelet aggregation rate (12.51%), but upon treatment with 400 mg/kg DAS, the platelet aggregation

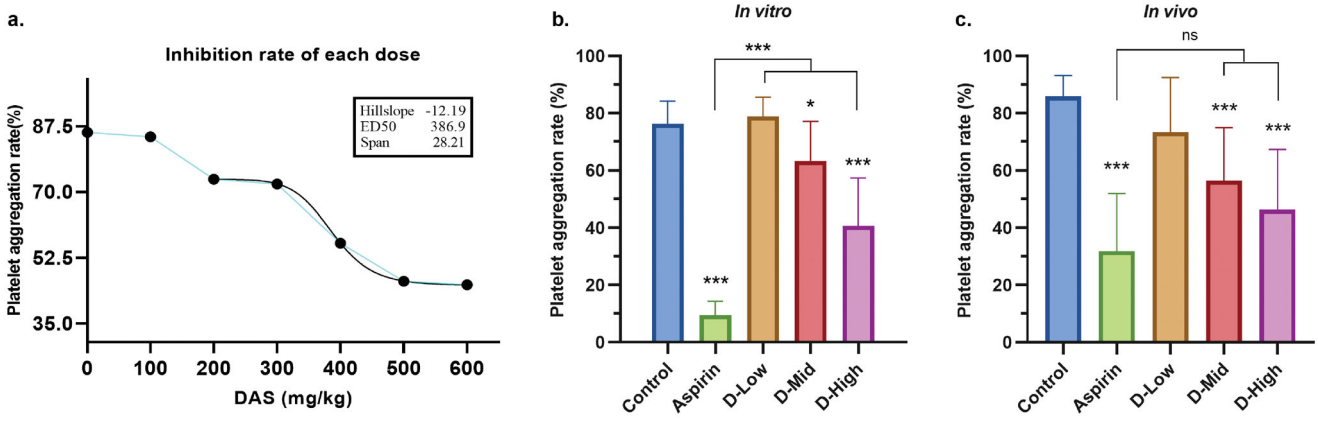


Figure 1. DAS administered to human blood *in vitro* and to SD rats *in vivo* inhibited arachidonic acid-induced platelet aggregation. (a–c) The platelet aggregation test, each group was $n = 10$. (a) DAS treatment inhibited platelet aggregation in SD rats. (b) *In vitro*, the aspirin group exhibited a significantly reduced platelet aggregation rate, and the effect was the strongest. Low-dose DAS had no effect, but the platelet aggregation rates were reduced in the medium- and high-dose groups, and the high dose exerted a significant effect. (c) *In vivo*, the aspirin group showed a significantly reduced platelet aggregation rate, whereas no change was observed in the low-dose DAS group. However, the platelet aggregation rates in the medium- and high-dose groups were significantly reduced. No difference was observed between the high-dose group and the aspirin group.

rate decreased 29.46%. With increasing concentrations, a marked reduction in the platelet aggregation rate was observed, particularly at concentrations of 500 mg/kg (39.61%) and 600 mg/kg (40.56%). Thus, DAS exerted an inhibitory effect on platelet aggregation, and the $ED_{50}$ of 386.9 mg/kg DAS was calculated in this paper. According to the dose–response curves shown in Figure 1(a) and the $ED_{50}$ value of DAS in SD rats, SD rats were treated with DAS at concentrations of 200, 400, and 600 mg/kg in subsequent experiments.

We first used whole blood from healthy people to conduct an arachidonic acid-induced platelet aggregation experiment *in vitro* and determine the effect of DAS on primary hemostasis. Compared with the control group, the platelet aggregation rates of the aspirin and medium- and high-dose DAS groups were significantly reduced ($p < 0.05$), and the antiplatelet aggregation effect of aspirin was significantly stronger than that of DAS ($p < 0.05$) (Figure 1(b)). Subsequently, DAS was administered to SD rats to test the inhibition of arachidonic acid-induced platelet aggregation. Compared with the control group, the platelet aggregation rates of the aspirin and medium- and high-dose DAS groups were reduced to varying degrees ($p < 0.05$), and the antiplatelet aggregation effect of aspirin was significantly stronger than that of DAS ($p < 0.05$) (Figure 1(c)). Based on these results, medium- and high-doses of DAS exerted similar but weaker antiplatelet aggregation effects than aspirin ($p > 0.05$) (Figure 1(b)).

