Interaction of detergent sclerosants with coagulation, antithrombotic and fibrinolytic mecanisms

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Interaction of Detergent Sclerosants with Coagulation, Antithrombotic and Fibrinolytic Mechanisms

Kurosh Parsi

A thesis submitted in fulfilment of the requirements

for the degree of Doctor of Philosophy

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Interaction of Detergent Sclerosants with Coagulation, Antithrombotic and Fibrinolytic Mechanisms

Abstract

The effects of detergent sclerosants, sodium tetradecyl sulphate (STS) and polidocanol (POL), on coagulation, antithrombotic and fibrinolytic mechanisms were investigated *in vitro*.

All samples were spiked with each sclerosant at therapeutic concentrations. Coagulation was investigated in clotting tests and functional assays for clotting factors in plasma. Fibrinogen was measured by the Clauss method and factor (F) XIII by ELISA. At low concentrations, sclerosants shortened phospholipid-dependent clotting times. At high concentrations, STS prolonged all clotting times and destroyed fibrinogen, FV, FVII, FX and FXIII.

Lytic activity in whole blood (WB), albumin and saline was investigated by absorbance densitometry. Both agents induced haemolysis, platelet, platelet microparticle (PMP) and endothelial lysis at high concentrations. The lytic effect was neutralised by albumin and plasma proteins.

Antithrombotic mechanisms were investigated in functional assays for activated protein C (APC), PC, protein S, antithrombin and FXa in normal plasma (NP). High concentration STS demonstrated anti-IIa, anti-Xa and anti-Va activity and potentiated the anticoagulant effects of APC. POL induced APC resistance.

Fibrinolytic enzymes/inhibitors were measured by ELISA in WB and plasma, and plasminogen by a chromogenic assay in NP. Inhibitors of fibrinolysis were elevated at low concentrations of sclerosants. Fibrinolytic enzymes/inhibitors were destroyed by high concentration STS.

Clot formation was assessed by thromboelasotometry in WB. Both agents induced strong clots at low concentrations, weak clots at mid-range and prevented clot formation at high concentrations. In turbidity measurements, neither agent had a lytic effect on cross-linked fibrin but STS destroyed non-cross-linked fibrin.

Platelets and PMP counts were assessed by flow cytometry and platelet activation by ELISA for soluble markers and by flow cytometry for CD62p, CD63 and calcium. Platelet aggregation was assessed by light transmission and impedance aggregometry, and by flow cytometry for glycoprotein (GP)IIb/IIIa. At low concentrations, both agents induced platelet activation, released phosphatidylserine+ PMPs but inhibited aggregation by suppressing the activation of GPIIb/IIIa.

In conclusion, detergent sclerosants interfered with coagulation, antithrombotic and fibrinolytic mechanisms. Both agents activated platelets, released procoagulant PMPs and potentiated prothrombotic and antifibrinolytic mechanisms at low concentrations. At high concentrations, both agents prevented clot formation. High concentration STS exhibited more anticoagulant activity than POL.

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THIS SHEET IS TO BE GLUED TO THE INSIDE FRONT COVER OF THE THESIS

To the memory of my father, Behruz, and my grandmother, Mamani,

who inspired me to study;

and to my mother, Violet, and wife, Yana,

for your endless love and support

Supervisor Certification

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18th March 2011

This is to certify that as primary supervisor, I have confirmed that all co-authors of the following published or submitted papers agree to Kurosh Parsi submitting these papers as part of his doctoral thesis. Furthermore, apart from where specifically indicated in the introductory declaration made prior to each individual paper/chapter in this thesis, none of the co-authors has, or intends to submit, any of the work covered by these papers as part of a separate thesis.

This thesis contains 6 manuscripts, 5 of which have been published in peer-reviewed journals. The last manuscript has been submitted and is currently under review**.** With regards to the contents of the submitted manuscript, I certify that in my professional opinion, the work is complete and is of a standard and significance that is likely to be accepted for publication in a peer-reviewed journal.

1. Parsi K, Exner T, Connor DE, Ma DDF, Joseph JE. *In vitro* Effects of Detergent Sclerosants on Coagulation, Platelets and Microparticles. *Eur J Vasc Endovasc Surg.* 2007; 34,731-740. (Chapter 2)
2. Parsi K, Exner T, Connor DE, Herbert A, Ma DDF, Joseph JE. The Lytic Effects of Detergent Sclerosants on Erythrocytes, Platelets, Endothelial Cells and Microparticles are Attenuated by Albumin and other Plasma Components *in vitro*. *Eur J Vasc Endovasc Surg*. 2008;36:216-23. (Chapter 3)
3. Parsi K, Exner T, Low J, Ma DDF, Joseph JE. *In vitro* Effects of Detergent Sclerosants on Antithrombotic Mechanisms. *Eur J Vasc Endovasc Surg.* 2009;38:220-8. (Chapter 4)
4. Parsi K, Exner T, Ma DDF, Joseph JE. *In vitro* Effects of Detergent Sclerosants on Fibrinolytic Enzymes and Inhibitors. *Thromb Res*. 2010;126:328-36. (Chapter 5)
5. Parsi K, Exner T, Low J, Ma DDF, Joseph JE. *In vitro* Effects of Detergent Sclerosants on Clot Formation and Fibrinolysis. *Eur J Vasc Endovasc Surg.* 2011;41:269-79 (Chapter 6)
6. Parsi K, Connor DE, Pilotelle A, Low J, Ma DDF, Joseph JE. Low Concentration Detergent Sclerosants Induce Platelet Activation but Inhibit Aggregation *in vitro*. (Chapter 7 submitted to Journal of Thrombosis and Haemostasis)

Yours sincerely

Joanne Joseph

Dr Joanne Joseph

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I started my PhD as a part-time UNSW student back in 2004. I consider myself a 'high maintenance' student for my supervisors as they had to meet and guide me for more than 6 years! We completed 6 research papers, included in this thesis, and a number of clinical papers during this period. Regular meetings with Joanne Joseph, my supervisor, were a source of inspiration. I am most grateful to Joanne for her close supervision and interest in my projects and for setting the bar high. I am especially grateful for the numerous hours of discussion and revision of the experiments and publications and for Joanne's incredible attention to detail.

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Publications and Presentations

Publications and presentations arising from this thesis:

Published Manuscripts

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Parsi K, Exner T, Ma DDF, Joseph JE. *In vitro* Effects of Detergent Sclerosants on Fibrinolytic Enzymes and Inhibitors. *Thromb Res*. 2010;126:328-36. **(Chapter 5)**

Parsi K, Exner T, Low J, Ma DDF, Joseph JE. *In vitro* Effects of Detergent Sclerosants on Clot Formation and Fibrinolysis. *Eur J Vasc Endovasc Surg.* 2011;41:269-79 **(Chapter 6)**

Submitted Manuscripts

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Oral Presentations

Parsi K, Exner T, Ma DDF, Joseph JE. Antithrombotic Potential of Sclerosants: *In vitro* Effects on Coagulation, Red Cells and Platelets. Australasian College of Phlebology 9th Annual Scientific Meeting and Workshops. Coffs Harbour, NSW, May 2005.

Parsi K, Exner T, Ma DDF, Joseph JE. Antithrombotic Potential of Sclerosants: *In vitro* Effects on Coagulation, Red Cells and Platelets. XV World Congress of Phlebology, Union International Phlebologie (UIP). Rio, Brazil, October 2005.

Parsi K, Exner T, Ma DDF, Joseph JE. Antithrombotic Potential of Sclerosants: *In vitro* Effects on Coagulation, Red Cells and Platelets. Haematology Association of Australia and New Zealand, NSW Monthly Meeting. Sydney, NSW, March 2006.

Parsi K, Deep Vein Sclerosis: Diagnostic Criteria. Australasian College of Phlebology 10th Annual Scientific Meeting and Workshops. Gold Coast, QLD, August 2006.

Parsi K, Neutralising the Sclerosants: What Works and What Doesn't. Australasian College of Phlebology 10th Annual Scientific Meeting and Workshops. Gold Coast, QLD, August 2006.

Parsi K, Exner T, Herbert A, Ma DDF Joseph JE. Effect of Sclerosants on Antithrombotic Mechanisms, Endothelial Cells and Fibrinolysis. World Congress of the International Union of Phlebology, Asian Chapter Meeting. Kyoto, Japan, June 2007.

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Parsi K, Exner T, Connor DE, Herbert A, Ma DDF, Joseph JE. The Effect of Detergent Sclerosants on Erythrocytes, Platelets and Endothelial cells is Attenuated by Plasma Proteins. World Congress of the International Union of Phlebology, Asian Chapter meeting. Kyoto, Japan, June 2007.

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Parsi K, Exner T, Connor DE, Herbert A, Ma DDF, Joseph JE. *In vitro* Effects of Detergent Sclerosants on Fibrinolytic Mechanisms. XVI World Congress Union Internationale Phlebologie (UIP). Monte Carlo, September 2009.

Parsi K. The Interaction of Detergent Sclerosants with the Thrombotic Systems. XVI World Congress Union Internationale Phlebologie (UIP). Monte Carlo, September 2009.

Parsi K. Sclerosants and the Coagulation. Australasian College of Phlebology 12th Annual Scientific Meeting and Workshops. Auckland, New Zealand, February 2010.

Parsi K, Exner T, Ma DDF, Joseph JE. *In vitro* Effects of Detergent Sclerosants on Fibrinolytic Mechanisms. Australasian College of Phlebology 12th Annual Scientific Meeting and Workshops. Auckland, New Zealand, February 2010.

Pilotelle A, Du D, **Parsi K.** Detergent Sclerosants Interfere with Platelet Activation and Aggregation. Australasian College of Phlebology 12th Annual Scientific Meeting and Workshops. Auckland, New Zealand, February 2010.

Du D, Pillotelle A, **Parsi K.** Overall Thrombotic Activity of Detergent Sclerosants: Thromboelastographic Data. Australasian College of Phlebology 12th Annual Scientific Meeting and Workshops. Auckland, New Zealand, February 2010.

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Parsi K, Exner T, Low J, Ma DDF, Joseph JE. Trimodal Effects of Detergent Sclerosants on Clot Formation *in vitro*. Australasian College of Phlebology 13th Annual Scientific Meeting and Workshops. Melbourne, VIC, March 2011.

Connor D, Pilotelle A, Low J, Ma DDF, Joseph JE, **Parsi K**. *In vitro* Effects of Detergent Sclerosants on Platelets and Microparticles. Australasian College of Phlebology 13th Annual Scientific Meeting and Workshops. Melbourne, VIC, March 2011.

Poster Presentations

Parsi K, Exner T, Ma DDF, Joseph JE. Prothrombotic Potential of Sclerosants: *In vitro* Effects on Coagulation, Red Cells and Platelets. XXth International Society for Thrombois and Haemostasis (ISTH) Congress, Sydney, NSW, August 2005. *Journal of Thrombosis and Haemostasis* 2005, Volume 3, Supplement 1: P0113.

Connor DE, **Parsi K,** Exner T, Ma DDF, Joseph JE. Detergent Sclerosants Cause Cell Lysis and the in vitro Formation of Platelet-Derived Microparticles with Procoagulant Phospholipid Activity. Australasian Society of Thrombosis and Haemostasis Annual Meeting, Sydney, NSW, October 2005.

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Sclerosants on Fibrinolytic Mechanisms. PhD Training Course. University of South Denmark. Ebeltoft, Denmark. April 2009 .

Exner T, **Parsi K**, Herbert A, Ma DDF. Effect of Sclerosants on Antithrombotic Mechanisms, Endothelial Cells and Fibrinolysis. XX1st International Society for Thrombosis and Haemostasis (ISTH) Congress; Geneva, Switzerland, August 2007. Journal of Thrombosis and Haemostasis 2007; Volume 5, Supplement 2: P-T-400.

Exner T, **Parsi K.** Are TEG Pattern Interpretations Reliable? XXII International Society for Thrombosis and Haemostasis (ISTH) Congress; Boston, USA, July 2009. Journal of Thrombosis and Haemostasis 2009; Volume 7, Supplement 2: PP-MO-671.

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Parsi K, Exner T, Low J, Ma DDF, Joseph JE. *In vitro* Effects of Detergent Sclerosants on Clot Formation and Fibrinolysis. International Society for Fibrinolysis and Proteolysis (ISFP). Amsterdam, the Netherlands, August 2010. *Journal of Thrombosis and Haemostasis* 2010; Volume 8, Supplement 1: P1-08.

Table of Contents

CHAPTER 8

CHAPTER 9

Abstract

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Platelet and PMP counts were assessed by flow cytometry and platelet activation by ELISA for soluble markers and by flow cytometry for CD62p, CD63 and calcium. Platelet aggregation was assessed by light transmission and impedance aggregometry, and by flow cytometry for glycoprotein (GP)IIb/IIIa. At low concentrations, both agents induced platelet activation, released phosphatidylserine+ PMPs but inhibited aggregation by suppressing the activation of GPIIb/IIIa.

In conclusion, detergent sclerosants interfered with coagulation, antithrombotic and fibrinolytic mechanisms. Both agents activated platelets, released procoagulant PMPs and potentiated prothrombotic and antifibrinolytic mechanisms at low concentrations. At high concentrations, both agents prevented clot formation. High concentration STS exhibited more anticoagulant activity than POL.

CHAPTER 1

Introduction

A. SUMMARY AND STRUCTURE

Sclerotherapy is a non-invasive procedure performed to treat varicose veins and vascular malformations.[1, 2] Sclerosing agents are used as a liquid or foam to occlude target vessels or lesions. These agents are commonly administered by a series of intra-vascular injections or less commonly via catheters.[3] It has been known for decades that injecting the sclerosants in a relatively 'empty' vein achieves a better outcome.[4] However, achieving an empty vein is technically difficult and hence the treatment generally results in substantial mixing and diffusion of the sclerosant in blood.

The usual target vessels for sclerotherapy of varicose veins are superficial veins, situated above the deep fascia. However, sclerosants can inadvertently enter the deep venous system during treatment, as visualised by ultrasound.[5] Furthermore, echocardiographic and transcranial Doppler monitoring have demonstrated entry of foam-derived bubbles into the cardiac chambers and cerebral circulation.[6] This occurs following the entry of bubbles into the adjoining deep veins and their subsequent diffusion into the central central venous system and the right heart. In the presence of a right-to-left shunt such as a patent foramen ovale (PFO), bubbles then enter the cerebral circulation.[7] Significant thromboembolic complications of sclerotherapy such as deep vein thrombosis (DVT), pulmonary embolism (PE) and stroke are reported to be rare while superficial thrombophlebitis (STP) is more common.[2, 8, 9] These adverse events have occasionally followed the use of small volumes of low concentrations of sclerosants.[8] Equally surprising, rare cases of stroke were reported to occur immediately after liquid sclerotherapy.[10, 11] The aetiology of these rare complications remains mostly unknown.

Detergent sclerosants function by inducing endothelial lysis to expose the underlying collagen with the ultimate aim of achieving endovascular fibrosis. However, whether this is achieved via a transitional state of thrombosis, direct induction of endovascular fibrosis or induction of an inflammatory state is unclear and debated.[12-15] Prior to the present work, very little was known about the basic interaction of detergent sclerosants with coagulation mechanisms. The two most commonly used detergent sclerosants, sodium tetradecyl sulphate (STS) and polidocanol (POL), were investigated in these studies. Given the scarcity of research in this field, we first aimed to explore whether these agents had any effect if any, on basic coagulation parameters such as clotting factors and clotting assays (Chapter 2). In these studies, we demonstrated that low concentrations of both agents shortened phospholipid-dependent clotting tests and released platelet-derived microparticles (PMP), indicative of procoagulant activity. At higher concentrations, STS prolonged most clotting tests and destroyed clotting factors in a concentration-dependent manner.

