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ORIGINAL ARTICLE

Oral administration of dermatan sulphate reduces venous thrombus formation in vivo: potential use as a formulation for venous thromboembolism

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Abstract

Dermatan sulphate (DS) is a sulphated polysaccharide that displays complexity in constituent sulphated disaccharides and interacts with proteins and signalling molecules to modulate numerous biological processes, including inhibition of the coagulation cascade and regulation of blood clotting and fibrinolysis. This study shows the antithrombotic and anticoagulant effects of DS prepared from bovine collagen waste liquor following oral and intravenous administrations in a deep vein thrombosis (DVT) rabbit model. In vitro, the prothrombin time, activated partial thromboplastin time, and thrombin citrated plasma clotting assays revealed that bovine DS had strong antithrombotic and anticoagulant effects comparable to low-molecular-weight heparin [Clexane® (enoxaparin sodium)]. In a DVT rabbit model, animals received intravenous and oral administrations of bovine DS and Clexane® providing further evidence that both agents had strong antithrombotic and anticoagulant effects by significantly reducing or preventing clot formation. Thromboelastography (TEG) assays revealed further that both bovine DS and Clexane® substantially prolonged the clotting time of recalcified citrated whole blood, but only bovine DS could retain clot strength suggesting that bovine DS had less effect on platelet–fibrin interactions. In conclusion, this is the first report that oral administration of DS from bovine collagen waste liquor reduces experimental venous thrombus formation warranting further research into bovine DS as an oral antithrombotic therapeutic.

Keywords Oral anticoagulant · Antithrombotic · Bovine collagen waste liquor · Dermatan sulphate · Venous thromboembolism · In vivo DVT model

I/We give this posthumous dedication and this work to our friend and colleague Dr Paul Masci who recently passed. This last September we farewelled a dear friend and colleague. In all the time I knew Paul I will always remember a man who was of good cheer and easy going, as evidenced by his love of snakes as only Paul could. His work on venom toxicology and the impact of snake venom on coagulation, became an important part of his academic-research life. A gentle man who befriended many, we will miss his friendship and collegiate input.

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Abbreviations

aPTT	Activated partial thromboplastin time
CTAB	Cetyl trimethyl ammonium bromide
DS	Dermatan sulphate
DVT	Deep vein thrombosis
GAGs	Glycosaminoglycans
LMWH	Low-molecular-weight heparin
IV administration	Oral administration
PT	Prothrombin time

TCT	Thrombin citrated clotting
TEG	Thromboelastography
VTE	Venous thromboembolism
UFH	Unfractionated heparin

Introduction

Dermatan sulphate (DS) displays a complexity in constituent sulphated disaccharides and interacts with proteins and signalling molecules to modulate numerous biological processes, including inhibition of the coagulation cascade and regulation of blood clotting and fibrinolysis (Raman et al. 2005). Through a basic glycosaminoglycan (GAG)-binding domain within heparin cofactor II (HCII), DS displaces the N-terminal acidic peptide sequence and causes HCII to unfold producing a DS-HCII-thrombin ternary complex that inhibits thrombin activity (Baglin et al. 2002; Casu et al. 2004) potentiating the anti-thrombotic and anti-coagulant properties of DS. Oral administered GAGs, such as DS, have been shown to be metabolized by intestinal bacteria (Kawai et al. 2018). The processing of DS by intestinal bacteria leads to the formation of DS derivatives that in patients with IBD, significant inhibition of P-selectin expression on the surface of platelets is reported that enhances protein C activity in blood (Ji et al. 2004). Hence, DS can potentially influence an important anticoagulant pathway. The anticoagulant effect exerted by the protein C system is directed at regulating the activities of FVIIIa and FVa in the tenase and prothrombinase complexes (Boissier and Semerano 2019).

Deep vein thrombosis (DVT) is one of the most prevalent venous thromboembolism (VTE) presentations that is among the leading causes of death worldwide from cardiovascular disease (Fernandes et al. 2016). DVT can lead to acute pulmonary thromboembolism. Inflammation shifts the haemostatic balance to a procoagulant state by increasing platelet reactivity, impairing anticoagulation and fibrinolysis, and triggering the coagulation process (Cezarette et al. 2020). Thrombo-inflammation is commonly used to describe the complex interplay between blood coagulation and inflammation, in relation to the pathophysiology of cardiovascular diseases (CVD), including atherosclerosis and acute atherothrombotic complications like myocardial infarction, ischemic stroke and venous thromboembolic disease (d'Alessandro et al. 2020).

Treatment of VTE generally requires full anticoagulation that in the short term involves intravenous or subcutaneous administration, and in the long term, oral therapy. Heparin and warfarin have been widely used as intravenous and oral anticoagulants over decades due to the immediate and easily monitored action of these agents, short half-life, and cost-effectiveness. However, these treatments require frequent monitoring and adjustment to produce varying degrees of anticoagulation (Canales and Ferguson 2008) that can result in side effects including bleeding and heparin-induced thrombocytopenia. Their side effects (Kelton and Warkentin 2008) highlight the need to identify therapeutic alternatives.

DS belongs to the same family of GAGs as heparin and has attracted significant research attention in the past 20 years. DS selectively inhibits the action of thrombin through HCII at the cell surface and can effectively inhibit thrombin bound to fibrin or at the surface of injured blood vessels (Bendayan et al. 1994). This mode of action supports the early observations that DS acts as a non-systemic anticoagulant that has much less bleeding side effects than heparin (Brister and Buchanan 1995; Hoppensteadt et al. 1990).

Studies have employed antithrombotic and anticoagulant DS from a variety of sources including porcine intestinal mucosa (Halldorsdottir et al. 2006) and skin (Maimone and Tollefsen 1990), marine invertebrates (Pavao et al. 1998; Volpi and Maccari 2009), shark fin (Higashi et al. 2015), several bovine tissues and bovine collagen waste liquor (Ofosu et al. 1987; Osborne et al. 2016, 2008).