## DAS inhibited TXB2 production

We tested the endogenous arachidonic acid metabolites that may affect platelet aggregation to explore the detailed mechanism by which DAS inhibited platelet aggregation. Based on the results of serum ELISAs *in vivo*, the MIC of 200 mg/kg DAS was tested. Compared with the control group (1531.95 ± 649.90 pg/mL), the aspirin group (27.02 ± 35.62 pg/mL) and the low- (589.27 ± 515.28 pg/mL), medium- (511.08 ± 411.82 pg/mL), and high-dose (714.57 ± 636.06 pg/mL) DAS groups showed significantly reduced contents of $TXB_2$ ($p < 0.05$), and the effect of aspirin was significantly stronger than that of DAS ($p < 0.05$, Figure 2(a)). In addition, the aspirin group exhibited a significantly reduced 6-keto-$PGF_{1\alpha}$ content (control: 3602.68 ± 1479.11 pg/mL, aspirin: 353.53 ± 153.57 pg/mL, $p < 0.05$), while the low- (1964.19 ± 1102.13 pg/mL), medium- (4169.17 ± 2011.74 pg/mL), and high-dose DAS groups (6032.05 ± 4738.57 pg/mL) showed an increasing trend but did not differ from the control ($r = 0.352$, $p > 0.05$, Figure 2(b)).

## DAS enhanced coagulation function

We first carried out *in vitro* experiments using whole blood from healthy people to detect the activities of PT, APTT, Fg and TT before and after the administration of medications and to determine whether DAS altered the secondary hemostasis system. Experimental results showed no significant change in PT after the administration of low (Table 1) and medium doses (Table 2) of DAS, but PT was significantly shorter after the administration of high-dose DAS ($p < 0.05$) (Table 3). The APTT and TT times of the three groups were significantly shorter after the addition of the medication ($p < 0.05$). Moreover, the Fg levels of the low- and high-dose DAS groups were significantly increased ($p < 0.05$). Subsequently, we used DAS to detect its effect on

**Table 1.** Effect of low-dose DAS on coagulation function in human blood *in vitro*.

| Test items | n | Before medication (mean ± SD) | After medication (mean ± SD) | *p* |
|---|---|---|---|---|
| PT (s) | 10 | 10.46 ± 0.36 | 10.57 ± 0.36 | 0.317 |
| APTT (s) | 10 | 32.29 ± 4.05 | 28.89 ± 1.70** | 0.005 |
| Fg (g/L) | 10 | 2.68 ± 0.59 | 2.86 ± 0.61*** | 0.000 |
| TT (s) | 10 | 18.48 ± 0.74 | 17.80 ± 0.49*** | 0.001 |
| AT-III (%) | 10 | 97.50 ± 5.31 | 99.57 ± 4.53 | 0.057 |

**Table 2.** Effect of medium-dose DAS on coagulation function in human blood *in vitro*.

| Test items | n | Before medication (mean ± SD) | After medication (mean ± SD) | *p* |
|---|---|---|---|---|
| PT (s) | 10 | 10.98 ± 1.03 | 10.93 ± 0.71 | 0.851 |
| APTT (s) | 10 | 31.58 ± 4.05 | 27.69 ± 2.32*** | 0.000 |
| Fg (g/L) | 10 | 2.85 ± 0.30 | 2.92 ± 0.39 | 0.070 |
| TT (s) | 10 | 17.39 ± 0.71 | 16.71 ± 0.28** | 0.002 |
| AT-III (%) | 10 | 84.91 ± 9.01 | 95.07 ± 10.83*** | 0.000 |

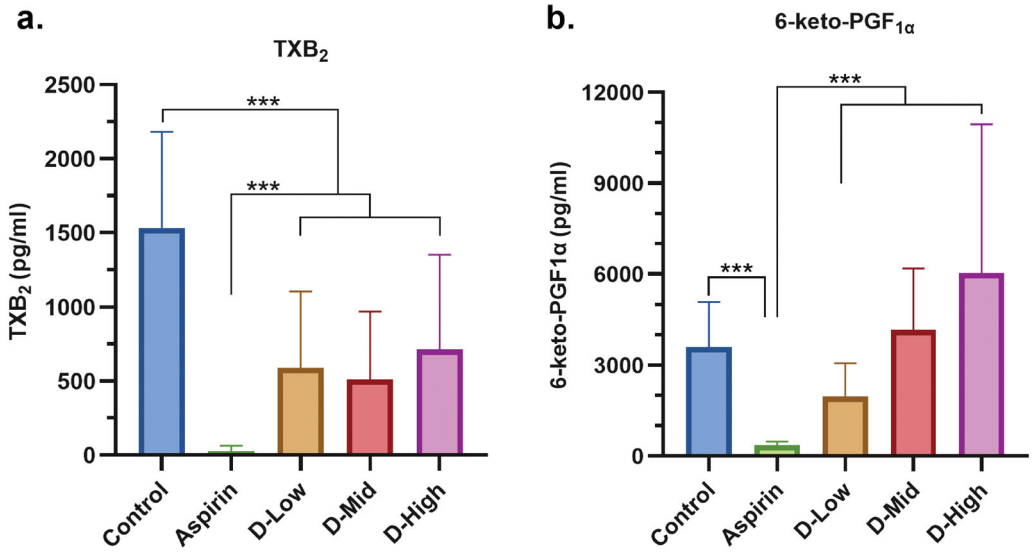


**Figure 2.** DAS regulates arachidonic acid metabolites. (a,b) The ELISAs test, each group was $n = 10$. (a) All medications reduced the TXB2 content in SD rats. No difference was observed between the three DAS groups. However, the TXB2 content in the DAS groups was higher than that in the aspirin group. (b) Compared with the control, the 6-keto-PGF1α content in SD rats from the DAS groups was not altered ($p > 0.05$), but the content in the aspirin group was significantly altered.