One important finding derived from experiments described in Chapter 2 was that the effect of sclerosants was concentration-dependent and that the effective concentration was influenced by the medium in which the measurements were performed. Clinically, the sclerosing potential of these agents in the liquid format is thought to be limited to a short distance from the point of entry and usually more than a single injection is required to occlude a vein segment which is more than a few centimetres long. The lack of distal sclerosing power was presumed to be due to dilution of these agents in blood.[16, 17] Similarly, the low thrombotic complication rate of sclerotherapy and the low incidence of thrombosis in deep veins has been presumed to be related to dilution effects. In Chapter 3, we examined the interaction of sclerosants with plasma proteins by investigating their effects on cell lysis in different sample types. Here we discovered that both agents are neutralised by albumin and plasma proteins and that POL is significantly neutralised in whole blood (WB) when compared with saline. Our findings were recently confirmed by another study that further quantified the neutralisation of STS by blood proteins.[18] These *in vitro* findings have provided some insight into why sclerosants lose potency when infused along large venous segments and mixed with significant volumes of blood. It may also be inferred that these findings explain the low incidence of distant thromboembolic complications of sclerotherapy.

Given the anionic nature of STS, this detergent would be expected to denature proteins such as activated clotting factors and hence potentiate the anticoagulant activity of antithrombotic proteins such as activated protein C (APC) or anticoagulant drugs such as heparin. In Chapter 4, we investigated the effect of sclerosants on plasma coagulation inhibitors protein C (PC), protein S (PS) and antithrombin (AT). High concentration STS mimicked AT and demonstrated significant anti-IIa and anti-Xa activity. It also demonstrated anti-Va activity and enhanced the anticoagulant effects of APC. This agent potentiated the anticoagulant effect of heparin and in comparative studies, demonstrated a similar anticoagulant profile to heparin but was 1000x weaker. By contrast, POL had less effect in general and induced APC resistance.

Experiments described in Chapters 2 and 4 examined the effects of sclerosants on coagulation and antithrombotic processes. In Chapter 5, we investigated the effects of these agents on fibrinolytic mechanisms essential in eliminating fibrin to recanalise an occluded vessel. Occlusion of target vessels is the ultimate aim of sclerotherapy and recanalisation constitutes treatment failure. Conversely, occlusion of deep veins exposed to the sclerosants is an unwanted complication of sclerotherapy and their recanalisation and restored competence are the desired outcomes. Here we discovered that both agents exhibit anti-fibrinolytic activity which was prothrombotic at low concentrations of sclerosants. This was evident by a rise in the inhibitors of fibrinolysis such as plasminogen activator inhibitor 1 (PAI-1), antiplasmin and thrombin-activatable fibrinolysis inhibitor (TAFI).

Historically, sclerosants were thought to be thrombogenic and assumed to function by forming a clot, the organisation of which could lead to vessel fibrosis.[19] In experiments detailed in Chapter 6, we investigated the effects of both sclerosants on clot formation and lysis. Both agents demonstrated a tri-modal effect on clot formation where at low concentrations they induced strong clots, at mid-range concentrations induced weak clots susceptible to lysis and at higher concentrations prevented clot formation. We also showed that neither agent had a lytic effect on cross-linked fibrin but high concentration STS destroyed non-cross-linked fibrin.

In experiments described in Chapter 6, we demonstrated that low concentration sclerosants initiate a platelet-dependent process of clot formation. We also had prior evidence as detailed in Chapters 2 and 3 that PMP were released at low concentrations of both agents. In Chapter 7, we investigated the effects of sclerosants on platelet integrity and function. Here we demonstrated that both agents induced platelet and PMP lysis at high concentrations. At low concentrations, both agents induced platelet activation but inhibited aggregation by suppressing the activation of glycoprotein (GP) IIb/IIIa. The sclerosant-induced PMP demonstrated a phosphatidylserine+ phenotype potentially capable of initiating clots at distant sites.

These findings have provided us with a basic understanding of the effects of detergent sclerosants on the haemostatic systems and have paved the way for clinical and *in vivo* studies currently underway by the candidate and co-authors.

B. LITERATURE REVIEW

1. Venous Disease and Sclerotherapy

1.1 Superficial and Deep Venous Systems

The peripheral venous system is comprised of a network of inter-connecting superficial and deep veins that communicate at numerous levels. Multiple small tributaries join to ultimately form large trunks that direct the venous return to the central venous system. The one-way valves in the venous system normally allow uni-directional flow. The mechanical pump required for venous return from the lower limbs is provided by muscular contractions. Deep veins, situated below the deep fascia, are responsible for up to 90% of the overall return.[20] The superficial venous system ultimately drains into the deep venous system at junctions with the deep veins or via 'perforating' veins (veins that perforate through the fascia to reach the deep veins).

The great and small saphenous veins (GSV and SSV respectively) are the main superficial venous trunks of the lower limbs. Each trunk normally terminates at a junction with deep veins. The GSV originates anterior to the medial malleolus and ultimately drains into the common femoral vein (CFV) at the sapheno-femoral junction (SFJ) (Fig. 1). The GSV may be duplicated or rarely may not be present. This vein has multiple tributaries and communicates with the SSV via a number of inter-saphenous veins.

The SSV originates posterior to the lateral malleolus (Fig. 2). This vein may also be duplicated or totally absent. The termination of the SSV is variable, as it may drain into the popliteal vein at the sapheno-popliteal junction (SPJ) or communicate with this vein via a perforator. Less commonly, the SSV has no communication with the popliteal vein at all and may drain into the great saphenous system via its cranial extension (the vein of Giacomini) or into the femoral vein via posterior thigh perforators.[21] Rarely, the SSV may terminate in the inferior calf and drain via gastrocnemius perforators or inter-saphenous communications.

The lateral aspect of the leg is served by the lateral superficial venous system that drains into the deep veins at multiple levels utilising perforators or via its communications with the small and great saphenous systems (Fig. 2).

The deep venous system of the lower limbs is comprised of a network of interconnecting intra- and inter-muscular veins (Fig. 3). Intra-muscular veins, such as the gastrocnemius and soleal veins, drain the muscle tissue and empty into inter-muscular veins. The peroneal, posterior and anterior tibial veins are the inter-muscular veins of the calf that unite to form the popliteal vein. In general, the deep veins of the calf are paired but the individual members of the pair communicate with each other at multiple levels.

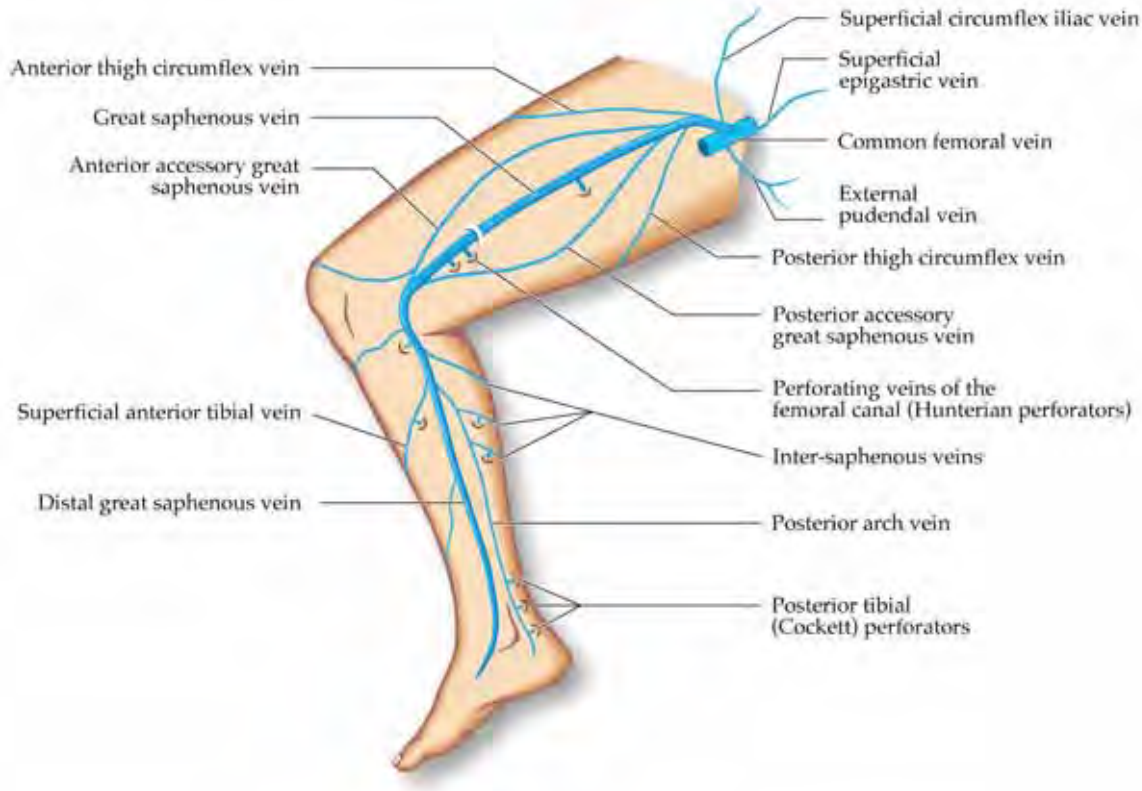

Figure 1. An idealised schematic representation of the Great Saphenous vein and its major tributaries and perforators.

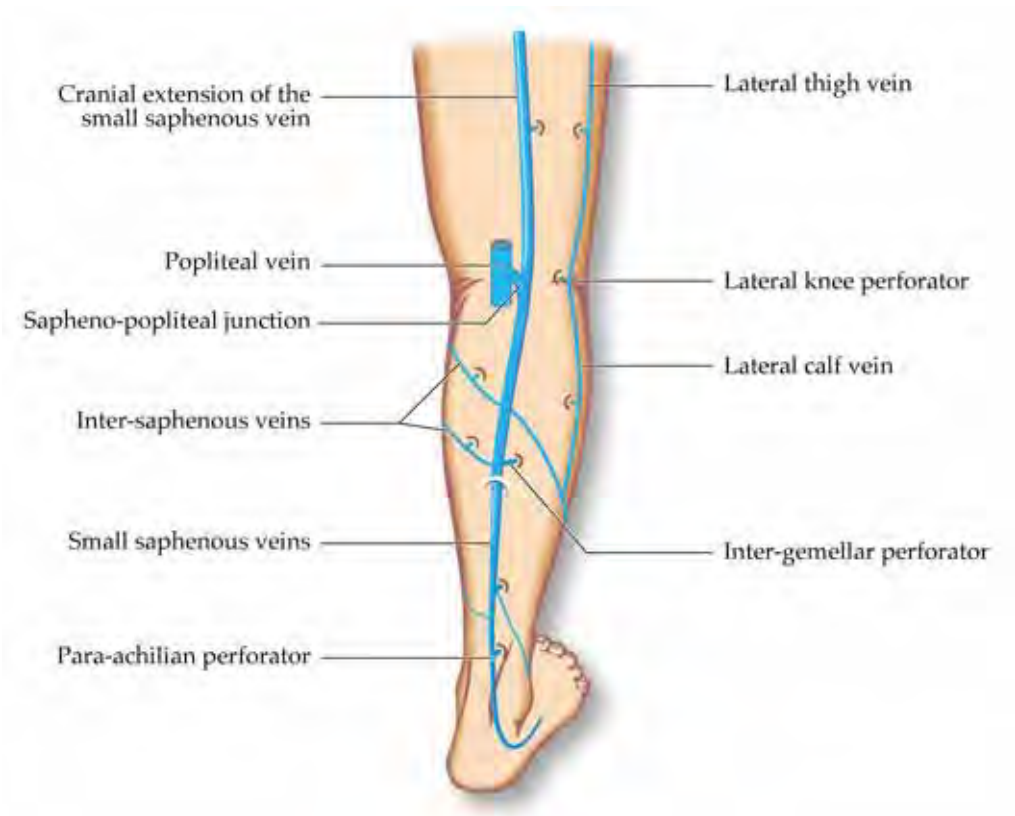

Figure 2. An idealised schematic representation of the Small Saphenous vein and its major tributaries and perforators.

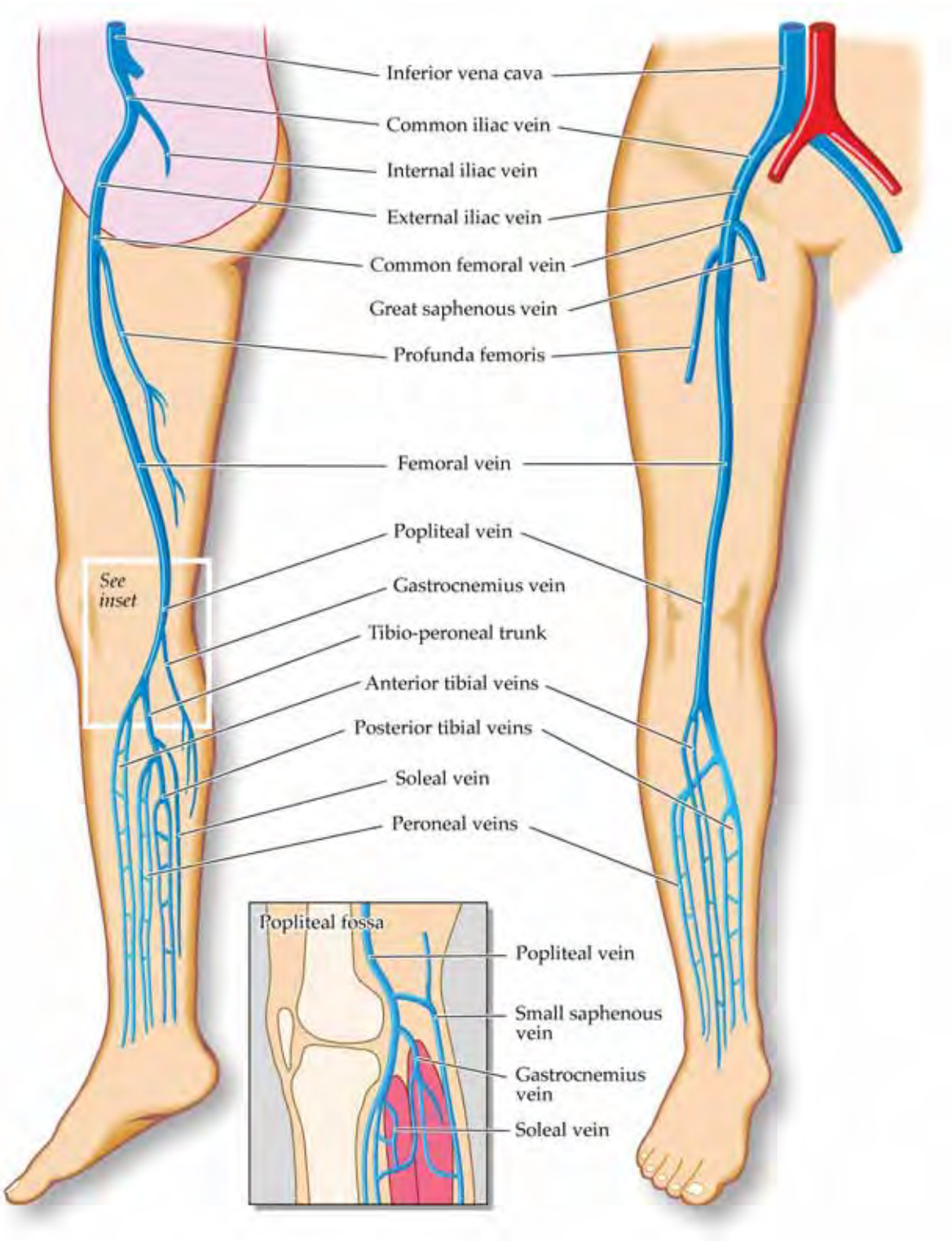

Figure 3. An idealised schematic representation of the deep venous system of the lower limbs.