This study extracted and purified DS from bovine collagen waste liquor to investigate its antithrombotic and anticoagulant property effects in vitro. Then, through a DVT rabbit model to determine intravenous and oral efficacy of bovine DS in vivo. This study builds knowledge that was previously reported (Osborne et al. 2008) to further confirm that purified DS from bovine hide waste liquor has antithrombotic and anticoagulant actions.

Methods

Animal research ethics

Animal research ethics approval (AEC No. PAH/108/08) for carrying animal experiments in this study was obtained from the University Animal Ethics Committee (Health Sciences) of The University of Queensland.

Materials

All the reagents used for preparing DS and purchased from different suppliers have been described previously (Osborne et al. 2016, 2008).

Fibrinogen concentration at 2.5 mg/mL and platelet count 250–450 $\times 10^9$/L were obtained from Princess Alexandra Hospital (PAH), Haematology Department. Low-molecular-weight heparin (LMWH) (100 IU/mL) was from Kabi Diagnostica (Sweden). Therapeutic-grade LMWH (Clexane®) from Aventis (10,000 IU/mL, 40 mg/0.4 mL) was prepared as 1:100 dilution of stock (100 IU/mL) in sterile normal saline and stored at 4ºC. Thromborel® S was from Dade

Behring Marburg Gmbh (Germany). Platelin® LS was from bioMerieux Inc. (USA).

Elaboration and further characterization of DS from bovine hide.

Dermatan sulphate (~ 100 g) was prepared from bovine hide processing as described previously (Osborne et al. 2016, 2008). The average molecular weight and disaccharide composition of the DS were determined as previously described (Osborne et al. 2008).

Plasma preparation and clotting parameters

Plasma was prepared from rabbit citrate whole blood for clotting assay in vitro. Also, plasma prepared from human citrate whole blood from normal volunteer donors with consent was used as a control for the clotting assay. Normal citrated plasma has two clotting parameters: prothrombin time (PT) at 10–11 s and activated partial thromboplastin time (aPTT) at 30–35 s.

PT assay

50 µl of plasma (human plasma containing added DS or LMWH, or rabbit plasma from the DVT in vivo model) was added to 150 µL Thromborel S (containing calcium). Clotting was determined by the change in absorbance at 880 nm using an ACL FUTURA, V3.6 (Beckman).

aPTT assay

50 µL of plasma (human plasma containing added DS or LMWH, or rabbit plasma from the DVT model) was added to 50 µL Platelin LS and 50 µL of 25 mM $CaCl_2$. Clotting was determined by the change in absorbance at 880 nm using an ACL FUTURA, V3.6 (Beckman).

Thrombin citrated clotting (TCT) assay

100 µL of plasma (either human plasma containing added DS or LMWH) was added to 100 µL thrombin (final concentration 1.3 IU/mL) containing 10 mM Ca^{2+}. Bovine thrombin (10 000 IU) was reconstituted in 50% glycerol/saline (500 IU/mL stock), and stored at − 70 °C. A working thrombin solution of 5 IU/mL with 0.03 M $CaCl_2$ was prepared in sterile normal saline. Clotting was measured as described previously (Zhao et al. 2019a).

Thromboelastography (TEG)

Studies were performed using a TEG® Haemostasis Analyser 5000 series (Haemoscope, Niles, USA) according to manufacturer's instructions. Two important parameters: *R* time and MA values were obtained from the TEG assay, which measured the mechanical properties of the blood clots. The details for this assay have been previously described (Zhao et al. 2019b).

DVT rabbit model: anaesthetisation and thrombogenic challenge

All experimental protocols were approved by The University of Queensland Animal Welfare Office. The anticoagulant effects of DS and Clexane® were studied in a modified stasis thrombosis model (Hladovec 1986) using male white New Zealand rabbits. The animals were safely restrained and anaesthetized with a premedication of 300 µg/kg medetomidine and 0.5 mL fentanyl using a No. 22 butterfly needle in the marginal ear vein. A total anaesthesia regime was achieved with 3.5–4% isoflurane in 2 L/min oxygen followed by a maintenance anaesthesia dosage of 2–3% isoflurane in 2 L/min oxygen. Following anaesthesia, the rabbits were weighed, and their neck and right upper leg areas shaved and positioned for surgery. The catheterization was done by carefully isolating the femoral artery under the tissues and loosely tying No. 4 Mersilk sutures (Ethicon, Somerville, USA) around 1 cm of the artery. After cutting a small part of the artery vessel, a short length (30–40 cm) of PE 60 tubing was inserted into the femoral artery and tied with Mersilk sutures. The tubing was attached to a No. 20 needle to enable blood sampling and was also connected to a Gilson pump to flush 0.5 mL/min saline to keep the artery clear between sample collections and for administration of intravenous saline, DS or Clexane®. A vertical incision in the neck area was made to surgically isolate the jugular veins from the fascia. The dissection was performed carefully using a scalpel, forceps and cautery so as not to damage or traumatize the vessels. Collateral vessels were cauterized and 1 cm of each jugular vein (including the bifurcation) was isolated. Mersilk sutures were loosely tied around each branch of the jugular vein without interfering with blood circulation. The exposed neck area was kept moist with saline-soaked gauze patches.