coagulation function in SD rats. The MIC of PT and APTT was 100 mg/kg and 200 mg/kg respectively, TT and Fg was 100 mg/kg *in vivo*. Compared with the control group (PT = 10.42 ± 0.44 s, APTT = 16.43 ± 1.4 s, Fg = 2.18 ± 0.39 g/L), the low- (PT = 9.32 ± 0.31 s, APTT = 14.07 ± 0.75 s, Fg = 2.88 ± 0.24 g/L), medium- (PT = 9.35 ± 0.27 s, APTT = 14.45 ± 0.73 s, Fg = 3.32 ± 0.31 g/L), and high-dose (PT = 9.22 ± 0.21 s, APTT = 14.33 ± 1.21 s, Fg = 3.61 ± 0.37 g/L) DAS groups exhibited significantly shorter PT and APTT and an increased Fg content ($p < 0.05$), but no significant changes were observed in the aspirin group (PT = 9.84 ± 0.70 s, APTT = 14.45 ± 0.73 s, Fg = 2.17 ± 0.41 g/L). At the same time, the aspirin group (TT = 34.80 ± 2.16 s) and the medium- (TT = 34.11 ± 1.76 s) and high-dose (TT = 32.68 ± 1.29 s) DAS groups displayed a shorter TT (control = 37.04 ± 2.13 s, $p < 0.05$) (Figure 3). These results indicated that medium and high doses of DAS enhanced blood coagulation function.

### DAS significantly activated at-III

We first tested the activity of AT-III *in vitro* and *in vivo* after medication to explore the detailed mechanism by which DAS inhibited secondary hemostasis. The activity of AT-III after the addition of medication to the whole blood of healthy people was significantly higher than before treatment ($p < 0.05$) (Tables 2 and 3). In addition, after the administration of medication *in vivo*, compared with the control group (103.22 ± 16.22%), the low- (134.71 ± 8.70%), medium- (134.77 ± 9.96%) and high-dose (146.46 ± 8.96%) DAS treatments significantly increased the activity of antithrombin III (AT-III) ($p < 0.05$), while no change was observed in the aspirin group (117.12 ± 15.18%) (Figure 4). The $EC_{50}$ of 133.3 mg/kg DAS was calculated *in vivo*. Therefore, DAS exerted an anticoagulant effect by enhancing AT-III activity.

Table 3. Effect of high-dose DAS on coagulation function in human blood *in vitro*.

| Test items | n | Before medication (mean ± SD) | After medication (mean ± SD) | *p* |
|---|---|---|---|---|
| PT (s) | 10 | 13.08 ± 1.23 | 11.44 ± 0.78** | 0.005 |
| APTT (s) | 10 | 40.71 ± 11.32 | 29.37 ± 4.06** | 0.002 |
| Fg (g/L) | 10 | 2.66 ± 0.47 | 3.03 ± 0.50*** | 0.000 |
| TT (s) | 10 | 20.73 ± 2.39 | 16.83 ± 1.04*** | 0.000 |
| AT-III (%) | 10 | 89.78 ± 7.27 | 96.63 ± 8.15*** | 0.000 |

### DAS activated some coagulation factors

We tested the activity of multiple coagulation factors in intrinsic and extrinsic pathways to explore the detailed mechanism by which DAS promoted secondary hemostasis. Through *in vivo* medication experiments in SD rats, we found that the MIC of FX was 100 mg/kg, the MIC of FV and FVII was 200 mg/kg, the MIC of FVIII was 300 mg/kg, and the MIC of FIX and FXI was 400 mg/kg. Compared with control conditions (FIIa = 89.21 ± 21.72%, FVa = 304.12 ± 79.65%, FVIIa = 324.19 ± 48.03%, FVIIIa = 524.79 ± 115.47%, FIX = 85.49 ± 27.87%, FXa = 34.90 ± 7.40%, FXIa = 38.12 ± 10.33%, FXIIa = 180.00 ± 25.33%), low-dose DAS activated the coagulation factors FV (470.84 ± 45.28%), FVII (600.46 ± 50.81%) and FX (89.93 ± 11.97%) ($p < 0.05$); medium-dose DAS activated the coagulation factors FV (443.44 ± 75.04%), FVII (632.12 ± 84.58%), FVIII (679.92 ± 143.34%), FIX (119.17 ± 31.12%), FX (89.88 ± 19.70%), and FXI (65.08 ± 15.39%) ($p < 0.05$); and high-dose DAS activated the coagulation factors FII (114.76 ± 23.92%), FV (414.84 ± 100.65%), FVII (790.66 ± 225.56%), FVIII (672.59 ±