The femoral vein is a continuation of the popliteal vein that unites with the profunda femoris to form the CFV. The CFV changes name to the external iliac vein at the lower border of the inguinal ligament to ultimately drain into the inferior vena cava (IVC).

The superficial venous system utilises a large number of perforating veins to drain into the deep venous system. Perforating veins contain one-way valves that allow uni-directional flow of blood into the deep veins. *Direct perforating veins* drain into the underlying inter-muscular veins whereas *indirect perforating veins* communicate with intra-muscular veins. (Fig. 4).

1.2 Venous Drainage of Skin and Subcutaneous Tissue

The skin is supplied by a rich network of arteries and arterioles that branch to ultimately form the capillary loops (Fig. 4).[22] As the capillary loops descend towards the subpapillary plexus, they take on venous characteristics. Post-capillary venules form the venous aspect of the sub-papillary plexus and function as the site where inflammatory cells migrate from the vascular to the extravascular compartment. Dilatation of the post-capillary venules results in clinically evident telangiectasias seen in venous disease. Post-capillary venules drain into descending venules that run perpendicular to the surface of skin to join the deep dermal plexus. The deep dermal veins drain into the smaller sub-dermal veins or the larger subcutaneous reticular veins. Other than most veins in the head and neck, small to medium-sized veins contain bicuspid valves oriented to prevent reflux back into the dermal vessels. Reticular veins join the larger venous tributaries or may drain into major superficial venous trunks, such as the saphenous veins, or via perforators directly into the deep veins.

The subcutaneous venous network forms a large-capacitance reservoir for venous blood. Sluggish flow in this network leads to the purplish colour seen in venous congestion. The abundance of superficial vessels is significantly more than what is metabolically required and a key function of superficial vascular networks is the regulation of core temperature.[23]

1.3 Varicose Veins

Varicose veins are common and may be found in up to 50% of the Caucasian population.[24] Histologically, the affected veins demonstrate a loss of elastic fibre and an increased muscle content.[25] There is debate whether the primary pathology of varicose veins is valvular failure or whether valvular failure is the result of a weakness in the vessel wall.[26-30] There is also debate whether the pathology in the affected veins progresses in an ascending or descending fashion or both.[31, 32] Haemodynamically, the affected veins demonstrate retrograde flow (away from the heart), referred to as 'reflux'. Veins demonstrating reflux are termed 'incompetent'. The smaller superficial vessels can also demonstrate reflux and clinically present as incompetent reticular veins and telangiectasias. Reflux in incompetent

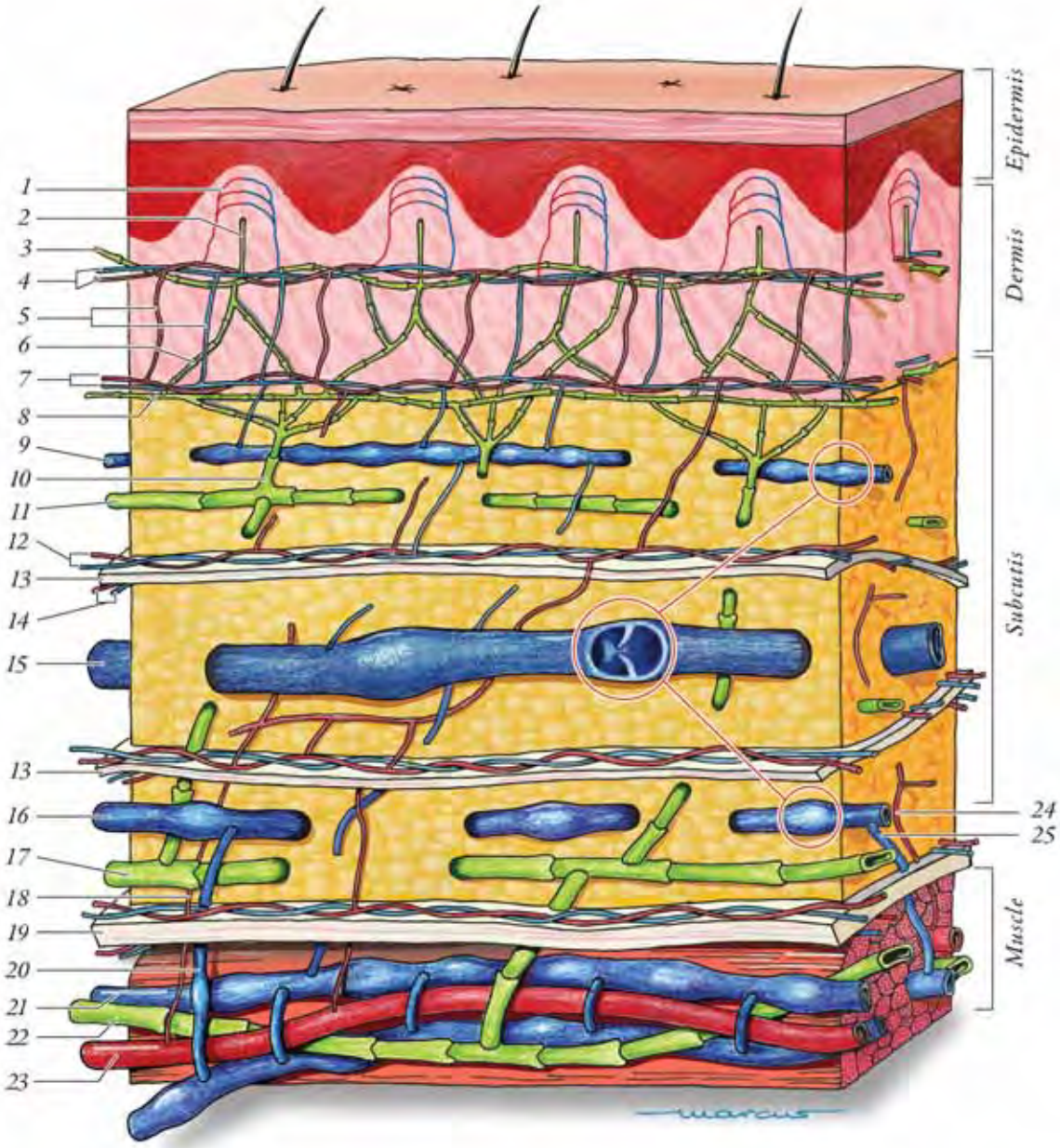

Figure 4. Cross section of skin and the underlying subcutis and muscle.

(1) Capillary network, (2) blind lymphatic capillary, (3) dermal lymphatic plexus, (4) subpapillary microvascular plexus, (5) ascending arteriole and descending venule, (6) cutaneous lymphatic pre-collector, (7) deep dermal microvascular plexus, (8) sub-dermal lymphatic plexus, (9) reticular vein, (10) subcutaneous lymphatic pre-collector, (11) subcutaneous lymphatic collector (superficial), (12) supra-fascial vascular plexus, (13) superficial fascia, (14) subfascial plexus, (15) superficial venous trunk, (16) tributary vein, (17) subcutaneous lymphatic collector (deep), (18) septo-cutaneous artery, (19) deep fascia, (20) direct perforating vein, (21) deep inter-muscular vein, (22) deep lymphatic, (23) deep inter-muscular artery, (24) musculo-cutaneous artery, (25) indirect perforating vein. Reproduced with permission from Parsi K *et al* [22].

veins is usually detected and quantified by Doppler measurements as part of a venous incompetence ultrasound study. The pathway of incompetence is usually documented to generate a venous incompetence 'map'. The diameter of the vessels and any anatomical variations are noted during the ultrasound study and the information is then used to recommend an appropriate management plan.

Prolonged venous incompetence can lead to a state of functional failure termed 'chronic venous insufficiency (CVI)'. CVI is characterised by progressive changes including oedema, red cell extravasation, peri-vascular fibrin deposition, fibrosis of the skin and subcutaneous fat (lipodermatosclerosis), impaired arterial inflow, cutaneous infarcts (atrophie blanche) and ultimately skin ulceration. Severe changes can lead to fixed joint deformities and substantial tissue necrosis. Varicose veins also increase the risk of thromboembolic events and in particular STP. CEAP (Clinical Etiological Anatomical Pathophysiological) classification was devised to standardise the staging of CVI.[33]

1.4 Treatment of Varicose Veins

The traditional approach to the treatment of varicose veins has involved some form of surgery to physically remove these vessels. This has generally included high ligation and stripping of major incompetent trunks such as the GSV followed by multiple stab avulsions of the visible varicosities. Less invasive alternative treatments have gained popularity in the past 20 years and have included ablative techniques such as sclerotherapy, endovenous laser ablation (EVLA) and radiofrequency ablation (RFA).[34, 35]

Sclerotherapy involves introduction of a sclerosing agent into the lumen of a target vessel to induce endoluminal chemical ablation and ultimately endovascular fibrosis. Sclerotherapy can be performed under ultrasound guidance to treat major superficial trunks such as the GSV as well as tributary veins and perforators.[36, 37] Direct vision sclerotherapy is used to treat the visible varicose veins, reticular veins or telangiectasias. EVLA and RFA are primarily used to ablate saphenous trunks.

1.5 Treatment of Vascular Lesions

Apart from treating incompetent veins, sclerotherapy is also used to occlude vascular malformations, vascular ectasias, haemorrhoids and oesophageal varices. Vascular malformations are anomalies of the peripheral vascular system that result from a developmental arrest during various stages of embryogenesis.[38] These are classified into venous, lymphatic, arteriovenous or a combination of these abnormalities.[39] Sclerotherapy is routinely used to treat venous and lymphatic malformations.[38] In contrast to vascular malformations, vascular ectasias are acquired lesions that present later in life. A common example is the so-called venous lake, which is a dilatation of superficial vessels secondary to actinic damage, also responsive to treatment with sclerotherapy.

1.6 A Brief History of Sclerotherapy

The German alchemist and physician, Johann Sigismund Elsholtzii (1623-1688) is credited for having introduced the concept of intravenous injections. In his 1667 book *Clysmatica nova*, Elsholtzii discussed possible methods for infusion therapy and even blood transfusion.[40] The Swiss scientist, Zollikofer of St. Gallen performed the first known sclerotherapy procedure when in 1683 he injected acid into a vein to induce clot formation.[41] Nearly two hundred years later, perchlorate of iron injections were used to treat varicose veins. In 1854, iodine and tannin were used in treatment of 16 cases of varicose veins. By the end of the century, this treatment was abandoned due to high incidence of adverse reactions.

In the early 20th century, carbolic acid, perchlorate of mercury and quinine were all tried as sclerosing agents but were also abandoned due to the high rates of complications. French phlebologists developed the use of sodium carbonate and then sodium salicylate during and after the First World War. In the 1950s, ethanolamine oleate was used as a sclerosing agent to occlude the distal segments of the divided saphenous veins. [42] This agent is now routinely used to treat oesophageal varices.

STS was first developed as a sclerosing agent in 1946 and was registered in the United Kingdom (UK) in 1967. In the 1960s, George Fegan reported treating over 13,000 patients with sclerotherapy using STS. Fegan is credited with defining the ultimate end-result of sclerotherapy, as endovascular fibrosis, as against thrombosis. He also emphasised the importance of controlling significant points of reflux and compression of the treated legs. The pioneering work of George Fegan resulted in a wave of enthusiasm for sclerotherapy as a substitute for surgery. However a decade later, this procedure lost its popularity in the UK following the publication of a prospective randomised study by Hobbs which demonstrated high recurrence rates.[43]

The history of POL goes back to the 1930s, when scientists working on textile chemistry discovered that compounds called alkylpolyglycolethers have local anaesthetic properties.[44] In the 1950s, these agents were injected intravenously to assess their systemic toxicity. It was observed that the injections may cause occlusion of the exposed veins and the agents were found unsuitable for systemic use. The Medical Director of Kreusssler Pharma at the time, Otto Henschel, explored the sclerosing properties of one particular member of this group, hydroxy polyethoxy dodecane, later named polidocanol. Henschel performed experiments on himself and then recommended this sclerosant to other medical practitioners. In 1963, the German physician E. Lukenheimer performed the first known sclerotherapy procedure with polidocanol using 2mL of Aethoxysklerol® foam. In 1966, This sclerosant was registered in Germany as a sclerosing agent.

The original Fegan approach, which was not performed under ultrasound guidance, involved treating the 'control points' which he located at the perforating veins filling distal vessels. This resulted in a 'distal first' approach which did not emphasize the need to obliterate the saphenous trunks.[45] By contrast, the 'French' approach advocated treating the most proximal sources of reflux first, before distal vessels were treated. This became achievable with the advent of duplex ultrasound in the late 1980s which allowed visualisation of the saphenous trunks and deeper tributaries. The French phlebologist, Michel Schadeck, is credited with developing ultrasound guided sclerotherapy (UGS) which involved injection of a sclerosant in the great or small saphenous vein near the junction, followed by a number of subsequent injections along the length of the trunk and eventually the distal tributaries.[37] Further preliminary reports of similar techniques emerged later that decade and the procedure gained popularity as an alternative to surgery in the 1990s.[46-48] Catheter directed sclerotherapy was introduced in the mid-1990s to increase the safety and efficacy of this procedure especially when treating the saphenous trunks.[3]

With the re-introduction of foam sclerosants in the late 1990s, sclerotherapy gained substantial popularity.[49] The concept of using froth rather than liquid goes back to 1944 when Orbach found this format to be 4.5 times more effective.[4, 45] The use of foam sclerosants was resurrected by Juan Cabrera who published his results of treating saphenous trunks and vascular malformations.[50] He reported to have successfully treated large veins and vascular malformations with a POL-derived foam sclerosant and proposed this method as a suitable alternative to surgery. The procedure probably owes its popularity to Lorenzo Tessari who introduced a simple way of making foam at the patient's bedside (see 1.8 Foam Sclerotherapy). Further studies demonstrated foam sclerosants to be more effective than the liquid agents and currently foam sclerotherapy is considered the method of choice for treatment of larger veins.[1, 45] The increased efficacy of foam sclerosants has been attributed to displacement of blood and increased exposure of the endothelium to the active sclerosing agent. Liquid sclerotherapy continues to be used for smaller reticular veins and telangiectasias, although foam sclerosants are also used to treat such small vessels.

Currently, STS and POL remain the most popular sclerosing agents used worldwide. Other sclerosants including osmotic agents (such as hypertonic saline) and irritant chemicals (such as sodium iodide and absolute alcohol), are less commonly used.[51]

1.7 Ultrasound Guided Sclerotherapy (UGS)

UGS involves introduction of a sclerosant into target vessels under ultrasound guidance. Imaging allows assessment of the size of the target segment, its communications with adjoining vessels, an accurate placement of the needle or catheter, monitoring the flow of the sclerosant and the subsequent spasm and non-compressibility of the target vessel.

The injected sclerosant, especially when in foam format, can be traced in the target vein and the next injection point can be selected. Ultrasound guidance has enhanced the safety and effectiveness of sclerotherapy.[52]

UGS is not a universally standardised procedure and may be influenced by multiple variables.[53, 54] There is no broad consensus or strong evidence to help define appropriate patient selection criteria or what is considered to be an absolute or relative contraindication. One important controversial issue is the requirement for cessation of the oral contraceptive pill or hormone replacement therapy to prevent thromboembolic complications.[40, 55-60] There is also no broad agreement on the necessity, grade (level of compression), type (bandages vs. stockings) or duration of post-treatment compression therapy. There are multiple technical variations including the choice (STS vs. POL), concentration and volume of the sclerosants used for a particular vessel of a certain diameter, as well as the format (foam vs. liquid) and the method by which it is administered (direct injections vs. catheters).[1, 45]

The treatment approach has also followed multiple trends. In the original 'French' approach advocated by Schadeck, the sclerosant is injected proximally, a few centimetres away from the junction. The flow of the sclerosant and its vasospastic effect on the target vein is followed distally to choose the next injection site. This proximal to distal approach was followed by most Australasian phlebologists who commenced performing sclerotherapy in the 1990s. The technique of Cabrera was adopted by most Australian surgeons who commenced sclerotherapy in the early 2000s. Here the vein is injected or cannulated relatively distally (for example at the knee for the treatment of the GSV) and the foam is injected to fill the proximal segment of the vein. Both approaches may be accompanied by further modifications such as leg elevation before, during or after the procedure, manual compression of the junctions at the time of the procedure and manual 'massage' of the injected foam.