IV administration of DS and Clexane®

Three animals were challenged for each treatment and dose ($n = 3$). The first 3.5 mL blood sample (A) was taken from the femoral artery before any IV administration as a control blood sample, and then flushed with 1 mL of sterile saline. Intravenous administration included three doses of DS: dose 1 = 1 mg/mL in plasma (30 mg/kg for rabbit weight); dose 2 = 2 mg/mL in plasma, (60 mg/kg for rabbit weight); dose 3. 4 mg/mL in plasma, (120 mg/kg for rabbit weight); one dose of Clexane®. 150 IU/kg in 10 mL of saline (1.5 mg/kg for rabbit weight); and 10 mL of saline control, injected via the

marginal ear vein 10 min before flushing the ear vein with 1 mL saline. To initiate thrombogenic challenge to induce DVT formation, 30 mL hypotonic saline solution (2.25 g/L NaCl) was infused into the femoral artery for 20 min via a Gilson Pump (at approximately 2 mL/min). The second 3.5 mL blood sample (B) was taken 10 min after completion of the hypotonic saline injection and prior to 1 cm ligation of the external jugular vein (time = 0 for thrombogenic challenge, 40 min post-IV administration). The third and final 3.5 mL blood sample (C) was taken 30 min after thrombogenic challenge (70 min post-IV administration). At completion of the experiment, the animal was euthanized using 1 mL/kg of Lethabarb. The 1 cm of ligated jugular vein was removed. Clots were weighed and examined under a microscope (25X). The clots in the whole ligated jugular vein section were divided into five grades as previously described (Fareed et al. 1985). When difficulties were encountered with catheterisation or IV injection the femoral or jugular veins were used, and when ligation presented difficulties in the jugular vein, the femoral vein or artery was used.

Oral administration of DS and Clexane®

Animals were restrained using a large towel and dosed with saline, DS or Clexane® using a gavage needle. Seven animals were challenged for each treatment ($n = 7$). Following gavage and prior to anaesthetization, the animals were observed for 1 h for regurgitation. 2 h post gavage administration ($T = 120$), the first 3.5 mL blood sample (A) was taken. To initiate thrombogenic challenge, 30 mL hypotonic saline solution (2.25 g/L NaCl) was infused into the femoral artery for 20 min via a Gilson Pump (at approximately 2 mL/min). The second 3.5 mL blood sample (B) was taken 10 min after completion of the hypotonic saline injection and prior to 1 cm ligation of the external jugular vein ($T = 0$ for thrombogenic challenge and 160 min post-oral administration). The third and final blood sample (C) was taken 30 min after thrombogenic challenge ($T = 30$ for thrombogenic challenge and 190 min post-oral administration).

Glycosaminoglycan assay in serum

To determine the half-life of DS following IV administration and to estimate the bioavailability of oral DS and Clexane®, total GAGs were measured in serum using the Blyscan Sulphated Glycosaminoglycan Assay according to manufacturer's instructions. Prior to the assay, the serum samples were digested with Proteinase K (5 mg/mL final concentration) in 50 mM Tris-0.1 mM $Ca(CH_3COO)_2$ at 50 °C for 4 h, heat inactivated at 95 °C for 10 min and cooled to room temperature prior to an equivalent volume of chloroform being added. The samples were vortexed, centrifuged at 9000×*g* for 10 min with the aqueous (top) layer retained for GAG analysis.

Statistical analysis

All statistical analyses of the in vitro and IV experiments were conducted using a one-way ANOVA followed by post hoc comparisons using Dunnetts multiple comparison tests. All statistical analyses of the oral experiment were conducted using unpaired *t* tests. *p* values less than 0.05 were considered significant. These calculations were performed using GraphPad Prism 5 Software for Windows (GraphPad Software, San Diego California USA, https://www.graphpad.com).

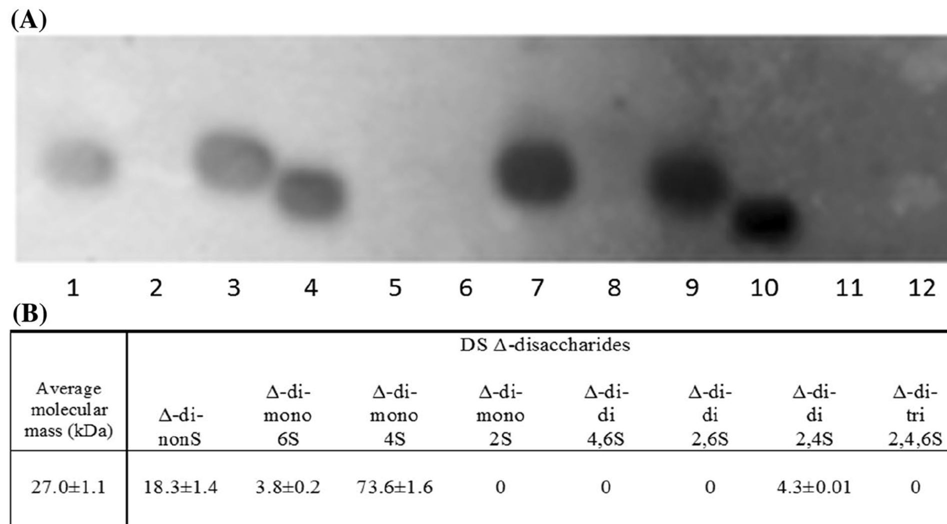
Results

Extraction of DS from bovine collagen waste liquor

Results showed first that ABC lyase completely digested the extracted DS while ACII lyase did not affect it (Fig. 1a). A similar pattern was also achieved following digestion of commercial DS (CSB), further confirming the extracted GAGs to be DS. The GAG purity was estimated to be 86.5 ± 3.0% w/w (data not shown). HPLC analysis estimated the average molecular mass to be 27.0 kDa whilst FACE mapping revealed the DS to be rich in the predominant mammalian disaccharide, GlcA/IdoA → $GalNAc4SO_3$ (Fig. 1b). Almost 74% of the extracted DS was 4-*O*-mono-sulphated with the remaining disaccharides being non-sulphated (18%), 6-*O*-mono-sulphated (4.0%) and importantly for antithrombotic activity, di-sulphated with approximately 4.0% of the disaccharide composition being $IdoA2SO_3$ → $GalNAc4SO_3$ (Fig. 1b). The disaccharide composition of DS was consistent with previous reports (Osborne et al. 2016, 2008).