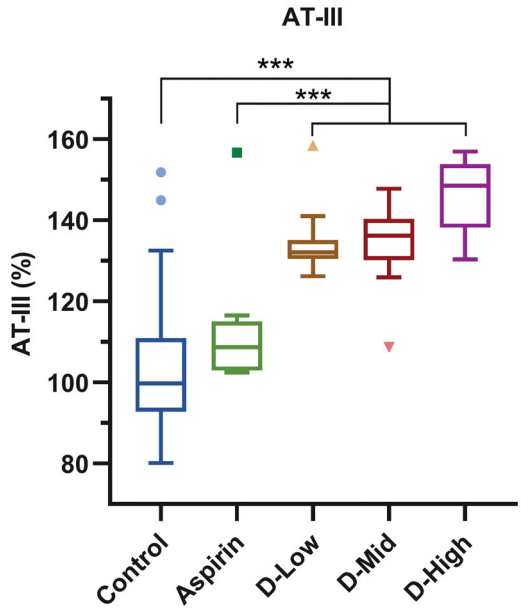


Figure 4. DAS significantly activated AT-III. The activity test of AT-III, each group was $n = 10$. Compared with the control group, all DAS groups showed significant activation of AT-III, but the aspirin group showed no change. Changes in AT-III were not observed different DAS dose groups.

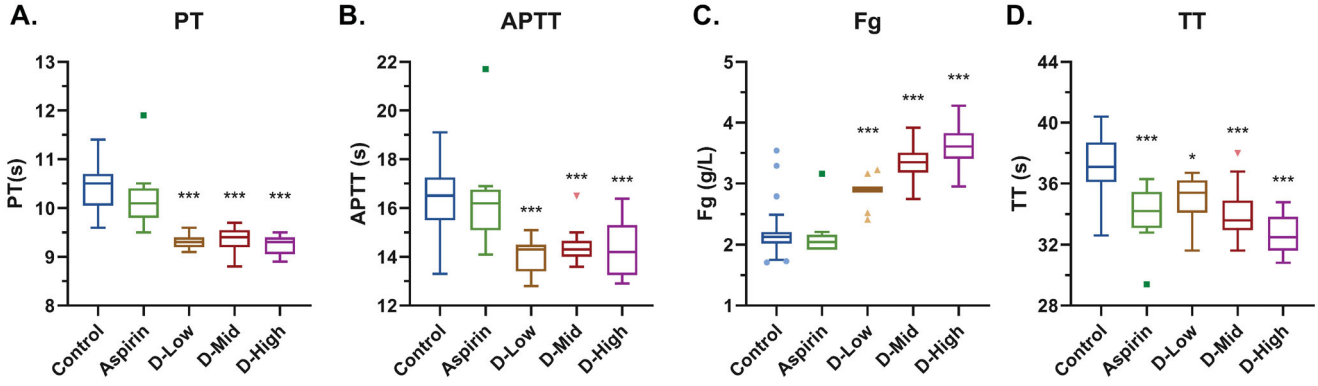


Figure 3. DAS enhanced coagulation function. (a–d) Coagulation function test, each group was $n = 10$. (a,b) All DAS groups of SD rats exhibited a shorter PT and APTT, but the aspirin group showed no changes in these parameters. (c) The Fg content increased gradually from the low- to high-dose DAS groups, but the aspirin group showed no changes. d. All DAS and aspirin groups had shorter TTs, and the TT in the high-dose DAS group was significantly reduced.

179.16%), FX (102.76 ± 29.41%), FXI (65.47 ± 34.08%), and FXII (311.86 ± 146.20%) ($p < 0.05$). Aspirin had no effect on the activity of most coagulation factors, but it reduced the activity of FII (61.83 ± 8.95%) and FVIII (306.60 ± 29.96%) ($p < 0.05$) (Figure 5). Therefore, DAS might exert a potential procoagulant effect by enhancing the activity of various coagulation factors, and aspirin inhibited coagulation factors.

### *DAS did not cause gastric mucosal haemorrhage*

As aspirin has the potential to cause gastrointestinal haemorrhage during clinical use, we further assessed whether similar adverse reactions would occur after DAS administration. The results of HE staining of gastric tissue obtained after dosing in SD rats showed that hyperaemia and haemorrhage of gastric mucosa and submucosal tissue were not present in the control group or in the low-, medium- and high-dose DAS groups. Meanwhile, in the aspirin group, hyperaemia was observed in the gastric body mucosa and gastric fundus submucosa along with haemorrhaging in the gastric body mucosa (Figure 6).