1.8 Foam Sclerotherapy

Detergent sclerosants are manufactured as liquid but are commonly mixed with various gases to prepare foam.[45, 49, 61, 62] The foam format is reported to be more effective than liquid presumably due to displacement of blood and a better exposure of the endothelium to the active sclerosant.[63]

Currently, foam sclerosants are not manufactured commercially but are prepared by individual practitioners at the bedside. Amongst many methods advocated to produce foam, that described by Lorenzo Tessari seems to be the most popular.[61] This method involves using two disposable syringes connected by a 3-way tap. One syringe contains gas (room air or CO_2 or a combination of oxygen and CO_2) and the other contains the liquid sclerosant in a ratio of 1 unit liquid to 4 units gas.[6, 54, 64] The liquid and gas are pumped

back and forth multiple times to generate a microfoam (containing small bubbles). The method of Monfreux published in 1997 involves pulling back the plunger in a capped glass syringe containing 0.3-0.5mL of liquid sclerosant.[65] This method is reported to make a longer lasting foam but contains larger bubbles. Sadoun and Benigni reported a method that involved rapid pulling and release of the plunger in a plastic syringe that contains gas and the liquid sclerosant.[66] The method described by Frullini is similar to that of Tessari but involved connecting an air-filled syringe to the sclerosant vial, turning the vial upside down, and then aspirating and injecting the liquid back into the vial to generate foam.[67] Given the large number of variables, generated foams may differ significantly depending on the technique, the foaming gas, the liquid/gas ratio, the base liquid sclerosant, the syringe type and other materials used.[54] These variations may affect the clinical outcome and have made comparative assessments exceedingly difficult.[5, 53, 68, 69]

Foam-derived bubbles can be observed on concurrent echocardiography and trans-cranial Doppler to enter the heart and cerebral circulation during foam sclerotherapy.[9, 70, 71] It is unknown whether a more stable foam is safer or whether the increased stability may lead to a higher incidence of vascular occlusive adverse events. It is also unknown whether foam-derived bubbles maintain a thin film of detergent at the blood-gas interface (which would reduce the surface tension) or whether such bubbles are devoid of a surfactant surface. Blood samples collected from the cardiac chambers in an animal study have shown no sclerosing activity.[72]

2. Surfactants and Biological Cell Membranes

2.1 Detergents and Surfactants

Both STS and POL are biochemically classified as surfactant detergents. Detergents are materials used for cleaning and hence water and soap can be defined as detergents. Detergents in common use may contain a range of compounds such as surfactants, softeners, abrasives, bleaching agents, buffers and foam-modifying substances.

Surfactants (surface active agents) are compounds that reduce the surface tension of a liquid. Surface tension (measured by N/m, force per unit length) is the cohesive force amongst the molecules at the surface of a substance that creates a surface film. For instance, surface tension maintains the shape of liquid droplets. The cohesive force between surface molecules makes it more difficult to move an object through the surface film than to move it within the liquid substance. Soaps are surfactants made of sodium or potassium salts of fatty acids.

Surfactants are amphiphilic compounds and in general contain a hydrophobic (non-polar) hydrocarbon tail and a hydrophilic (polar) head group (Fig. 5a). Therefore, surfactants are soluble in both oils (via the hydrophobic tail) and water (via the hydrophilic head). Surfactants reduce the surface tension by adsorbing at the interface between the two substances. Normally oil fragments suspended in water form larger masses. The presence of a surfactant would decrease the surface tension between oil and water surfaces, allowing more mixing and preventing the coalescence of oil droplets into a bigger mass. Therefore, the surfactant allows the break-up of oil into multiple minute droplets that can be washed away.

2.1.1 Aggregation of Surfactants

Surfactants mimic the lipid environment of a cell membrane but unlike lipids do not form bilayers. In polar solvents, for example water, surfactant monomers have limited solubility. Beyond a certain concentration specific for each compound, surfactants spontaneously form an ordered phase where they aggregate to form micelles. This concentration is referred to as the critical micellar concentration (CMC). Above the CMC, monomers and micelles exist in dynamic equilibrium. The destructive effect on endothelial cells is increased when surfactants act as aggregates rather than monomers.[51] CMC is dependent on size, charge and length of the surfactant molecule and varies with buffer/salt. For detergents with the same head group, the CMC decreases with increasing hydrocarbon chain length by approximately one order of magnitude for every two methylene units.[73] The addition of buffer additives significantly affects the solubility, CMC, cloud point and aggregation number of a detergent (Table 1). For example, high salt concentrations strongly decrease the CMC of ionic detergents.

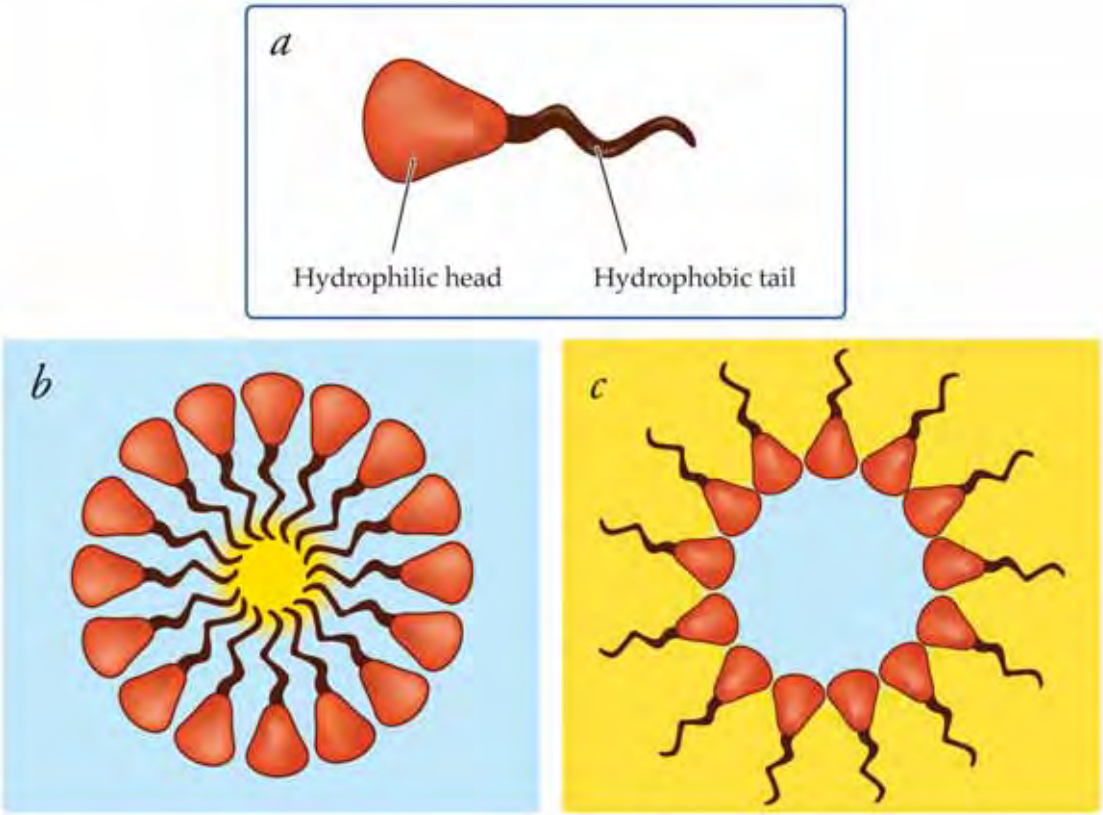

Figure 5. Aggregation of surfactants.

(a) An individual surfactant molecule, (b) a surfactant micelle in an aqueous solution (normal phase micelle), and (c) in an oily medium (reverse micelle).

When micelles form in an aqueous solution (such as water), the hydrophobic tails form a core that can encapsulate an oil droplet, and the hydrophilic (ionic/polar) heads form an outer shell that maintains contact with the aqueous solvent. This is called a normal phase micelle (oil-in-water micelle) (Fig. 5b). Normal phase micelles are soluble in water and maintain equilibrium with soluble monomers, resulting in a constant exchange of individual surfactant molecules between individual micelles.

When surfactants assemble in oil, the aggregate is referred to as a reverse micelle. In a reverse micelle, the heads are in the core which can contain an aqueous environment and the tails maintain contact with the oily medium (water-in-oil micelle) (Fig. 5c). Other than the monomeric and micellar states, surfactants can form a liquid phase that is immiscible with the water phase. This process is called phase separation and represents aggregation of a large number of micelles.[73] Cloud point refers to the temperature at which phase separation occurs and is specific for each type of surfactant (Table 1).

Table 1. Glossary of Detergent Properties

Term	Definition
Micelle	A spherical aggregate of defined size, made up of detergent monomers
Aggregation Number (N)	The number of detergent monomers in a micelle
Critical Micellar Concentration (CMC)	The minimum concentration above which detergents form micelles
Critical Micellar Temperature	The minimum temperature at which micelles form
Cloud Point	The temperature at which phase separation occurs
Csat	Saturation concentration: the minimum concentration at which detergent molecules co-operatively interact to produce large membrane fragments
Critical Solubilisation Concentration (CSC)	The minimum concentration at which lipid and protein containing units start to become solubilised

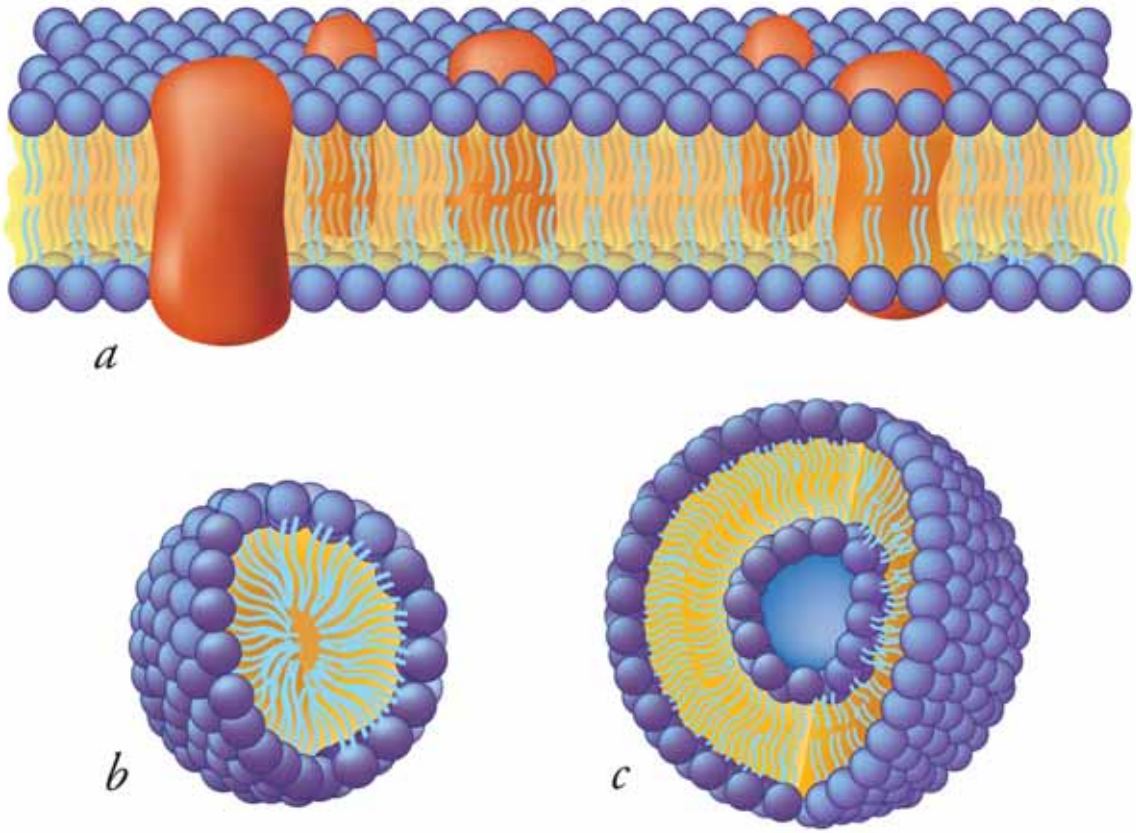

Figure 6. Structural organisation of phospholipids.

(a) Phospholipids bilayer containing membrane proteins, (b) micelle and (c) liposome.

2.1.2 Classification of Surfactants

Surfactants can be classified into four groups depending on the surface charge. Non-ionic surfactants have no charge groups whereas anionic agents carry a net negative charge and cationic surfactants carry a net positive charge. STS is an anionic surfactant whereas POL is a non-ionic agent.

Surfactants containing a head with two oppositely charged groups are called zwitterionic. These are characterized by their (net) uncharged, hydrophilic headgroups. Such surfactants are also called amphoteric (capable of reacting chemically either as an acid or a base) and ampholytic (cationic in acidic solutions and anionic in basic solutions).

Anionic surfactants normally contain a sulfate, sulfonate or carboxylate group. STS contains a sulfate group. Non-ionic agents include fatty alcohols and alkyl polyethylene oxides such as polysorbates. POL is a polyethylene oxide detergent. Cationic agents contain a quaternary ammonium group and zwitterionic agents contain a quaternary ammonium cation and a sulfonate, carboxylate or a phosphate anionic group.

2.2 Biological Cell Membranes

Cell membranes are composed of lipid bilayers. Similar to surfactants, membrane phospholipids are amphiphilic and contain a hydrophilic polar head group and a hydrophobic tail group. Phospholipids usually have two hydrocarbon chains forming the tail group while surfactants may have one or two such chains.

Molecules with large hydrocarbon chains tend to form bilayers as the alkyl chains are too bulky to fit into smaller structures like micelles. Thus phospholipids with two hydrocarbon chains are likely to form bilayers while detergents tend to form micelles. The polar heads of phospholipids face the aqueous solutions on both sides of the bilayer structure (Fig. 6a). One layer of polar heads faces the external environment of the cell (exoplasmic) while the other layer faces the cell cytoplasm (cytoplasmic). The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer.

The membrane structure is quite fluid and held together by non-covalent interaction of hydrophobic tails. Disruption of cell membrane phospholipids would lead to formation of monolayer micelles or bilayer liposomes (Fig. 6b and 6c). The phospholipid monolayer micelle has a hydrophobic lipid core. The bilayer liposomes have a hydrophilic polar external surface and also a hydrophilic polar internal surface that can contain an aqueous solution.

2.2.1 Composition of Membrane Lipids

Phospholipids such as phosphatidylethanolamine and phosphatidylcholine are the most common membrane lipids. Other membrane lipids include glycolipids and cholesterols but phospholipids in general are the most abundant membrane lipids. The cytoplasmic

and exoplasmic leaflets of the cell membrane maintain an asymmetric distribution of phospholipids. In the resting state, the cytoplasmic surface is enriched in anionic and primary amine containing phospholipids such as phosphatidylserine and phosphatidylethanolamine. The exoplasmic surface and the equivalent luminal surface of internal organelles, are enriched in choline containing neutral phospholipids such as phosphatidylcholine, sphingomyelin and sugar-linked sphingolipids.

This asymmetrical distribution of phospholipids on cell membranes is tightly controlled and maintained by groups of enzymes collectively referred to as 'flippases and floppases'. These enzymes use ATP to transport phospholipids to either side of the membrane. The transport of anionic phospholipids into the cytoplasmic aspect is catalysed by flippases whereas floppases catalyse the transport of choline-containing phospholipids back to the exoplasmic surface. Therefore, these enzymes actively function to maintain membrane lipid asymmetry during the resting state.