DS has anticoagulant activities in vitro

To estimate the amount of DS required to produce an anticoagulant effect in vivo, anticoagulant activity of DS and LMWH (as a positive control) was determined in vitro using PT, aPTT, and TCT assays (Table 1). Significant clotting inhibition was observed in the PT assay at all concentrations except for the lowest DS dose (dose 1 = 0.2 mg/mL). DS and LMWH significantly prolonged aPTT at all concentrations compared to the saline control. In the PT assay, the ratios of DS and LMWH to saline were calculated to determine the concentration of DS and LMWH required to produce an anticoagulant effect in vivo (Table 1). A ratio greater than 2.0 was observed in response to DS doses 8–10



Average molecular mass (kDa)	DS Δ-disaccharides							
	Δ-di-nonS	Δ-di-mono 6S	Δ-di-mono 4S	Δ-di-mono 2S	Δ-di-di 4,6S	Δ-di-di 2,6S	Δ-di-di 2,4S	Δ-di-tri 2,4,6S
27.0±1.1	18.3±1.4	3.8±0.2	73.6±1.6	0	0	0	4.3±0.01	0

Fig. 1 Characterisation of dermatan sulphate (DS) prepared from bovine collagen waste liquor. **a** Agarose gel electrophoresis of commercial and DS prepared from bovine collagen waste liquor. 15 µg of DS and chondroitin sulphate A (CSA), chondroitin sulphate B (CSB), chondroitin sulphate C (CSC) commercial standards, were digested with chondroitin ABC lyase (Chase ABC) and chondroitin ACII lyase (Chase ACII) and separated by 0.5% agarose/50 mM diaminopropane electrophoresis run at 80 V for 90 min. The glycosaminoglycans were fixed using 0.1% (w/v) cetyl-trimethyl-ammonium bromide (CTAB) solution and stained using 0.1% toluidine blue (w/v) /50% (v/v) ethanol/1% acetic acid (v/v) solution. (1) DS (2) DS-Chase ABC (3) DS-Chase ACII (4) CSA (5) CSA-Chase ABC (6) CSA-Chase ACII (7) CSB (8) CSB-Chase ABC (9) CSB-Chase ACII (10) CSC (11) CSC-Chase ABC (12) CSC-Chase ACII. **b** Disaccharide composition and molecular mass of DS and LMWDS prepared from bovine collagen waste liquor. Disaccharide composition was determined using fluorophore-assisted carbohydrate electrophoresis. Molecular weight was determined via HPLC analysis using a TSK-GEL G4000SW (7.5 × 30 cm) and a G2000SW (7.5 × 30 cm) connected in series

Table 1 In vitro antithrombotic activity of bovine DS, LMWH and Clexane® expressed as PT, ratio of PT, aPTT and TCT

In vitro	Plasma concentration (mg/mL)	PT (s)	Ratio of PT	aPTT (s)	TCT (s)
DS					
Dose 1	0.2	10.8 ± 0.3	1.1 ± 0.02	66.3 ± 7.3[a]	15.3 ± 0.2[a]
Dose 2	0.4	11.9 ± 0.3[a]	1.2 ± 0.02	93.5 ± 1.8[a]	–
Dose 3	0.6	12.6 ± 0.3[a]	1.6 ± 0.02	112.7 ± 5.0[a]	22.4 ± 0.5[a]
Dose 4	0.8	13.1 ± 0.04[a]	1.3 ± 0.0	119.4 ± 4.0[a]	–
Dose 5	1	13.6 ± 0.04[a]	1.4 ± 0.01	135.8 ± 0.9[a]	34.9 ± 0.8[a]
Dose 6	1.5	–	–	–	36.2 ± 3.6[a]
Dose 7	2	18.0 ± 0.6[a]	1.8 ± 0.01	221.4 ± 7.9[a]	No clot
Dose 8	4	21.6 ± 0.1[a]	2.1 ± 0.01	282.5 ± 0.5[a]	–
Dose 9	6	24.3 ± 1.1[a]	2.3 ± 0.1	286.3 ± 5.2[a]	–
Dose 10	8	27.8 ± 0.1[a]	2.6 ± 0.01	No clot	–
LMWH					Clexane®
Dose 1	0.02	12.2 ± 0.0[a]	1.2 ± 0.0	219.1 ± 10.0[a]	14.6 ± 0.1
Dose 2	0.04	14.9 ± 0.4[a]	1.5 ± 0.04	No clot	–
Dose 3	0.06	17.3 ± 0.3[a]	1.7 ± 0.02	No clot	31.7 ± 0.8[a]
Dose 4	0.08	18.9 ± 0.4[a]	1.8 ± 0.04	No clot	–
Dose 5	0.1	21.3 ± 0.7[a]	2.04 ± 0.06	No clot	112 ± 1.2[a]
Dose 6	0.15	–	–	–	No clot
Dose 7	0.2	–	–	–	No clot
Saline	–	10.5 ± 0.2	–	33.8 ± 1.3	–
Citrate	–	–	–	–	11.7 ± 0.2

[a]Denotes mean values in columns that are significantly different ($p < 0.05$) to the saline or citrate negative control using a one-way ANOVA followed by post hoc comparisons using Dunnetts multiple comparison tests

(4.0–8.0 mg/mL), and in response to LMWH dose 5 (0.1 mg/mL) (Table 1).

TCT was extended by the presence of both DS and Clexane® with a significant dose-dependent pattern, except for the lowest Clexane® concentration (dose 1 = 0.02 mg/mL) (Table 1). Higher concentrations of DS (2.0 mg/mL) and Clexane® (0.15 and 0.2 mg/mL) completely prevented clot formation during the time course of the TCT assay (Table 1). Based on the results from aPTT, PT, and TCT assays, a DS plasma concentration of 4.0 mg/mL could be a therapeutic dose to achieve a PT ratio greater than 2.0 in vivo and prevent clot formation in a hypertonic saline injected DVT rabbit model. Furthermore, a 0.1 mg/mL plasma concentration of Clexane® could achieve similar anticoagulation in vivo.