## Discussion

According to recent studies, many plant compounds have been reported to have some antithrombotic effects. For example,

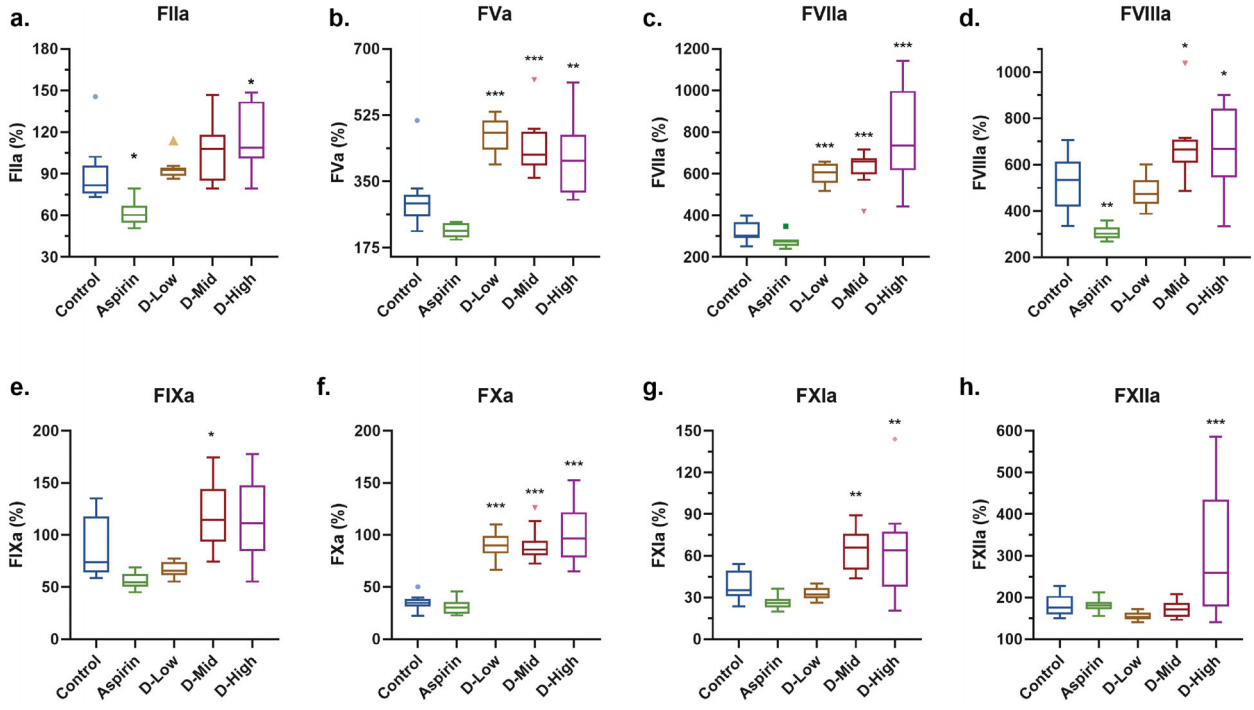


Figure 5. DAS strengthened the activity of coagulation factors, each group was $n = 10$. (a,h) The high-dose DAS treatment activated FII and FXII, and aspirin inhibited FII. (b,c,f) All DAS treatment significantly activated FV, FVII and FX. (d,g) The medium- and high-dose DAS treatments activated FVII and FXI, and aspirin inhibited FVII. (e) The medium-dose DAS treatment activated FIX.

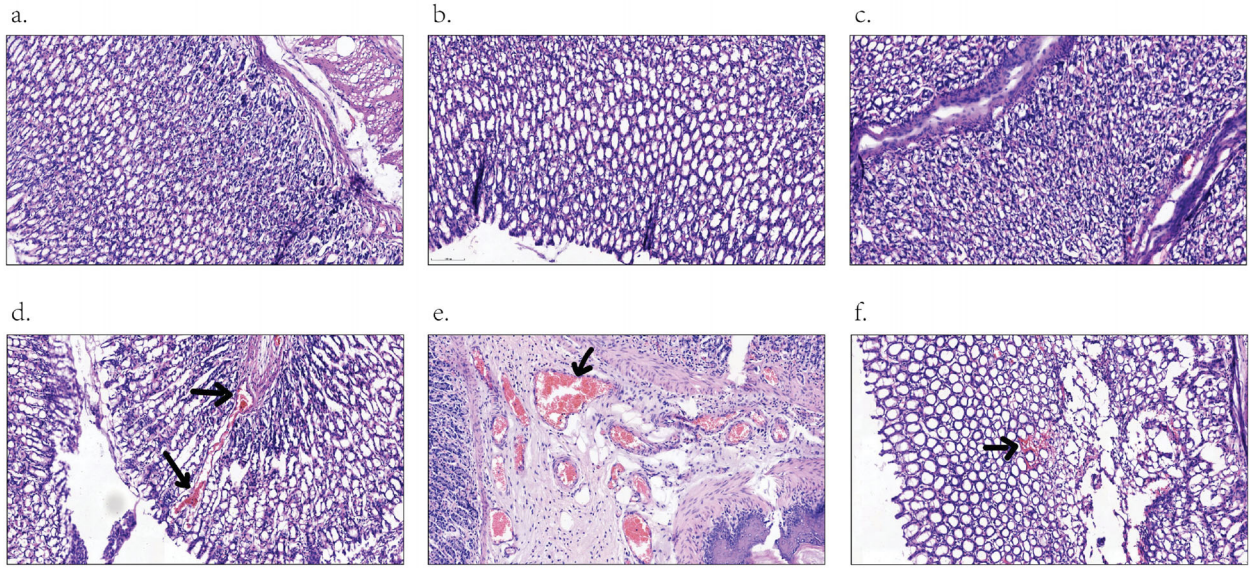


Figure 6. Comparison of the damaging effects of DAS and aspirin on gastric mucosa after medication. (a–c) The stomach in the low-, medium- and high-dose DAS groups did not show obvious hyperaemia; (d) Gastric body mucosal hyperaemia in the aspirin group; (e) Gastric fundus submucosal hyperaemia in the aspirin group; (f) Gastric body mucosal haemorrhage in the aspirin group.