Cell activation and apoptosis can lead to exposure of negatively-charged phospholipids such as phosphatidylserine on the exoplasmic surface of the cell membrane. This is mediated by another group of enzymes referred to as 'scramblases'. These enzymes are not ATP-dependent but depend on a rise in the cytoplassmic calcium for their function. Scramblases re-shuffle the phospholipids between the two membrane surfaces and generate a symmetric distribution of the negatively-charged phospholipids.

Exposure of phosphatidylserine on the cell membrane provides the negatively-charged surface required for the initiation of the coagulation cascade. This is facilitated by the phosphatidylserine-induced activation of the coagulation complexes such as tenase and prothrombinase. Coagulation reactions otherwise occur very slowly on membrane surfaces that do not contain phosphatidylserine and therefore resting cells are essentially incapable of supporting the coagulation cascade (see 4.1.2 The Cell-based Model of Coagulation).

2.2.2 Membrane Proteins

Membranes contain a variety of proteins. Integral membrane proteins such as receptors, transporters, channels and adhesion proteins are permanently attached to the cell membrane. Integral membrane proteins can be transmembrane structures where they span the entire membrane or monotopic when permanently attached to the membrane from one side. Such proteins are solubilised under conditions which disrupt the lipid bilayer and normally require a detergent or an apolar solvent to be displaced.

Peripheral membrane proteins attach to integral membrane proteins, or penetrate the peripheral regions of the lipid bilayer using a combination of non-covalent interactions. Conditions including high or low salt and alkaline pH allow the differential extraction of such proteins as they induce the disruption of protein-protein interactions while leaving the lipid bilayer intact.

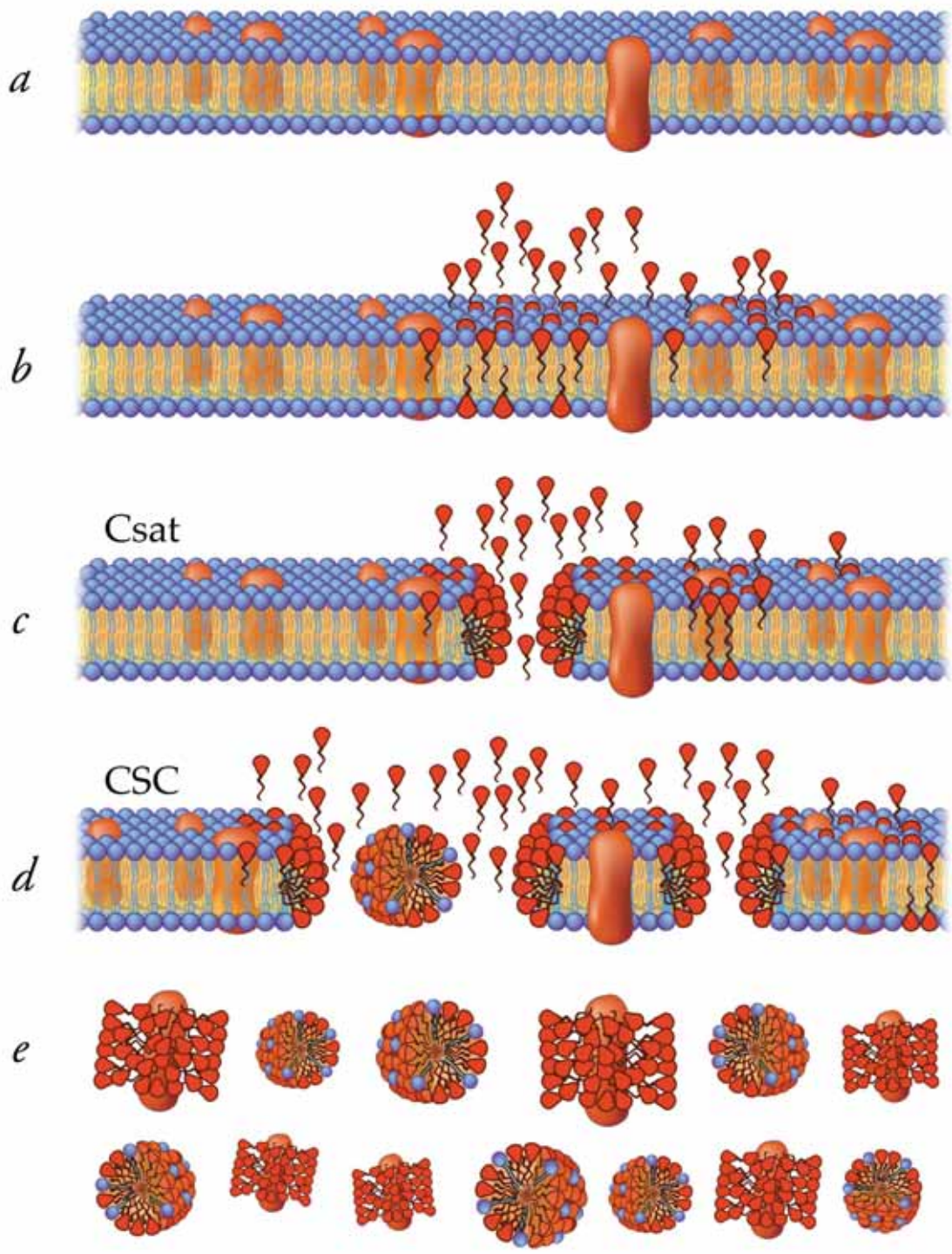

Figure 7. Interaction of surfactants with the lipid bilayer.

(a) Normal cell membrane, (b) non-co-operative interaction at low concentrations, (c) co-operative interaction producing large membrane fragments sealed at the edges by toroidal complexes occurring at a minimum saturation concentration (Csat), (d) initiation of solubilisation at the critical solubilisation concentration (CSC) and (e) the final mixed lipid-detergent micelles and detergent covered protein units.

2.3 Interaction of Detergents with Cell Membranes

Detergents are used to solubilise membrane proteins and lipids, leading to disruption of cell membranes at high enough concentrations. In membrane biochemistry, relatively high concentrations of detergents with low CMCs are used to solubilise large amounts of lipids.[73] Although removal of lipids can also be achieved by using organic solvent extraction, these agents in general destroy the native structure of membrane proteins. Solubilisation using detergents is a milder method that may yield stable membrane proteins depending on the detergent used.[73]

2.3.1 Intermediary States of Membrane Solubilisation

Solubilisation of biological membranes involves a number of intermediary states determined by the free detergent concentration (Fig. 7). It is initiated by destabilisation of the lipid component and follows four overlapping phases:[74]

1. *Initial phase:* Non-co-operative interactions. In this phase, the detergent is taken up in non-micellar form by the lipid phase (Fig. 7b).

2. *Second phase:* Co-operative interactions. Beyond a certain free detergent concentration, the detergent molecules co-operatively interact in the membrane and produce large membrane sheets sealed at the edges by toroidal (doughnut-shaped) assemblies of detergent molecules. (Fig. 7c). This concentration is referred to as 'critical saturation concentration (Csat)'. For most detergents, the onset of co-operative interactions is an essential condition for efficient solubilisation.

3. *Third phase:* Solubilisation. Once the concentration of the detergent has reached beyond a 'critical solubilisation concentration (CSC)'. In this phase, small membrane sheets or mixed lipid-detergent micelles are formed (Fig. 7d).

4. *Final phase.* In this phase, only mixed lipid-detergent micelles and detergent solubilised protein units are present. In this stage, membrane segments are covered by detergent (Fig. 7e).[74, 75]

2.3.2 Factors Influencing the Interaction of Detergents with Cell Membranes

Detergents may form mixed micelles with lipids, bind to hydrophobic portions of proteins or cause protein denaturation. The interaction of surfactants with cell membranes and the eventual outcome is influenced by physical and chemical characteristics such as charge, CMC and aggregation number.[76]

In general, detergents with a neutral large head group and longer hydrocarbon chains (such as POL) have milder properties. Detergents with a charged, small head group and short alkyl chain (such as STS) have harsher properties and more frequently denature

proteins or disrupt membrane protein complexes (Table 2).[77] The effect on membrane proteins is also influenced by the ionic nature of the polar head of the detergent molecule. Non-ionic detergents (such as POL) solubilise membrane proteins without affecting important structural features.[74] Zwitterionic detergents are more inactivating than non-ionic detergents but stabilise membrane proteins better than ionic detergents. Ionic detergents (such as STS) are in general denaturing.

Non-ionic detergents are considered 'mild detergents'. Such agents in general do not interact to any noticeable degree with most water-soluble proteins, except those which like serum albumin can accommodate small amounts of detergents inside their hydrophobic pockets. In general, polyoxyethylene detergents with a short (C7-10) hydrocarbon chain are more inactivating than those with an intermediary (C12-14) hydrocarbon chain length such as POL.[78] Non-ionic detergents with long hydrocarbon chains are usually inefficient solubilisers of biological membranes.[74]

The extent to which efficient solubilisation occurs is likely to be influenced by the degree with which detergents are able to penetrate and cross the membrane. Due to the hydrophilic-hydrophobic properties of the polyoxyethylene chains, detergents like POL flip-flop rapidly across the membrane.[74] On the other hand, detergents with strongly hydrophilic heads such as STS can be expected to flip-flop at a slow rate, resulting in delayed solubilisation. A similar anionic agent, sodium dodecyl sulphate (SDS), only slowly solubilises pure liposomal membranes.[79] The solubilisation which eventually takes place with SDS may be caused by extraction of phospholipid molecules directly from the membrane into preformed detergent micelles (Fig. 8a and 8b).[74]

Table 2. Structural Properties of Detergents.

	Harsh	Mild
Head Group	Charged (ionic) Small	Neutral Large
Hydrocarbon Tail Group	Short	Long
Effect on Proteins	Denaturing	Solubilising
Flip-flop Rate	Slow	Rapid
Membrane Solubilisation	Delayed	Rapid
Examples	Ionic detergents such as sodium tetradecyl sulphate (STS)	Non-ionic detergents such as polidocanol (POL)

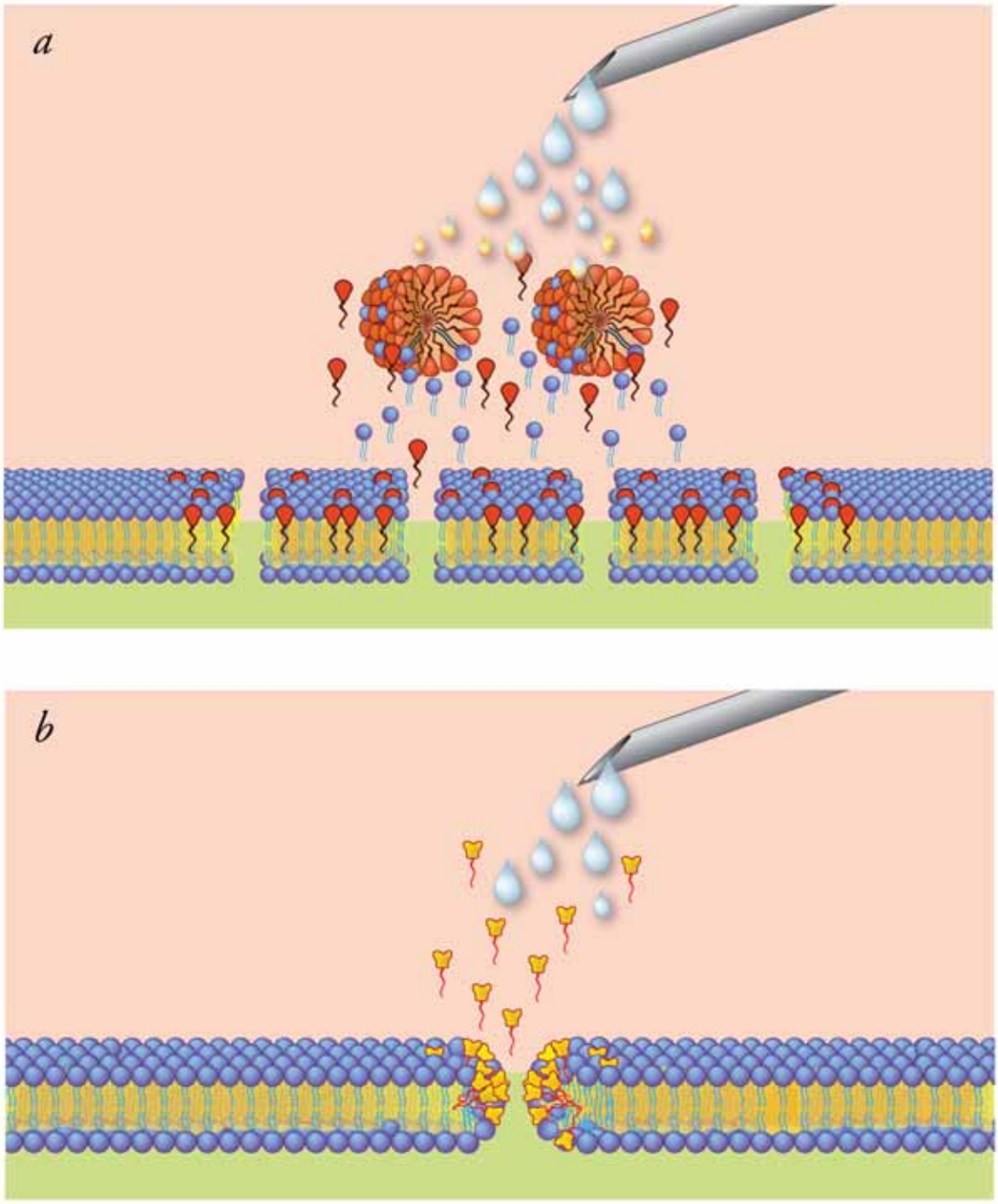

Figure 8. Anionic surfactants such as sodium dodecyl or tetradecyl sulphate may solubilise membranes by extraction of phospholipid molecules directly from the membrane into preformed detergent micelles (a). Non-ionic detergents such as polidocanol are more likely to form toroidal complexes (b).

3. *Detergent Sclerosants*

Detergent sclerosants function by damaging the endothelial lining of target vessels to expose the underlying collagen with the ultimate aim of inducing endovascular fibrosis. The most commonly used detergent sclerosants are STS and POL (Table 3) but other detergent agents less commonly used include sodium morrhuate and ethanolamine oleate. Clinically, STS is considered to be at least three times more potent than POL.[58]

Table 3. Sodium tetradecyl sulphate (STS) and polidocanol (POL) synonymous chemical names.