TEG studies were used to investigate the mechanism of DS anticoagulation and to determine whether normal blood clotting could be achieved post DS treatment. For the first time, recalcified citrated rabbit whole blood was used for this analysis to show the global effect on blood clotting in this model. Table 2 shows the in vitro whole blood antithrombotic activities of both DS and Clexane® by TEG assay. Compared with the citrated blood control, *R* times significantly increased following three doses of DS and Clexane®, suggesting that both DS and Clexane® delayed blood clotting (Table 2). Interestingly, MA values associated with all DS doses were statistically similar to that of the citrated blood control and were significantly higher than Clexane® (Table 2), indicating that DS could produce a stronger clot than Clexane®. This would suggest that platelets are fully functional, while in the Clexane® group, there may still be some inhibition of platelet function consistent with the reduced clot strength. The significant decrease of the α-angles in all DS and Clexane® treatments further indicated that the two anticoagulants inhibited formation of the blood clot by reducing the rate of thrombin formation in whole blood (Table 2).

Intravenous DS anticoagulant activities

Based on in vitro data (Tables 1, 2), it was predicted that a 4 mg/mL DS plasma concentration (i.e. 2.0 mg/mL DS blood concentration) could produce a therapeutic ratio greater than 2.0 in vivo, preventing clot formation whilst retaining clot strength in blood samples taken post treatment. Three doses of DS (100, 200, and 400 mg/per animal) and one dose of Clexane® (3.3 mg/per animal) were chosen to treat rabbits, respectively, with one dose administered to three rabbits ($n = 3$). Table 3 shows that no clots were observed in any of the Clexane®-treated rabbits based on visual observation and clot subjective rating. Comparatively, small clots were observed in one rabbit in each DS dosage group. The control animals had the highest clot subjective rating (6.3 ± 1.3) (Table 3) with clots observed in all animals in this group. No significant change in PT was observed in both DS and Clexane®-treated animals compared to control animals at the three time points (data not shown). While aPTT did not change in any of the control animals, a significant increase in aPTT was observed in blood samples B and C from DS, and in blood sample B from Clexane®-treated rabbits.

TEG recalcified citrated whole blood assays showed base line changes in *R* times of control animal blood samples during the 70 min experimental procedure. These changes from 376.3 to 273.8 s were expected in response to the hypotonic saline challenge. The *R* time significantly increased in doses 3 of DS and Clexane®-treated rabbits (Table 3), indicating that the two agents reduced the propensity for whole blood clotting. Consistently, over the 70 min experimental procedure, *R* time remained prolonged after treatment at a stable ratio of 3–3.5 times for dose 2 and 3 of DS, and 4 times for Clexane. Furthermore, the changes in MA value were from 64 to 50 mm for the three doses of DS and from 60 to 17 mm for the Clexane® suggest a strong effect on rabbit platelets with Clexane® treatment but not with DS (Table 3), further supporting the assumption that DS will still produce a firm clot. Compared with saline-treated rabbits, the α-angle degrees in all DS and Clexane®-treated rabbits were

Table 2 In vitro antithrombotic activity defined by TEG measurements (MA value, *R* time and α-angle) of bovine DS and Clexane®

In vitro TEG	Blood concentration (mg/mL)	MA value (mm)	*R* time (s)	α-Angle (°)
DS				
Dose 1	0.6	54.9 ± 0.8	842.5 ± 59.5[a]	39.6 ± 0.6[a]
Dose 2	1.2	54.9 ± 2.3	1483.3 ± 82.5[a]	14.7 ± 1.6[a]
Dose 3	1.8	56.3 ± 2.4	1515.0 ± 115.0[a]	18.0 ± 3.6[a]
Clexane®	0.06	40.6 ± 3.1[a]	957.5 ± 84.0[a]	11.9 ± 1.3[a]
Citrate	–	60.8 ± 2.4	456.0 ± 18.0	53.9 ± 5.5

[a]Denotes mean values in columns that are significantly different ($p < 0.05$) to the citrate negative control using a one-way ANOVA followed by post hoc comparisons using Dunnetts multiple comparison tests

Table 3 In vivo antithrombotic activity of bovine DS and Clexane® in rabbit blood following intravenous administration in the DVT model assessed using aPTT and TEG measurements

IV dose	Clot subjective rating	Blood sample	aPTT (s)	MA value (mm)	*R* time (s)	*α*-Angle (°)
DS dose 1 (30 mg/kg)	2.5 ± 2.5	A	59.9 ± 5.7	63.2 ± 1.6	410.0 ± 21.0	60.9 ± 5.9
		B	*150.0 ± 33.2*	45.9 ± 3.0	1257.0 ± 287.0	27.6 ± 10.2[a]
		C	139.2 ± 14.6	49.1 ± 2.6	781.7 ± 211.2	25.8 ± 5.9[a]
DS dose 2 (60 mg/kg)	2.5 ± 1.4	A	76.2 ± 8.2	64.3 ± 2.0	422.5 ± 37.9	60.4 ± 5.7
		B	*198.0 ± 2.0*[a]	48.4 ± 3.8	1297.0 ± 239.5	21.6 ± 6.7[a]
		C	*187.9 ± 10.2*[a]	47.9 ± 2.6	1258.0 ± 227.7	22.1 ± 2.3[a]
DS dose 3 (120 mg/kg)	1.7 ± 1.7	A	76.9 ± 5.9	65.7 ± 2.3	399.2 ± 88.18	58.7 ± 7.4
		B	*199.1 ± 0.9*[a]	45.3 ± 2.3	1511.0 ± 299.7[a]	25.6 ± 1.7[a]
		C	*180.2 ± 19.8*[a]	49.9 ± 3.7	1300.0 ± 232.4	23.6 ± 6.1[a]
Clexane® (1.5 mg/kg)	0.0 ± 0.0*	A	74.5 ± 6.9	60.5 ± 2.3	430.8 ± 119.5	47.7 ± 8.0
		B	*179.2 ± 20.8*[a]	*13.3 ± 13.3*[a]	*1780.0 ± 220.0*[a]	*3.5 ± 3.5*[a]
		C	*170.6 ± 29.4*	*17.3 ± 17.3*[a]	*1692.0 ± 308.3*[a]	*12.9 ± 12.9*[a]
Saline[b]	6.3 ± 1.3	A	81.4 ± 11.5	62.1 ± 0.9	376.3 ± 11.3	58.6 ± 11.3
		B	74.5 ± 23.3	56.8 ± 0.3	326.3 ± 143.8	57.4 ± 1.0
		C	83.7 ± 22.2	62.2 ± 2.0	273.8 ± 48.8	64.2 ± 5.0