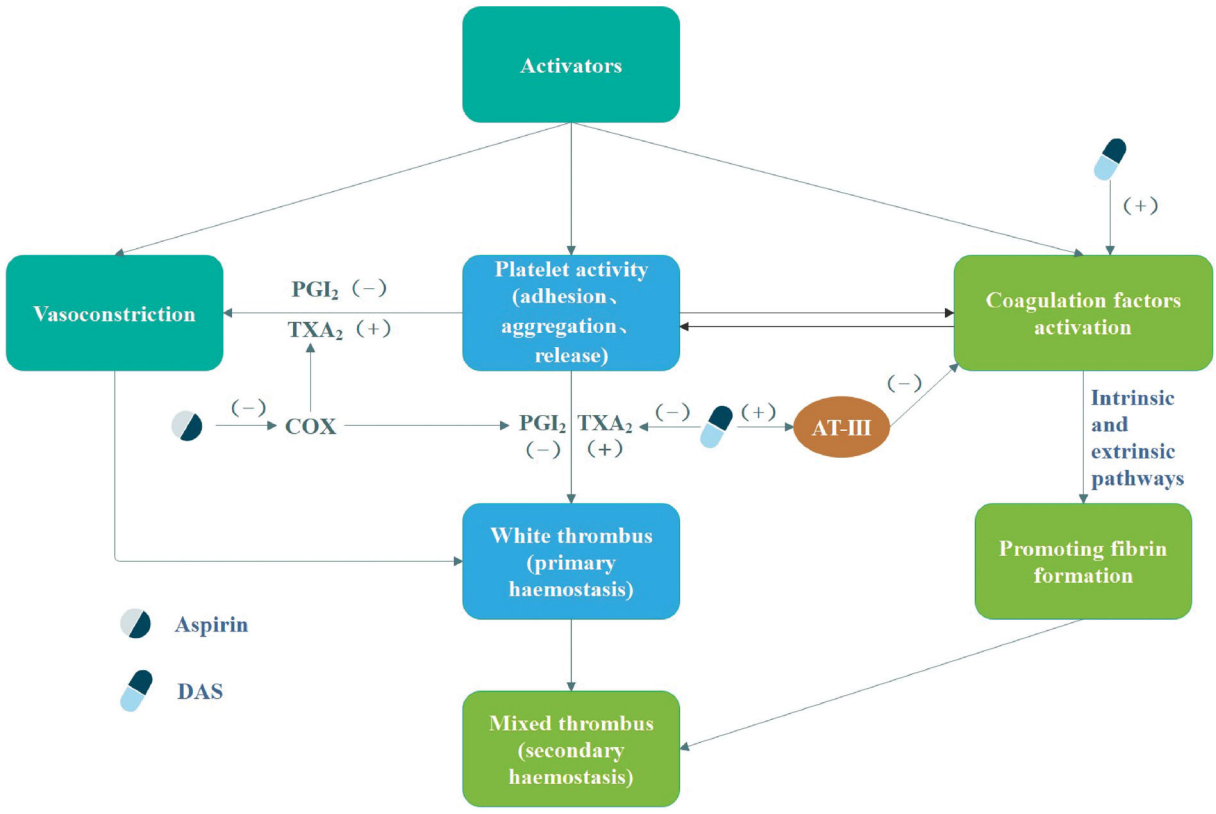


Figure 7. The main mechanism by which DAS inhibits platelet aggregation. In this figure, vascular factors are shown in dark green, platelet-induced primary hemostasis is shown in blue, and coagulation factor-induced secondary haemostatic reinforcement is shown in light green on the right. Through the coordinated actions of the three processes, platelets will eventually form a stable and irreversible mixed thrombus. The experimental results are summarised below. (1) DAS directly inhibits platelet aggregation by inhibiting $TXA_2$, thus preventing primary hemostasis. (2) DAS indirectly inhibits thrombin activity by enhancing AT-III activity, reduces thrombin-induced platelet aggregation, and weakens the effect of primary hemostasis. (3) DAS indirectly inhibits the cascade reaction of coagulation factors by enhancing AT-III activity and weakens the reinforcing effect of secondary hemostasis on platelet aggregation.