STS	POL
Sodium 1-isobutyl-4-ethyloctyl sulphate	Hydroxy polyethoxy dodecane
Monotetradecylsulphate sodium salt	Polyoxyethylene (9) lauryl ether
Sodium myristyl sulphate	PEG-9 lauryl ether (Laureth 9)
Tetradecyl hydrogen sulphate	Nonaoxyethylene monododecyl ether
Tetradecyl sodium sulphate	Nonaethylene glycol monododecyl ether
Tetradecyl sulphate	Dodecylnonaoxyethylene glycol monoether
Tetradecyl sulphuric Acid	Dodecylpolyethyleneglycolether
4-undeconal,7-ethyl-2-methyl-sulphate, sodium salt	Oxypolyethoxydodecane

3.1 *Sodium Tetradecyl Sulphate (STS)*

STS ($C_{14}H_{29}NaSO_4$) is a negatively-charged sulphated surfactant with a molecular weight of 316.44 (Fig. 9a). STS as a sclerosing agent is manufactured by STD Pharmaceutical Products Ltd., Hereford, UK and available in Australia as FIBRO-VEIN® (Australian Medical and Scientific Ltd., Chatswood, NSW, Australia). The CMC of STS in FIBRO-VEIN® is not published but the CMC of the parent product, Niaproof 4 is 2.1mM in water (direct communication with STD Pharmaceutical). At 3%, each mL of FIBRO-VEIN® contains 30mg of STS and 2% benzyl alcohol as a bactericidal excipient. The solution is buffered using phosphates (dibasic sodium phosphate, monobasic potassium phosphate) and

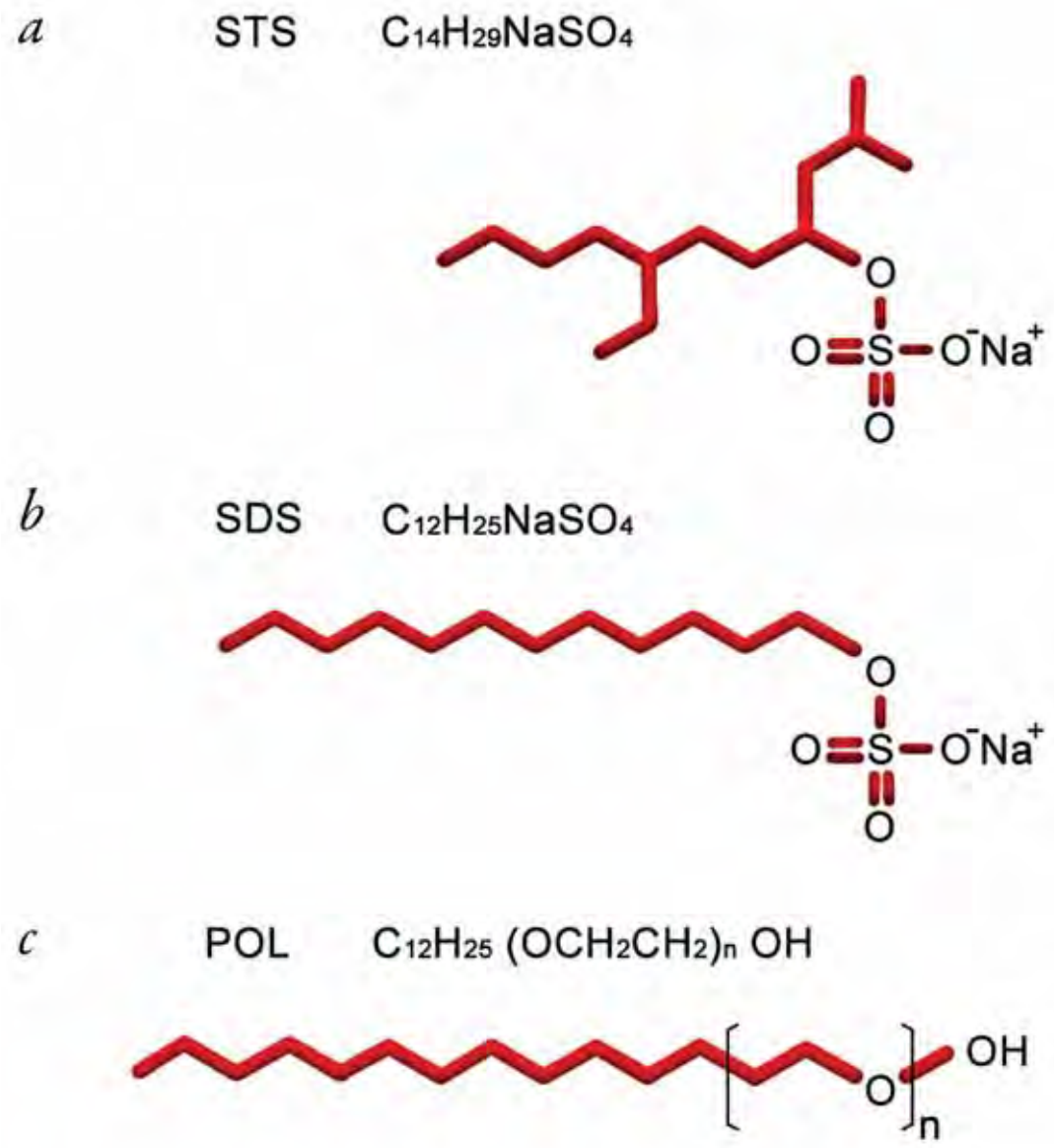

Figure 9. The chemical structure of detergents.

(a) Sodium tetradecyl sulphate (STS), (b) sodium dodecyl sulphate (SDS) and (c) polidocanol (POL). The mean extent of POL polymerization n≅9.

sodium hydroxide in water to a pH of 7.6. In the United States (USA), this product is available as SOTRADECOL® (manufactured by Bioniche Teo. Inverin, Co. Galway, Ireland and distributed by AngioDynamics, Queensbury, NY, USA). SOTRADECOL® contains similar ingredients to FIBRO-VEIN but the pH is set at 7.9 rather than 7.6.

Clinically, STS at 0.05%-0.2% is used to treat telangiectasias and venulectasias, at 0.2-0.5% to treat reticular veins and at 1%-3% to treat varicose veins. It is generally used as foam to treat larger veins and as liquid or foam to treat reticular veins and telangiectasias.

STS is distilled from Niaproof anionic surfactant 4 (also known as NAS 4 and Niaproof 4, Niacet, Niagara Falls, New York, USA), a high-grade detergent. This detergent is manufactured to contain 26%-28% STS and up to 20% diethylene glycol ethyl ether (carbitol). This compound has many applications in a range of industries including textile processing and dyeing, household and industrial cleaners, pickling (reduces the amount of the required acid) and molding (provides a more uniform sand for use in casting molds and cores). In pharmaceuticals, it may be used to enhance bactericidal properties of antiseptics and rapid fixing of histological specimens. In latex preparations, it is used as an emulsifying agent and is the preferred emulsifier for the polymerisation of acrylic ester monomers and vinyl esters of higher fatty acids.

NAS 4 contains 27% STS and 20% carbitol and is hence purified further to generate the STS for injection. FIBRO-VEIN® contains 0.02% to 0.045% (wt/vol) of carbitol. Carbitol has a toxicity profile similar to ethylene glycol, is mutagenic in bacteria, teratogenic in mice and can induce an acute or delayed cutaneous hypersensitivity reaction in humans.[80] Before the release of SOTRADECOL®, a number of compounded products containing STS were used in the USA with discrepancies in the stated and actual concentrations of STS and a carbitol content of up to 4.1%.[80]

STS has a similar structure to SDS, the most frequently used detergent in protein biochemistry (Fig. 9b). SDS is a strong and harsh detergent used commonly to induce cell lysis or in biochemical techniques such as SDS polyacrylamide gel electrophoresis (SDS-PAGE). Being an anionic detergent, SDS is an efficient solubiliser but almost always denatures most proteins.[74]

In certain cases, reactivation of SDS-solubilised proteins is possible.[81,82] A class of membrane proteins that is frequently resistant to SDS denaturation is beta-barrel proteins from the outer membranes of Gram-negative bacteria. SDS precipitates at low temperatures and in the presence of cations.

3.2 Polidocanol

POL ($C_{12}H_{25}O(CH_2CH_2O)_nH$, Lauromagrogol 400, hydroxy polyethoxy dodecane) is a polyethylene glycol ether of lauryl alcohol (Fig. 9c). As a sclerosing agent, POL is available in Australia as AETHOXYSKLEROL 3% (Chemische Fabrik Kreussler & Co GmbH, Wiesbaden, Germany). The CMC of Kreussler POL is approximately 0.084 g/L or 0.148mM (direct communication with the manufacturer). At 3%, each ml of AETHOXYSKLEROL® contains 30mg of polidocanol in water for injection with 5% (v/v) ethanol. The solution is buffered using phosphates (disodium hydrogen phosphate dehydrate and potassium dihydrogen phosphate) to a pH of 6.5-8.0. In the USA, this sclerosant is available from the same manufacturer at 0.5% and 1% and distributed as ASCLERA®. Clinically, POL at 0.1-0.5% is used to treat telangiectasias and reticular veins and at 1-3% to treat varicose veins.

POL is a non-ionic emulsifying agent consisting of two components, a polar hydrophilic (dodecyl) head and an apolar hydrophobic (polyethylene oxide) chain (Fig. 9c). POL belongs to the group of alkylpolyglycolethers, commonly called alcohol ethoxylates (AE). AEs are manufactured commercially by the reaction of an alcohol and ethylene oxide. This reaction generates mixtures of ethoxylates of different ethylene oxide units. For the synthesis of polidocanol, natural fatty alcohols or synthetic alcohols are converted with ethylene oxide.

These detergents are commercially available in many different chain lengths, either as pure substances or mixtures of a certain size distribution. They have the general formula CxEy according to their alkyl chain length (x) and the number of polyoxyethyleneglycol units in the headgroup (y). POL, manufactured by Kreussler, has an average alkyl chain of 12 carbon atoms and an ethylene oxide (ethoxy, $-OCH_2CH_2$) chain of ≅ 9 units. Thus, POL as manufactured by Kreussler, can be presented as C_{12} E_9. The molecular weight of POL depends on the number of ethoxy units in the molecule. For POL manufactured by Kreussler (E_9), the molecular weight is approximately 600.

POL was first introduced in Germany in 1936 as a topical and local anaesthetic agent. The possible anaesthetic properties of POL (and other non-ionic surfactants) were investigated in human volunteers, in parallel with proven local anaesthetic agents.[83, 84] In contrast to therapeutically used anaesthetics (lidocaine and prilocaine), the tested surfactants, including POL, were found to have no significant effect on heat and cold sensation or significant local anaesthetic effects. POL in common cosmetic products is referred to as Laureth-9. In this setting, it is used in rinse-off products as an emulsifier and surfactant, especially in shampoos and hair conditioners in concentrations up to 4%. It is also used in leave-on products such as body and face creams in concentrations up to 3%.

4. A Brief Overview of the Haemostatic System

4.1 Coagulation System

4.1.1 The Classical Model of Coagulation

The classical model of coagulation was described simultaneously by two research teams in 1964. McFarlane[85] described the 'cascade' model published in *Nature* while Davie and Ratnoff[86] described the 'waterfall' model published in *Science*. This model consisted of two independent pathways, where the activation of each clotting factor would lead to the activation of another, ultimately resulting in the formation of fibrin (Fig.10). The majority of the steps in the cascade required phospholipids and calcium.

The extrinsic pathway involved the tissue factor (TF) thought to occur only extrinsic to the circulating blood. By contrast, members of the intrinsic pathway were considered to be intravascular. The activation of either pathway would lead to the common pathway which involved the activation of Factor (F) X (FX), the conversion of prothrombin to thrombin and the subsequent formation of fibrin. Prothrombin time (PT) test was designed to assess the extrinsic and common pathway whereas the activated partial thromboplastin times (APTT) would assess the intrinsic and common pathways. Thrombin time (TT) would assess the conversion of fibrinogen to fibrin.

In the classical model, the extrinsic and intrinsic pathways operated as two independent and redundant pathways. However, the clinical manifestations of individual factor deficiencies contradicted this concept. For example, although FXII deficiency causes marked prolongation of the APTT, this deficiency is not associated with a bleeding tendency in humans. In contrast, FVIII or FIX deficiency result in serious bleeding tendencies seen with haemophilia A and B, despite the fact that these patients have an intact extrinsic pathway. Similarly, FVII deficiency can be associated with bleeding, despite the presence of an intact intrinsic pathway. Finally, platelets and other cellular elements played a small role in the classical model which limited its widespread adoption and *in vivo* application.

4.1.2 The Cell-based Model of Coagulation

In 1992, Hoffman described a cell-based model of haemostasis where the coagulation reactions were localised to cell surfaces.[87] This model was based on experimental models using monocytes cultured with agents to induce TF. In this model, the cellular localisation acted as a control mechanism to prevent the spread of the clotting process throughout the vascular system.[88] The formation of trace amounts of thrombin stimulates platelet activation, allowing for a burst of thrombin generation on the platelet surface which leads to the formation of fibrin. This process occurs in a series of three overlapping steps (Fig. 11).

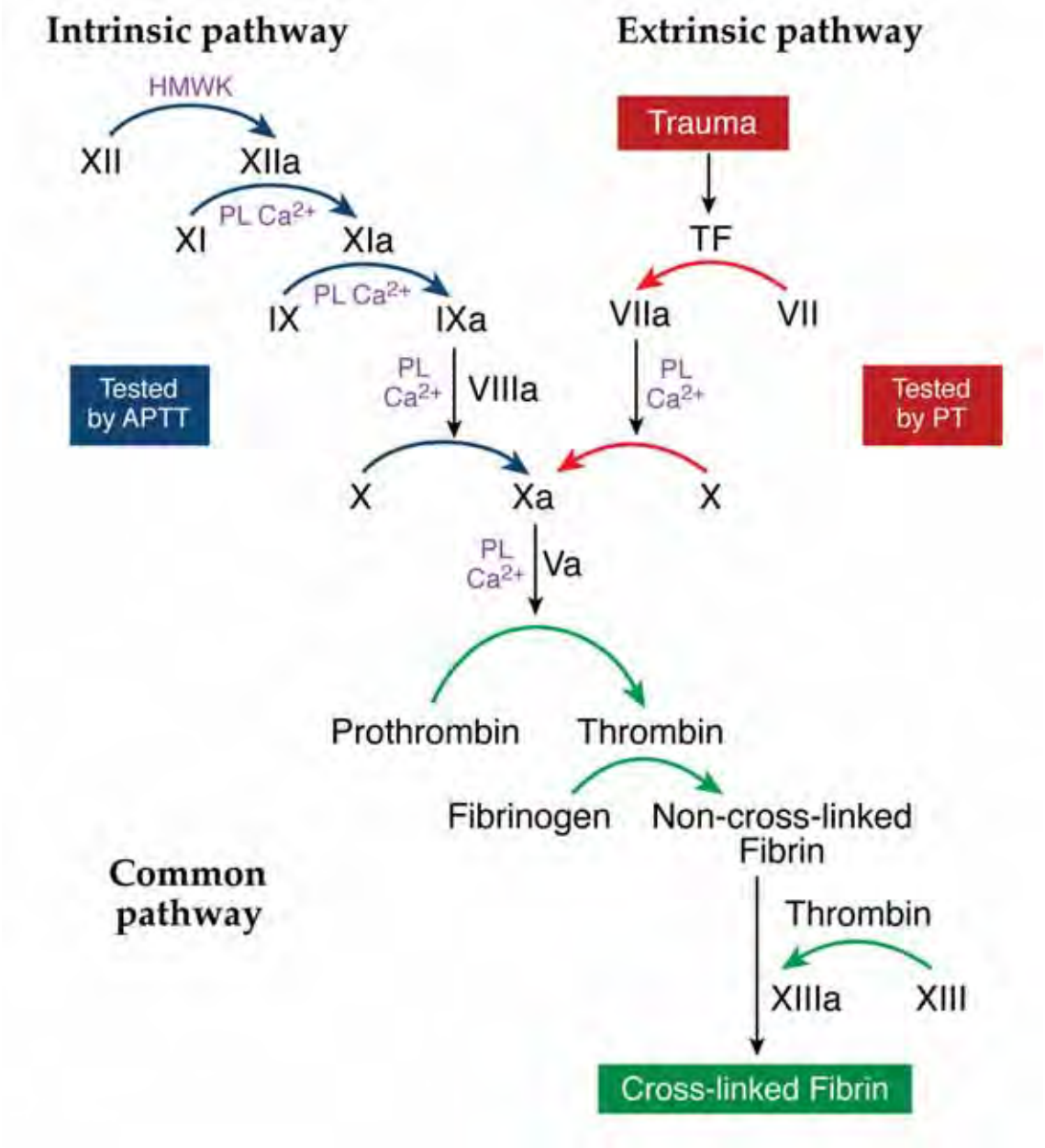

Figure 10. The classical model of coagulation.