Use of italics denotes assays where blood sample failed to clot preventing a defined measurement (was therefore denoted as ‘0’ in the MA value and *α*-angle, 200 s in the aPTT assay, and 2000 s in the *R* time measurements)

[a]Denotes mean values in columns that are significantly different ($p < 0.05$) to the saline negative control using a one-way ANOVA followed by post hoc comparisons using Dunnetts multiple comparison tests

[b]The saline control was limited to two replicates (due to loss of animal prior to sample C being taken)

significantly decreased, suggesting that the rates of thrombin production and fibrin formation were reduced.

To determine the half-life of IV DS, a single animal was administered by IV administration with 480 mg DS (dose 3 = 120 mg/kg) and blood samples were collected for preparing serum at 5 min intervals over a 100 min time course. The amount of glycosaminoglycan (GAG) was determined using the Blyscan™ Sulphated GAG Assay (Fig. 2). The basal GAG levels in serum were 40.7 ± 1.7 µg/mL (Fig. 2a) in a blood sample taken immediately prior to IV administration. After IV administration, the amount of total GAGs in serum substantially increased and reached a peak of 1500 µg/mL at 10 min—almost 40-fold higher than basal levels. The amount of total GAGs in serum then decreased to 570 µg/mL at 25 min post-intravenous administration. Figure 2b shows the amount of GAG continued to decrease and using a one-phase exponential decay of a non-linear data fit half-life, was predicted to be 19.2 min (855 µg/mL) following IV administration, or 9.2 min following peak GAG concentration (that was achieved at 10 min). A plateau concentration 189 µg/mL GAG was also predicted for the remaining time course and was four- to fivefold higher than basal GAG levels (measured at $T = 0$; 40.7 ± 1.7 µg/mL). The *R* time of recalcified citrated whole blood was measured over the time course. The control *R* time was 228 s at *T*0 and significantly increased to 1535, 1610, and 1640 s at 40, 70, and 100 min post-intravenous administration (Fig. 2).

Oral DS anticoagulant activity

To investigate whether DS could be considered as an oral presentation therapeutic molecule, the anticoagulant activities of oral formulations of DS and Clexane® in the same DVT rabbit model were investigated. Based on the results from both in vitro and IV DVT model experiments (Tables 3, 4), and the previously predicted 10% DS bioavailability (Dawes et al. 1991; Volpi 1996), a dose of 2500 mg DS was provided orally to seven animals. This dosage could produce ~ 2 mg/mL concentration in plasma, which doubled aPTT and *R* time. Similarly, given a predicted 10% Clexane® bioavailability, a dose of 120 mg Clexane® was orally administrated to seven rabbits, which could produce ~ 0.1 mg/mL in plasma, with a doubled aPTT and *R* time. Seven animals were also orally administrated with saline as a negative control. Following the thrombogenic challenge, six of the seven saline control animals presented medium to strong thrombus with a rating of 4.8 ± 1.2 (Table 4). Oral DS administration presented a minor thrombus in one animal producing an average rating of 0.7 ± 0.7; no thrombus was observed in any animals following the oral Clexane® treatment (Table 4; Fig. 3). No changes in both PT and aPTT were observed in any of the animals following the oral administrations of DS and Clexane® (data not shown).

Representatives of TEG traces of blood samples from saline, DS, and Clexane® orally administrated animals are

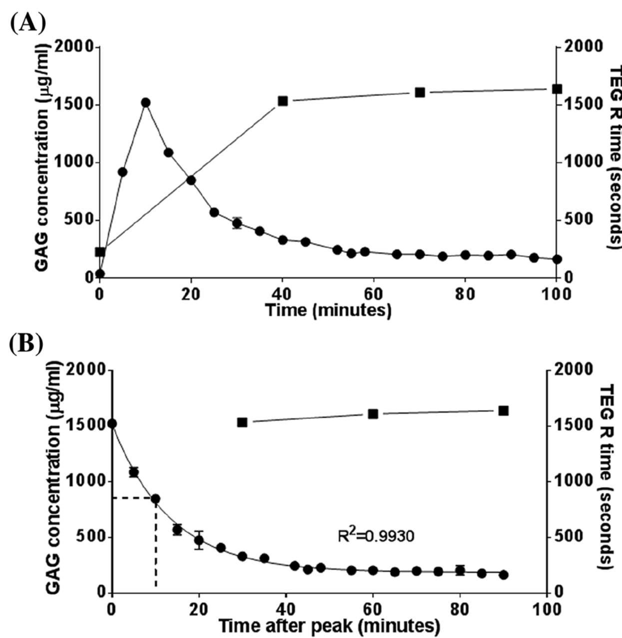


Fig. 2 Thromboelastography *R* time and half-life of glycosaminoglycans in serum following intravenous DS administration. To determine the half-life of intravenous DS, the amount of GAG in serum was determined in one animal every 5 min over a 100 min time-course post-intravenous DS administration. The blood was sampled from a 3.5 kg animal that received 480 mg of DS (dose 3 = 120 mg/kg). **a** Total GAGs were estimated in the serum using the Blyscan™ Sulphated Glycosaminoglycan Assay and expressed in µg/mL as the mean concentration ± standard error, over the 100 min time course. The *R* times of blood samples taken at time = 0, 40, 70 and 100 min were also measured. **b** GAG half-life was determined following peak concentration of GAG in serum via a one-phase exponential decay of a non-linear fit ($R^2 = 0.9930$) using GraphPad Prism 5 Software