*Spatholobus suberectus* Dunn (Leguminosae) and *Carthamus tinctorius* L. (Asteraceae) have potent anti-platelet properties in platelet-rich plasma induced by ADP or platelet activating factor (Yan et al. 2015). Few reports have explored whether AP has a similar effect, but many recent reports have confirmed that AP and its compounds regulate arachidonic acid metabolic pathways (Jiao et al. 2019; Gupta et al. 2020; Prasetyo et al. 2021) for anti-inflammatory purposes (Majumdar et al. 2020; Lim et al. 2021). In consequence, there is reason to believe that this pathway, while effective anti-inflammation, also affects platelet aggregation that shares this pathway. Although its antithrombotic description is not uniform. Some researchers speculated *in vitro* that the antiplatelet activity of andrographolide may involve an increase in cyclic GMP/PKG, followed by inhibition of the p38 MAPK/radical dotHO/NF-κB/ERK2 cascade in human platelets (Lu et al. 2012). However, as mentioned above, some other researchers only found that it had an inhibitory trend on platelet aggregation in patients, but did not find any changes in COX mRNA expression and arachidonic acid metabolites that caused this effect (Sirikarin et al. 2018). Therefore, it is necessary to systematically review the role of its compounds in haemostatic system. In this study, for the first time, we found that DAS also exerts a good antiplatelet effect and predicted its potential clinical application prospects.

In the hemostasis system, DAS mainly affects primary hemostasis and the early stage of secondary hemostasis, which is very important in preventing early thrombosis. Stable thrombosis results from the interaction of the vascular wall, platelets and plasma in different stages. The whole process includes two stages, from platelet aggregation to white thrombosis (primary hemostasis) and then gradually to mixed thrombosis (secondary hemostasis). The haemostatic system is sensitive and rapid, among which platelets play the most important role. They coordinate with the vascular wall through $TXA_2$, $PGI_2$ and other substances and further produce mixed thrombi through the interaction of Fg, FIIa (thrombin), PF3, P4 and other coagulation factors in plasma. Therefore, the exact role of DAS is to prevent thrombosis by inhibiting platelet aggregation (Figure 7).

### *DAS exerts an antiplatelet aggregation effect on primary hemostasis*

In the present study, the antiplatelet aggregation effect of DAS was first verified by conducting two experiments using whole blood from healthy people and drug administration to SD rats *in vivo*. Aspirin and the medium- and high-dose DAS treatments exerted antiplatelet effects, but the effect of DAS administration was milder than that of aspirin. We examined the metabolites of arachidonic acid that affect platelet aggregation to confirm the pharmacological mechanism of DAS. Platelet aggregation is bidirectionally regulated by the arachidonic acid metabolites $TXA_2$ (platelet aggregation-inducing factor) and $PGI_2$ (platelet aggregation-inhibiting factor). When platelets are activated by stimulation, platelet membrane phospholipids are cleaved to produce arachidonic acid. Prostaglandin $G_2$ and $H_2$ ($PGG_2/PGH_2$) are generated from arachidonic acid by COX, and then $TXA_2$ and $PGI_2$ are generated by thromboxane synthase and $PGI_2$ synthase. Finally, these molecules are eventually transformed into

the stable metabolites $TXB_2$ and 6-keto-$PGF_{1\alpha}$ (Kij et al. 2016). $TXA_2$ is a specific product released form platelets and an important inhibitor of adenylate cyclase that reduces the content of cyclic adenosine monophosphate (cAMP), thus promoting platelet aggregation and vasoconstriction. $PGI_2$ is an important agonist of adenylate cyclase. It increases the content of cAMP, thereby inhibiting platelet aggregation and vasodilation, which is the opposite function to $TXA_2$ (Yuhki et al. 2011). In the present study, we first found that DAS significantly inhibited $TXA_2$ production but had no effect on 6-keto-$PGF_{1\alpha}$ production, which may be related to the specific inhibition of thromboxane synthesis by DAS. These results support previous research suggesting that the mechanism of action of andrographolide and other diterpenoid lactones differs from that of NSAIDs (Amroyan et al. 1999). As a classic antiplatelet aggregation and nonsteroidal anti-inflammatory drug, aspirin significantly reduces the contents of $TXA_2$ and $PGI_2$ by inhibiting COX activity and platelet aggregation. These effects have also been confirmed in the present study.

### DAS exerts an antiplatelet aggregation effect on secondary hemostasis

We performed an *in vitro* experiment using whole blood from healthy people and an *in vivo* drug administration experiment in SD rats to detect the effects of DAS on the anticoagulation system and secondary hemostasis. The secondary hemostasis process is closely related to coagulation factor activity, and AT-III is the most important antithrombin agent in the body that inactivates many coagulation factors (Allingstrup et al. 2016).