This model consisted of a 'cascade' of reactions along two independent pathways where the activation of one factor would lead to activation of another. The two pathways eventually converged to generate thrombin and the subsequent formation of fibrin. Prothrombin time (PT) tests the extrinsic and common pathways, activated partial thromboplastin time (APTT) tests the intrinsic and common pathways and thrombin time (TT) tests the conversion of fibrinogen to fibrin. Ca^{2+}, calcium ion, HMWK, high molecular weight kininogen; PL, phospholipid; TF, tissue factor. Roman numerals refer to the individual clotting factor proenzymes and the activated (a) enzymes.

The *initiation phase* involves vascular injury and exposure of TF. This may occur on a variety of cells such as activated endothelial cells, fibroblasts and even apoptotic cells.[89] TF can also be delivered to sites of vascular injury by circulating microparticles and in certain hypercoagulable conditions via cells such as neutrophils and monocytes.[90] These cells and cell fragments can then contribute directly to the process of thrombus formation.[91] The initiation phase is characterised by the binding of TF to the circulating FVIIa which constitutes approximately 1% of the total circulating FVII.[92] All other coagulation proteins circulate solely as zymogens. The TF/FVIIa ('tenase') complex then activates additional FVII to FVIIa, allowing for more TF/FVIIa complex activity. The TF/FVIIa complex is responsible for the cleavage and subsequent activation of FIX and FX. FXa activates FV to form FVa and then combines with FVa to form the FXa/FVa ('prothrombinase') complex. This complex stimulates the production of trace amounts of thrombin from prothrombin.

In the *amplification phase*, surface-bound thrombin stimulates platelet activation resulting in a shape change, surface exposure of phosphatidylserine and release of granule contents. Release of agonists from platelet granules promotes further platelet activation. Thrombin activates FV and FVIII. Release of von Willebrand factor (vWF) from FVIII would promote further platelet adhesion and aggregation. Thrombin also activates FXI required for activation of FIX. This phase occurs mainly on platelets, although it may also occur on microparticles, activated endothelium and other cells.

In the *propagation phase*, the release of soluble factors from platelet granules results in recruitment of additional platelets to the site of injury. Expression of the fibrinogen receptor, GPIIb/IIIa, results in aggregation of platelets. In this phase, FIXa generated by TF/FVIIa complex in the initiation phase binds to FVIIIa. Additional FIX is activated by FXIa on the platelet surfaces. The FVIIIa/FIXa complex on activated platelets forms a tenase complex that would activate FX. This complex amplifies the generation of FXa which with its co-factor FVa forms the prothrombinase (FXa/FVa) complex. Both these complexes are localised to the platelet membranes and lead to a burst of thrombin generation and the subsequent conversion of fibrinogen to fibrin.[93, 94]

Thrombin initiates the formation of fibrin by converting the soluble fibrinogen to non-cross-linked fibrin. Fibrin monomers are first assembled in a non-covalent fashion and then covalently cross-linked by FXIIIa. Cross-linking enhances the mechanical strength of the fibrin polymer and leads to increased clot stability, stiffness and resistance to fibrinolysis and deformation.[95]

Thrombin plays multiple roles in the coagulation, antithrombotic and fibrinolytic systems. Thrombin's primary role is the conversion of fibrinogen to fibrin but it also plays an important role in the activation of platelets through protease activated receptors (PARs), activation of FXIII, activation of TAFI and in complex with thrombomodulin, activation of protein C.

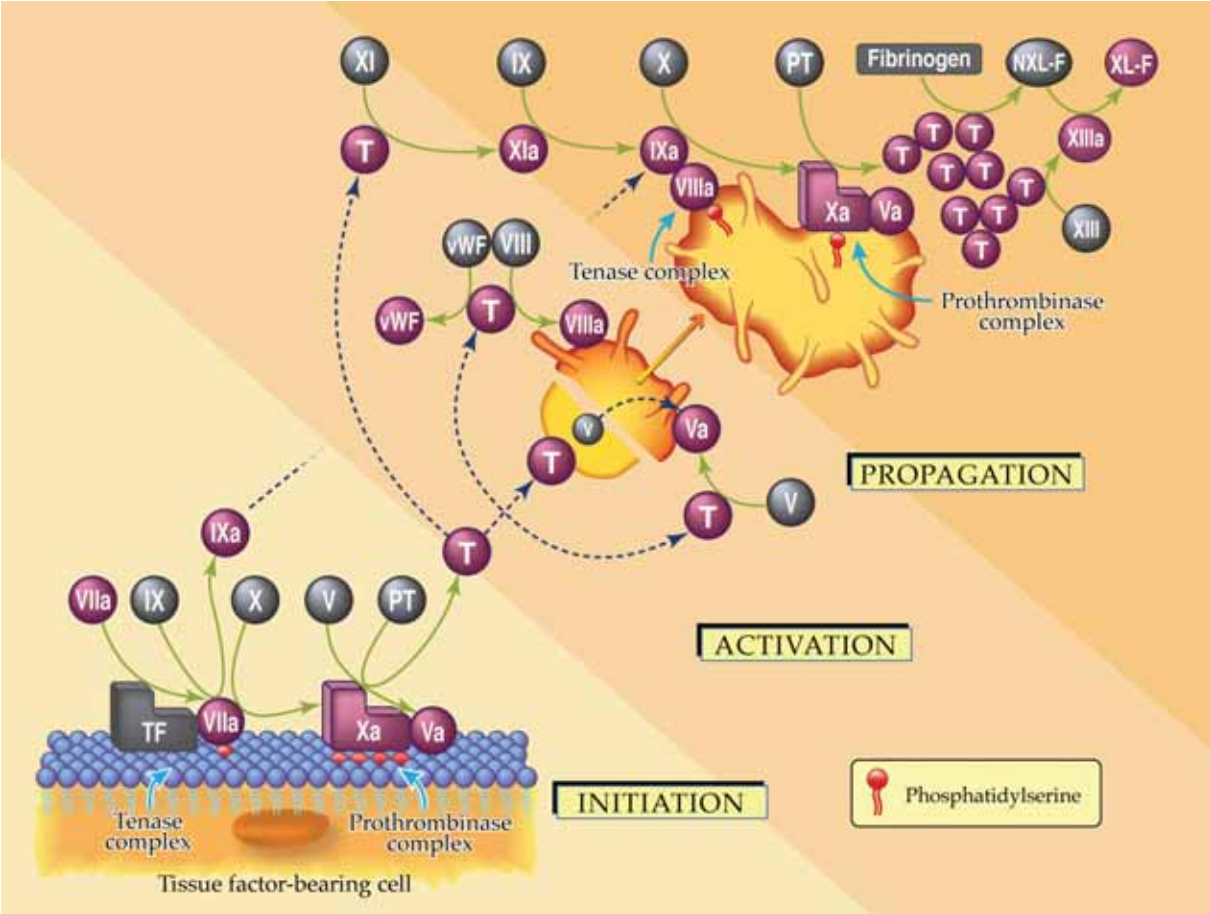

Figure 11. The cell-based model of coagulation.

This model involves three overlapping phases that incorporate the contribution of cells and negatively-charged membrane surfaces to clot formation. The *initiation phase* occurs when a tissue factor (TF)-bearing cell is exposed to the running blood. TF binds to the circulating factor VIIa. The TF/VIIa complex generates further factor VIIa and small amounts of factor IXa and thrombin (T). During the *amplification phase*, the trace amounts of thrombin generated during the initiation phase activates platelets, releases von Willebrand factor (vWF) and catalyses the activation of factors V, VIII, and XI. In the *propagation phase*, activated enzymes assemble on the negatively-charged surfaces of the activated platelets to form the tenase and prothrombinase complexes resulting in a burst of thrombin generation from prothrombin (PT). Thrombin would catalyse the activation of factor XIII and the conversion of fibrinogen to non-cross-linked fibrin (NXL-F). Activated factor XIII (XIIIa) mediates the conversion of non-cross-linked fibrin to cross-linked fibrin (XL-F). Roman numerals refer to the individual clotting factor proenzymes and the activated (a) enzymes.

4.2 Platelets and platelet microparticles

Normal blood flow is dependent on the integrity of the vessel wall. Interruption of endothelial lining leads to adhesion of platelets to the injured wall and the subsequent platelet activation and aggregation which results in the formation of a haemostatic plug (Fig. 12). Following damage to the vessel wall, vWF binds rapidly to the exposed sub-endothelium which enables the arrest of platelets from fast-flowing blood through the interaction of vWF A1 domain with the platelet GPIbα receptor. This bond has a short half-life. Receptors such as GPVI and integrin αIIbβ3 (GPIIb/IIIa) engage their respective ligands and mediate permanent adhesion, spreading and aggregation.

Platelets supply factors that support the activation of prothrombin and play a major role in localising and controlling the burst of thrombin generation. Following activation, phosphatidylserine expression is significantly increased on the exoplasmic surface of platelet membranes. Phosphatidylserine acts as a catalytic site for the formation of the tenase and prothrombinase complexes.

The platelet surface plays a central role in the promotion and regulation of thrombin generation. A direct comparison of platelets and phospholipids has shown that platelet surfaces provide specific coagulant activities not mimicked by phospholipids.[96, 97] This regulation extends beyond the expression of phosphatidylserine on the outer leaflet of the platelets and requires binding proteins and receptors that contribute to promoting and controlling the extent of thrombin generation.[98]

The activation of platelets results in formation of microparticles which are small membrane-bound vesicles typically less than 1μm in diameter. Microparticles are generated when activated or apoptotic cells shed small segments of their membranes. Thrombin, shear stress, hypoxia and certain cytokines (such as tumour necrosis factor-α) can stimulate microparticle formation. Microparticles contain cell surface proteins similar to those found on their parent cells. PMP express phosphatidylserine[99], adhesion receptors[100] and possibly TF[101, 102] on their external membranes and hence can provide the negatively-charged surface required for the coagulation reactions. Clinically, increased plasma levels of PMP have been linked with a large number of conditions and in particular vascular events such as arterial thrombosis,[103] stroke[70, 104, 105] and myocardial infarction.[106]

4.3 Antithrombotic Mechanisms

While platelets play a major role in supporting procoagulant reactions, endothelial cells play a key role in maintaining the anticoagulant mechanisms.[94] These cells express a number of anticoagulant proteins on their cell surface including heparin-sulfated proteoglycans (HSPG), thrombomodulin (TM) and tissue factor pathway inhibitor (TFPI). Plasma coagulation inhibitors such as PC, PS, AT and endothelial surface membrane proteins

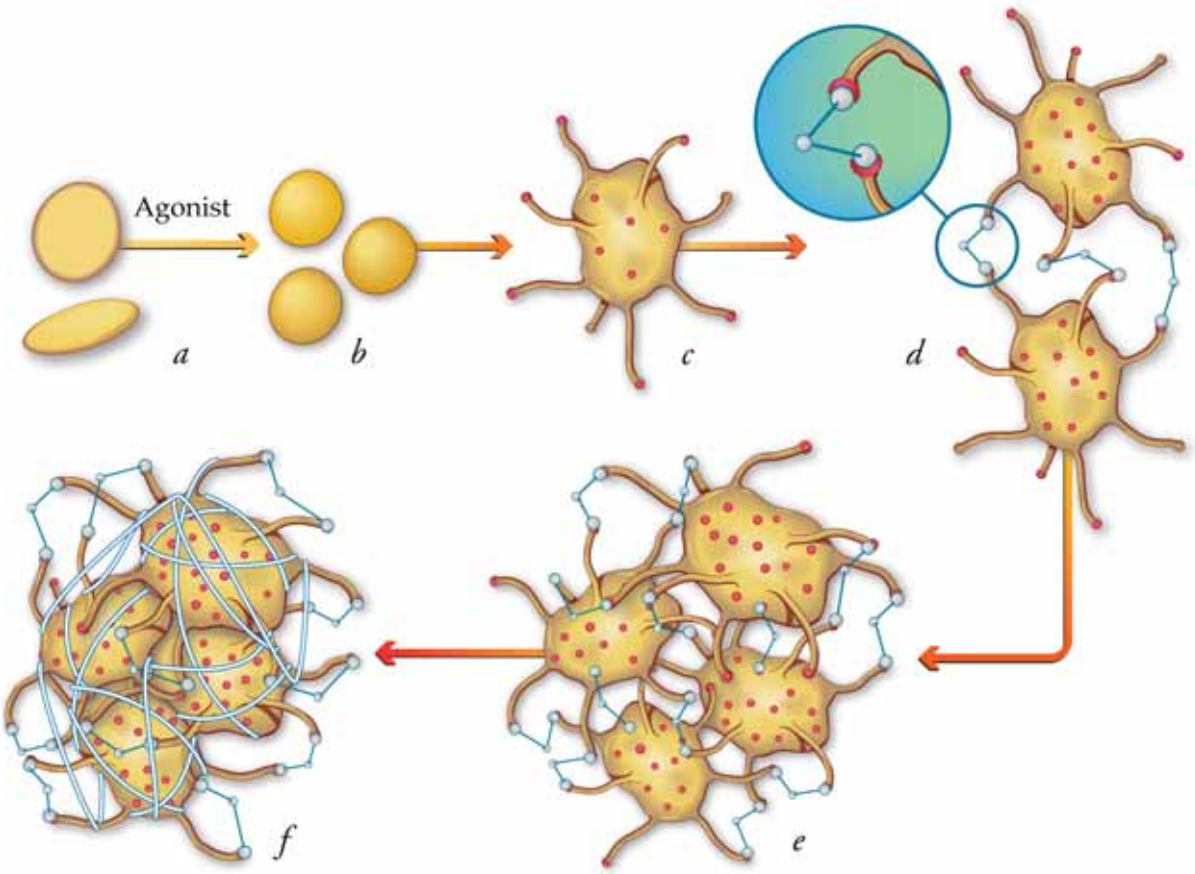

Figure 12. Steps leading to platelet activation and aggregation.

Following exposure to agonists, disc shaped platelets (a) undergo a shape change to a spherical shape (b) and then develop pseudopodia (c). Fibrinogen mediates platelet aggregation via glycoprotein (GP) IIb/IIIa receptor (d) which leads to platelet aggregation (e). The deposition of the fibrin is the final step in the formation of a fibrin clot (f).

play a critical role in limiting the process of thrombus formation to the site of vascular injury.[107] These antithrombotic proteins are dependent on the integrity of the endothelium surrounding the focus of injury.[108, 109]

PC is a vitamin K-dependent protein activated by thrombin-thrombomodulin complex. PS acts as a cofactor for PC. The APC/PS complex inhibits factors Va and VIIIa. TM expression is 100-fold higher in the capillary endothelium as compared with the endothelial lining of larger vessels. Therefore, excess amounts of thrombin in major vessels are rapidly taken up by the capillary TM when the blood flow reaches the capillary network. HSPG are expressed by resting endothelial cells and form a binding site for AT. Once bound, AT becomes fully capable of inhibiting thrombin and FXa in the vicinity of HSPG.

TFPI is expressed on the endothelial cell surfaces to prevent additional thrombin generation. It irreversibly binds to FXa and then forms a quaternary complex with FVIIa, and TF. Therefore, TFPI acts as an upstream inhibitor and prevents further participation of these clotting factors in the coagulation reactions.

4.4 Fibrinolytic Mechanisms

The fibrinolytic system is responsible for degradation of fibrin and recanalisation of occluded vessels.[110, 111] The key fibrinolytic enzyme, plasmin, degrades fibrin and activates matrix metalloproteinases responsible for degradation of collagen and the extra-cellular matrix. The main inhibitor of plasmin, alpha-2-antiplasmin (AP), competitively inhibits the binding of plasminogen to fibrin. In blood, AP protects the plasma proteins from the proteolytic activity of plasmin by forming a stable plasmin-antiplasmin (PAP) complex. In clots, AP is cross-linked to fibrin by FXIIIa which provides an additional measure of protection to the fibrin clot from proteolysis by plasmin.