shown in Fig. 4. TEG assays showed significant increases in *R* time in response to the oral DS and Clexane® administrations (Table 4). The MA values in DS-treated animals were lower than those in control animals, but, statistically, only blood sample A had a significantly lower MA values (Table 4). Conversely, all blood samples taken from the Clexane®-treated animals had significantly lower MA values (Table 4). The oral administrations together with in vitro and IV experiments all show that DS had fewer effects on MA values. Both DS and Clexane® treatments resulted in a significant decrease of α-angles, consistent with decreases in clot subjective ratings (Table 4). Total GAG in serum was also measured and as expected, the levels of total GAG in DS-treated animals were significantly increased, with 1.5-fold higher than those in Clexane®-treated animals and twofold higher than those in saline-treated animals (Table 4). Given that the oral dosage of DS was 20 times higher than that of Clexane®, it was not surprising that significantly higher levels of GAGs would be observed in DS-treated animals. However, these GAG levels indicate bioavailability and success of oral DS delivery.

Discussion

This study demonstrated that the anticoagulant and antithrombotic properties of native DS that was extracted from bovine collagen waste liquor may be an alternate therapeutic option to Clexane® a low-molecular-weight heparin derived from depolymerized porcine and bovine intestinal mucous and used as prophylaxis of venous thromboembolism. Presently, DS with antithrombotic and anticoagulant activity with a therapeutic grade profile has been reported sourced from porcine intestinal mucosa and employed as

Table 4 In vivo antithrombotic activity of bovine DS and Clexane® in rabbit blood following oral administration in the DVT model measured using TEG

Oral dose	Clot subjective rating	Blood sample	MA value (mm)	*R* time (s)	α-Angle (°)	GAG levels in serum (µg/mL)
DS	0.7 ± 0.7[a]	A	52.8 ± 2.3[a]	854.3 ± 88.6	47.1 ± 4.6[b]	13.2 ± 2.0[a]
		B	54.7 ± 1.8	726.8 ± 74.2[a]	52.9 ± 3.4[b]	13.6 ± 2.5[a]
		C	54.1 ± 1.9	722.1 ± 153.4[a]	54.1 ± 4.4[a]	15.7 ± 3.4[a]
Clexane®	0.0 ± 0.0[b]	A	51.3 ± 2.5[a]	1255.0 ± 318.2[a]	42.6 ± 8.7[a]	9.5 ± 1.3
		B	51.8 ± 1.6[b]	841.8 ± 167.2[a]	49.2 ± 6.3[a]	8.4 ± 1.8
		C	50.7 ± 1.7[b]	1011.0 ± 149.3[a]	42.4 ± 6.1[b]	9.5 ± 1.4
Saline	4.8 ± 1.2	A	60.5 ± 2.0	431.8 ± 38.9	63.9 ± 2.5	7.5 ± 0.7
		B	60.8 ± 2.3	413.6 ± 35.6	68.0 ± 1.4	6.7 ± 0.8
		C	60.0 ± 1.7	477.1 ± 46.9	67.3 ± 2.2	8.1 ± 0.7

[a]Denotes mean values in columns that are significantly different ($p < 0.05$) to the saline negative control for that blood sample using an unpaired *t* test

[b]Denotes mean values in columns that are significantly different ($p < 0.01$) to the saline negative control for that blood sample using an unpaired *t* test

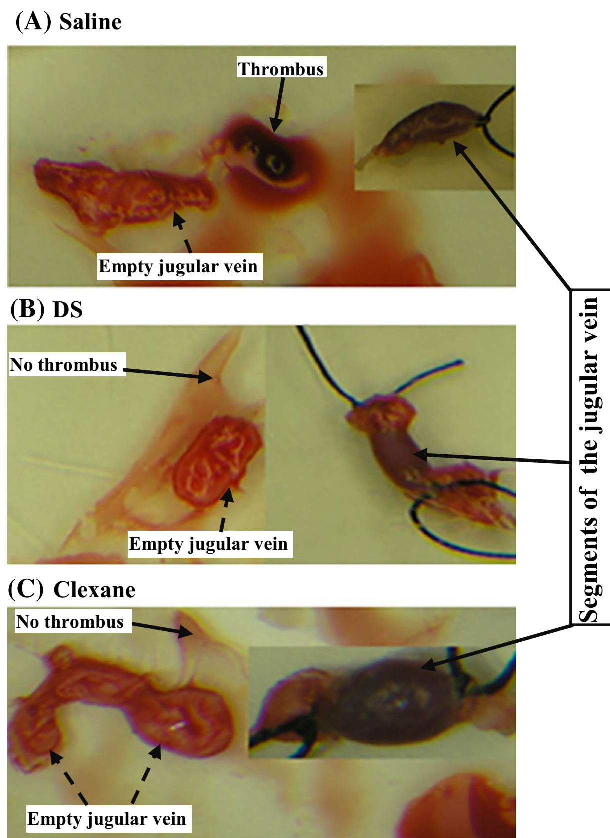


Fig. 3 Representative images of stasis blood thrombus removed from the 2-cm segments of jugular veins dissected from rabbits orally administrated with **a** saline; but not with **b** DS and **c** Clexane, respectively. Data of the blood clot rating from the excised jugular veins of three groups are shown in Table 4