In both the *in vitro* and *in vivo* experiments, AT-III activity increased by approximately 30–45%, suggesting that DAS effectively prevented the cascade reaction of the intrinsic and extrinsic pathways by enhancing AT-III activity. These results support previous research suggesting that andrographolide derivatives inhibit thrombin-induced platelet aggregation (Thisoda et al. 2006). Based on these findings, we indicated its exact antithrombin mechanism. This enables DAS to consolidate its anticoagulant effect by preventing secondary hemostasis through the inhibition of platelet aggregation. However, this enhancement is limited. On the one hand, antithrombin is responsible for inhibiting 60–70% of thrombin *in vivo*, and it mainly acts on FII, FIX, FX, FXI, and FXII. On the other hand, with increasing doses, the total amount of partially activated coagulation factors will gradually exceed the inhibitory capacity of AT-III. Therefore, with the increase in AT-III activity, coagulation factors with enhanced activity are still detected with an increasing dose, but the inhibitory effect of AT-III on FII effectively prevented the increase in the prothrombin complex and further inhibited thrombin-induced platelet aggregation. This process directly weakened the effect of secondary hemostasis on reinforcing platelet aggregation, and thus the final effect of DAS on secondary hemostasis was still attributed to the inhibition of platelet aggregation.

### DAS effectively avoids hypoactive coagulation function

In the detection of coagulation factors, we found that DAS activated many coagulation factors after administration to rats. The activation of FV, FVII, FVIII, FX and FXI was significant in the medium- and high-dose groups. The activation of these factors indicates that DAS effectively protects both the intrinsic and extrinsic pathways and confirms the results of coagulation function: PT and APTT are shortened. At the same time, a slight increase in the Fg content was observed both *in vivo* and *in vitro*. We speculated that the increase in Fg levels was due to the release of platelet α granules induced by related factors, which was verified by the shortening of TT *in vitro*. These phenomena in the secondary haemostatic system are encouraging. The current antiplatelet, anticoagulant and thrombolytic drugs all inhibit the haemostatic system, resulting in a low reaction within the coagulation system. In contrast, the appropriate protective effect of DAS on secondary hemostasis effectively avoids hypoactive coagulation function.

Notably, DAS does not activate the whole secondary hemostasis system but exerts a protective effect by strengthening the activity of some coagulation factors, avoiding the inhibition of the coagulation system. We drew this conclusion for three reasons, as described below. (1) DAS only enhanced the activity some coagulation factors. With increasing doses, the activity of other coagulation factors increased, which we speculated was caused by the interaction of upstream and downstream coagulation factors. (2) At different doses, all coagulation factors were not activated systematically in the form of a cascade reaction, especially FII (thrombin), which was basically not activated. (3) The increases in PT and APTT were limited to normal physiological levels, and thus the mild enhancement was potentially considered a protective effect. In contrast, aspirin inhibited FII and FVIII, which may explain the low responsiveness of the coagulation system and subsequent bleeding after long-term administration. In summary, DAS may be an antiplatelet aggregation drug that prevents hypoactive coagulation function.

### The risk of gastric bleeding caused by DAS is low

Aspirin is a commonly used antiplatelet aggregation agent in clinical treatment, but due to its potential low reactivity and serious side effects, including gastrointestinal haemorrhage, its wide application is limited (Gaziano et al. 2018). Does DAS also induce similar adverse reactions through the inhibition of platelet aggregation? We compared the pathological changes in the gastric mucosa of SD rats after administering low, medium and high doses of DAS with those observed after administering aspirin to answer this question. Neither hyperaemia nor haemorrhage of the gastric mucosa and submucosa were observed in rats from any of the DAS groups, while the aspirin group showed signs of gastric body mucosal hyperaemia, gastric fundus submucosal hyperaemia and gastric body mucosal haemorrhage, indicating that DAS had a lower risk of gastrointestinal haemorrhage than aspirin. Combined with the analysis of the anticoagulant mechanism described above, we reviewed the adverse reaction mechanisms of the two drugs. The principle of aspirin is to reduce the $TXA_2$ content through the non-selective inhibition of COX to inhibit platelet aggregation. However, as the $PGI_2$ content is also reduced, the gastric mucosa lacking the protective effect of $PGI_2$ will be directly damaged by gastric acid, thus causing gastric mucosal hyperaemia and haemorrhage. At the same time, as aspirin also exerted certain inhibitory effects on FII and FVIII, long-term aspirin use may induce coagulation disorders, significantly increasing the risk of bleeding. In contrast, DAS exerts a mild antiplatelet aggregation effect and the potential for $PGI_2$ protection, resulting in a low risk of bleeding. Even if a bleeding tendency appears, DAS activates various coagulation factors to enhance basic coagulation function, and secondary hemostasis can be restored in a timely manner to prevent the occurrence and development of bleeding events.

## Conclusions

In this study, we identified DAS has the roles of inhibiting $TXA_2$, enhancing AT-III and activating some coagulation factors, antithrombosis in clinical treatment. On the one hand, DAS significantly reduces the contents of procoagulant products by inhibiting thromboxane synthesis, subsequently inhibiting platelet aggregation. On the other hand, DAS significantly enhances the activity of AT-III and thus antagonises coagulation function. At the same time, it also consolidates coagulation function by activating various coagulation factors, ensuring that the coagulation function is not excessively inhibited and thus significantly reducing the risk of bleeding. Thus, DAS could potentially apply to thromboprophylaxis treatment, which provides a reliable theoretical and experimental basis for its clinical application.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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