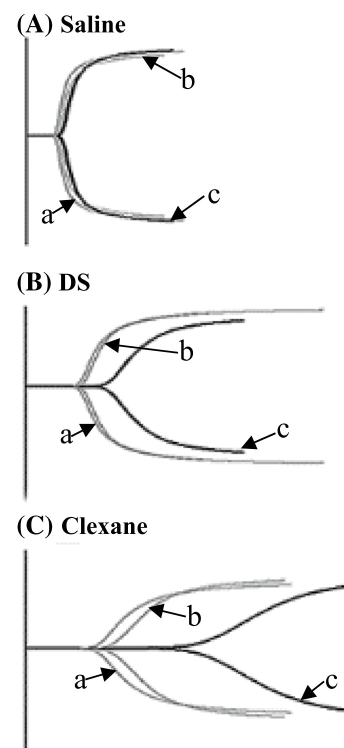


Fig. 4 Representatives of thromboelastography (TEG) traces of recalcified citrated whole blood from three time points: a at 120 min, b at 160 min and c at 190 min post administration that were orally administered with **A** saline; **B** DS; **C** Clexane, respectively

one component in a mixture of GAGs like danaparoid (de Pont et al. 2007) and mesoglycan (Tufano et al. 2010), or depolymerised into a low-molecular-weight form (Fabiana Alberto et al. 2008; Mungall 1999). It is important to note though that DS extracted from different sources all present with antithrombotic and anticoagulant activity.

A recent report concluded that there is a deficit of controlled studies limiting consistent anticoagulant effects of dietary supplements alone or in combination with drug therapy (Stanger et al. 2012). There is much ambiguity in the mechanistic understanding of the anticoagulant nature of natural compounds and, therefore, providing a robust appreciation of the anticoagulant activity of a novel compound, like the study presented here, may significantly reduce the future risk of serious adverse events.

In this study, TEG assays confirmed the anticoagulant and antithrombotic activities of Clexane® and DS following intravenous and oral administration in rabbits. The observed GAG absorption rates of Clexane® and DS were low and determined to be approximately 1 and 3% in serum. Based on these data, 240,000 U Clexane® and 50 g DS would be required for oral administration to a 70 kg person to produce similar anticoagulant and antithrombotic activities (due to the lower bioavailability of the two agents). Low oral bioavailability of unfractionated heparin (UFH) has been previously observed in a rat model (Hiebert et al. 1993). Thus, in the development of DS as a therapeutic alternative, an increase in oral bioavailability is extremely important.

Furthermore, PT and aPTT assays confirmed the anticoagulant and antithrombotic activities of Clexane® and DS following oral administration. No distinct changes in PT or aPTT were observed in any animals. As reported previously, PT and aPTT did not respond to the subcutaneous administration of ultra LMWH to healthy human volunteers (Rico et al. 2011). In addition, TEG parameters tightly correlate with the anti-FXa values in monitoring the anticoagulant level of LMWH (Artang et al. 2009; Klein et al. 2000). Therefore, TEG analysis was used to better determine the antithrombotic and anticoagulant effects of bovine DS and Clexane® in vivo. The significantly prolonged *R* times in the present study provide strong anticoagulant evidence for both Clexane® and bovine DS.

Overall Clexane® potentiated the largest increase in *R* time, but led to a significant decrease in MA value—a value

widely used to detect clot strength and platelet dysfunctions that determine the ability of platelet–fibrin interactions to facilitate clot formation. Surprisingly, the significant increase in R time observed in animals administrated either intravenously or orally with DS, was associated with unchanged MA values that were statistically similar to those from saline animals suggesting that bovine DS had less effect on platelet–fibrin interactions. Conversely, porcine intestinal mucosa DS increased R times but also decreased MA values (Senzolo et al. 2007). However, results from this study showed that bovine DS was able to not only delay clot formation but could also prevent a strong clot in the rabbit DVT model.

In vitro studies have revealed that DS inhibits thrombin through the formation of a HCII/DS complex (Baglin et al. 2002) while in vivo studies show that DS interacts with HCII distributed throughout the vessel endothelium following injury, consequently activating a natural anticoagulant pathway (Tollefsen 2007; Tovar et al. 2005). It has also been reported that intravenous injection of recombinant HCII with low affinity for heparin sulphate corrects abnormally short thrombosis times; however, this is not observed for DS in a HCII-deficient mice model, suggesting that HCII binding to DS is essential for correcting abnormally short thrombosis times (He et al. 2008). A previous study showed that porcine skin DS prolonged arterial thrombosis time in a HCII-dependent manner when both wild-type and HCII-deficient mice were intravenously administrated with DS, indicating that most DS disappeared from the bloodstream (Vicente et al. 2004). Based on these studies, it is apparent that administered DS confers antithrombotic activity after being transferred from plasma to injured sites within arterial endothelia.

The in vivo data here demonstrated oral delivery of DS with good efficacy in the 2-h DVT rabbit injury model. DS completely stemmed the formation of jugular vein thrombus with no evidence of haemorrhage in post-mortem tissue samples. Even though a clinical study would have to confirm initial impressions, nevertheless it is interesting to note that a daily oral dose of DS or Clexane® achieving serum levels of 1.2 and 0.06 mg/mL could prevent and alleviate vascular thrombosis particularly in high risk, cardiovascular compromised patients (Middeldorp et al. 2020; Pavoni et al. 2020).

In conclusion, we have demonstrated that DS purified from bovine collagen waste liquor has anticoagulant and antithrombotic potential. The in vitro and in vivo experiments provided proof of concept evidence that bovine DS may have strong anticoagulant effects comparable to Clexane®. As a result, bovine DS could be a potential anticoagulant drug used in clinics and that bovine collagen waste liquor represents a new and abundant source of biologically active DS that is currently unexploited.

Author contributions SAO contributed to the study concept and design and data acquisition, analysis and interpretation. PPM and QSD contributed to the study design, carried out all animal experimentation and the acquisition, analysis and interpretation of data, RAD contributed to the acquisition and analysis of data. KD contributed to the acquisition of data. KNZ and LV contributed to the interpretation of data. RBS contributed to the study concept and design and the interpretation of data. KNZ, SAO and LV contributed the drafting of the manuscript with input from the other authors.

Compliance with ethical standards

Conflict of interest All authors declare no conflicts of financial or commercial interest.

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