Plasmin is generated from plasminogen by the action of tissue-type plasminogen activator (t-PA) in blood and urokinase in tissue. Plasma t-PA is synthesized and released by endothelial cells when stimulated by clotting factors thrombin and FXa.[110, 112] Inhibitors of t-PA include PAI-1, AP and C-1 inhibitor. PAI-1 is expressed by activated platelets[112, 113], PMP[114, 115] and other sources. TAFI is another important inhibitor of fibrinolysis.[116, 117] Activated TAFI (TAFIa) acts on the partially-degraded fibrin resulting in a decrease in the binding of plasminogen to fibrin and its conversion to plasmin.

5. *Detergent Sclerosants, Thromboembolism and the Haemostatic System*

5.1 Clinical Thromboembolism

Although sclerosants may inadvertently enter the deep venous system via perforators or junctions (Fig. 13), the clinical incidence of significant thromboembolic complications of sclerotherapy remains relatively low.[34, 118, 119]

The true incidence of DVT following sclerotherapy is unknown and most publications include clinically (and not ultrasonically) detected DVTs. In the French registry of 12,173 procedures, only one proximal vein and 5 cases of calf vein thrombosis were reported.[2] In the Australian Polidocanol Study, three cases of DVT (0.02%) were detected following 16,804 sclerotherapy procedures.[120] In a clinical audit data of foam UGS in 7027 patients (11,537 procedures) from nine UK centres, 36 patients (0.5%) were diagnosed with DVT.[121]

The incidence of venous thromboembolic events following sclerotherapy is possibly higher as a significant number of procedural DVTs may be silent.[9] In a study by Gillet *et al*, 1025 patients underwent foam UGS.[8] Duplex ultrasound screening was performed between the eighth and thirtieth day in the majority of patients. 11 thromboembolic events (1.07%) were reported which included 10 DVTs and one pulmonary embolism (PE). 5 DVTs were asymptomatic. Similarly, Bergan has reported an incidence of 1.8%.[62] In a study by Myers *et al*, 489 patients underwent 1189 foam UGS procedures. Duplex ultrasound screening revealed 16 cases of DVT (3.2%) all of which were asymptomatic.[69]

The reported frequency of 1-3% is similar to the author's experience.[9] Treatment factors that might influence the risk of deep vein occlusion (thrombosis or sclerosis) following foam sclerotherapy have been reviewed.[5] STP has been reported to have a an incidence of 1-5%.[40, 122, 123] In the French registry, only 3 cases of STP were reported following 12,173 procedures.[2] However, post-sclerotherapy STP is possibly under-diagnosed and it may be difficult to differentiate between STP and an adequate treatment response.

In earlier publications, PE followed sclerotherapy of small superficial vessels with a reported incidence of 1 in 250 to 1 in 1000.[124-126] In the study by Gillet *et al*, one case of PE was reported in 1025 patients.[8] In the French registry of 12,173 procedures and the Australian Polidocanol Study (16,804 procedures), no cases of PE were reported.[2, 120] There is no data regarding the incidence of silent PE following sclerotherapy. Concurrent PE is found in 5% of the diagnosed cases of paradoxical embolism.[127] Patients with PE and a PFO >4mm have a 10-fold increased risk of death and 5-fold increased risk of systemic embolism.[128] Concurrent PE has been reported in only one case of stroke after sclerotherapy.[3]

The incidence of neurological complications of sclerotherapy is reported to be up to 2%.[2, 8] Serious neurological ischemic complications are less frequent but include rare cases of stroke and transient ischemic attacks.[10, 11, 70, 71, 129-132] The earlier reports implicated liquid

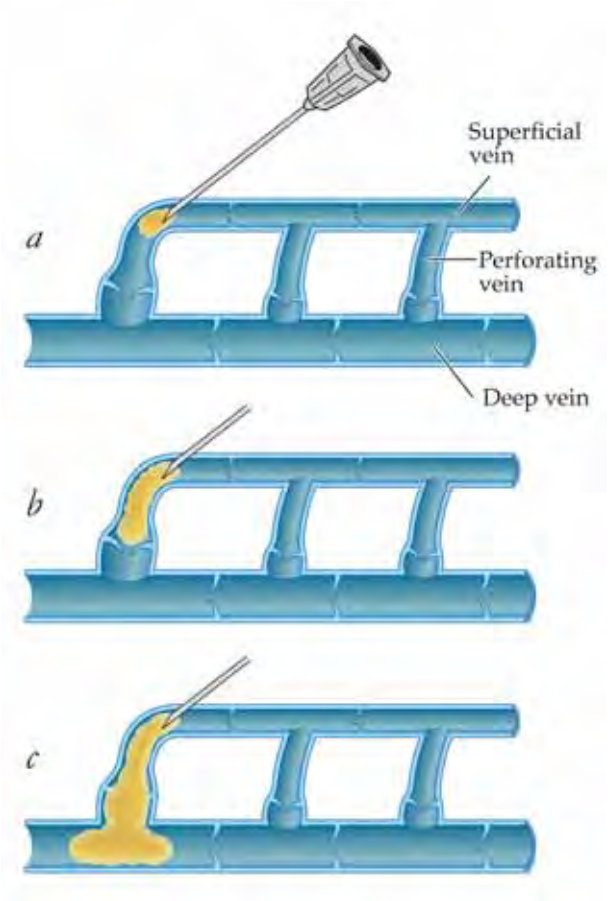

Figure 13. Schematic diagram demonstrating the possible inadvertent entry of sclerosant into the deep venous system via a junction.

(a) Entry of sclerosant into the superficial vessel, (b) the sclerosant flows towards the junction, (c) inadvertent entry of sclerosant into the deep vein.

sclerosants[10, 11, 129, 131, 132] but more recently, these events have followed foam sclerotherapy[70, 71, 130]. The aetiology of distant ischemic complications of sclerotherapy have been widely debated and various mechanisms such as a direct effect of sclerosants, a direct effect of the foam-derived bubbles and release of biological by-products of sclerosants have been proposed.[70, 133, 134]

5.2 Detergent Sclerosants and the Haemostatic Systems

Research focussing on the interaction of detergent sclerosants with the haemostatic system has been limited and the published reports have been often contradictory. Sclerosants were initially reported to have no effect on the coagulation system.[135, 136] Fegan reported that apart from a minor increase in the platelet count in some patients, STS had no effect on clotting times.[137] Minor changes were reported in later publications.[138-143] Others reported an effect on platelets as evident by the release of ADP.[144, 145] Some clinical studies have reported no change in coagulation parameters,[63] while others reported significant changes[146]. A number of *in vitro* studies demonstrated the suppressive effects of both sclerosants on the coagulation system and platelet function.[138, 147] A very limited number of *in vivo* studies have been performed which reported both potentiation as well as inhibition of coagulation and fibrinolysis.[148-150] Differences were also reported to be influenced by the choice of sclerosant.[138, 147-150]

In an elegant *in vitro* study by Jacobson *et al*, the effects of STS on coagulation and platelet function were found to be concentration-dependent.[144] Diluted STS induced a hypercoagulable state and initiated platelet aggregation. At higher concentrations, this agent inactivated the coagulation cascade, prolonged prothrombin time and partial thromboplastin time and lysed platelets completely. This group also reported a reduction in protein C levels induced by low concentration STS. Jacobson *et al* had previously reported significant derangement of clotting tests in an earlier study.[151]

In a study by Ariyoshi *et al*, 10 patients undergoing varicose vein surgery were compared with 10 patients undergoing surgery as well as sclerotherapy.[146] The sclerotherapy patients received 7.6+/-1.6 mL of POL 1%. Patients were evaluated at baseline and at post-operative days 1, 3, 7 and 30. No significant difference was found in the transient elevation of acute phase proteins, C-reactive protein or fibrinogen. In the surgery only group, thrombin anti-thrombin complexes peaked 3 days after treatment, whereas in the sclerotherapy group the elevation peaked at 7 days. Elevation of the markers of fibrinolysis, PAP complexes and fibrin degradation products (FDP), peaked at 7 days after treatment in both groups. Compared to the surgery only group, the sclerotherapy patients showed a significant decrease in the platelet count at 3 days and a significant rise in PAP at 7 days after the procedure. The authors concluded that sclerotherapy may prolong the activation of the coagulation system and enhance fibrinolysis after surgical procedures.

An *in vitro* study by Wupperman and Hass demonstrated the direct lytic toxicity of polidocanol on platelets. [147] This study also revealed the direct suppressive effects of polidocanol on the coagulation pathway through its inhibitory effects on FV and FVIII. Most other studies have reported an inhibitory effect of sclerosants including POL on platelet function and coagulation. [147, 149, 150] The inhibitory effect on coagulation observed in these *in vitro* studies was in contrast with the observed prolonged activation of the coagulation reported by Ariyoshi *et al*.[146] This group postulated that the discrepancy is most likely due to prolonged injury incurred upon the vascular endothelium rather than a direct effect of the sclerosants.

In an *in vivo* study lead by Patricia Burrows, 29 patients underwent sclerotherapy or embolisation of vascular malformations using a range of sclerosing or embolising agents.[141] Measurements were made at baseline, immediately after, and 24 hours after the procedure. A positive relationship was found between the use of dehydrated alcohol or STS and a disruption in coagulation profiles as evident by a decrease in the platelet count and fibrinogen levels, an increase in prothrombin time and an elevation of the D-dimer levels.

C. AIMS AND HYPOTHESES

Considering the conflicting literature on the haemostatic effects of sclerosants, the overall aim of this thesis was to examine the *in vitro* effects of detergent sclerosants, STS and POL, on coagulation, antithrombotic and fibrinolytic mechanisms. The general hypothesis of this thesis was that detergent sclerosants are not thrombogenic which may explain the relatively low incidence of thromboembolic complications of sclerotherapy.

The subsidiary aims and hypotheses of this thesis included:

1. To investigate the *in vitro* effects of detergent sclerosants on clotting factors, and their potential for inducing platelet lysis. It was hypothesized that sclerosants may destroy clotting factors and lyse platelets at high enough concentrations. This hypothesis was tested in experiments detailed in Chapter 2. These experiments were designed to explore whether sclerosants demonstrated any anticoagulant activity at all.

2. To investigate the potential inhibitory effects of serum albumin on the lytic activity of sclerosants *in vitro*. The relatively low incidence of deep vein thrombosis after sclerotherapy has been partially attributed to a loss of potency of sclerosants following dilution in blood. It was hypothesized that in addition to simple dilution, sclerosants would be further neutralised by albumin and other plasma proteins. This hypothesis was tested in experiments detailed in Chapter 3.

3. To investigate the *in vitro* effects of detergent sclerosants on antithrombotic proteins. It was hypothesized that sclerosants may enhance the anticoagulant effects of APC and potentiate other antithrombotic mechanisms. This hypothesis was tested in experiments detailed in Chapter 4.

4. To investigate the *in vitro* effects of detergent sclerosants on fibrinolytic mechanisms. It was hypothesized that sclerosants may enhance the activity of fibrinolytic enzymes which would potentiate the recanalisation of thrombosed vessels. This hypothesis was tested in experiments detailed in Chapter 5.

5. To investigate the *in vitro* effects of detergent sclerosants on clot formation and lysis. The experiments detailed in Chapters 2, 4 and 5 examined the individual components of the coagulation, antithrombotic and fibrinolytic pathways but did not provide a global understanding of how the sclerosants affect clot formation. Based on the results of earlier experiments, it was proposed that sclerosants would prevent clot formation at higher concentrations. It was also hypothesized that sclerosants may exhibit direct fibrinolytic activity. These hypotheses were tested in experiments detailed in Chapter 6.

6. To investigate the *in vitro* effects of detergent sclerosants on platelet activation and aggregation. Experimental findings detailed in Chapter 2 showed that sclerosants demonstrate procoagulant activity at low concentrations as evident by shortening of clotting tests and release of PMP. This concept was further reinforced by findings detailed in Chapter 6 where both agents enhanced strong clot formation as tested in the global assays of coagulation. It was hypothesized that the observed procoagulant activity is most likely due to activation of platelets at low concentrations of sclerosants. This hypothesis was tested in experiments detailed in Chapter 7.

Acknowledgements

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CHAPTER 2

In vitro Effects of Detergent Sclerosants on Coagulation, Platelets and Microparticles

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I was the first author on this paper which was published in the European Journal of Vascular and Endovascular Surgery in 2007 and has been cited 13 times. I was the major (>50%) contributor to this study and undertook experimental work under Dr Tom Exner's direct supervision. The data was analysed by myself, presented to the team and discussed in regular meetings. I wrote the manuscript, and was responsible for the submission process and dealing with the reviewers comments. Dr Exner performed the clotting factor measurements, Dr David Connor undertook the flow cytometry experiments on microparticles. Other co-authors contributed to discussion and the manuscript. David Connor was a PhD student at the time and included the microparticle analysis, a figure derived from Figure 6, in his PhD thesis. (PUBLISHED, IF= 3.007)

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In Vitro Effects of Detergent Sclerosants on Coagulation, Platelets and Microparticles

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Objectives. *To investigate the* in vitro *effects of Sodium Tetradecyl Sulphate (STS) and Polidocanol (POL) on clotting tests, clotting factors, platelets and microparticles.*
Materials and methods. *Platelet rich (PRP) and platelet poor (PPP) plasmas were incubated with varying concentrations of STS and POL. Clotting tests, platelet/plasma turbidity, and microparticle studies were performed. Specimens were mixed with individual factor deficient plasmas and clotting factor activities were studied.*
Results. *STS at high concentrations (>0.3%) destroyed platelets, microparticles and the clotting factors V, VII and X. It prolonged all clotting tests including prothrombin time (PT), activated partial thromboplastin time (APTT), non-activated partial thromboplastin time (NAPTT), thrombin time (TT), factor Xa clotting time (XACT) and surface activated clotting time (SACT). Higher concentrations of POL were required to achieve some anticoagulant activity. Low sclerosant concentrations shortened XACT and SACT, and induced release of procoagulant platelet derived microparticles. Increased exposure time resulted in increased procoagulant activity. STS at concentrations higher than 0.5% precipitated a complex containing apolipoprotein b and fibrinogen.*
Conclusions. *Detergent sclerosants affect the clotting mechanism by interfering with clotting factor activities, procoagulant phospholipids and platelet derived microparticles. STS has more anticoagulant activity than POL in high concentrations. Low concentration sclerosants demonstrate procoagulant activity.*

Keywords: Varicose veins; Sclerotherapy; Sclerosing solutions; Blood coagulation; Phospholipids; Detergents.

Introduction

One traditional approach to the treatment of varicose veins has been the surgical removal of these vessels. The modern alternative is to occlude incompetent vessels by endovenous laser ablation or ultrasound guided sclerotherapy (UGS). UGS was first introduced in 1989 to treat truncal superficial veins, tributary veins, and incompetent perforators.[1,2] Ultrasound guidance has significantly enhanced the safety and effectiveness of sclerotherapy by providing accurate visualisation of the target vessels, monitoring the effectiveness of the sclerosing agent and ultimately assessing the success or failure of the treatment.[3]

Modern sclerotherapy is performed using detergent sclerosants such as Sodium Tetradecyl Sulphate (STS) and Polidocanol (POL). The proposed mechanism of sclerotherapy is destruction of the endothelial lining of the target vessel, exposure of the basal layer collagen, induction of vasospasm and ultimately complete vessel fibrosis. With ultrasound monitoring, sclerosants have been observed to enter the deep venous system via the perforators or junctions, but the clinical incidence of post-sclerotherapy deep vein thrombosis or pulmonary embolism remains very low.[4–6]

Sclerosants have been reported to have no effect on the coagulation system in some publications[7,8] whilst others have reported minor changes *in vivo*.[9–14] Fegan noted that apart from a minor increase in the platelet count of some patients, STS had no effect on clotting times.[15] Others have reported the lytic effects of sclerosants on platelets and other cells and concluded that ADP release could stimulate platelet aggregation.[16,17]

Given the low incidence of post-sclerotherapy thromboembolic complications, we aimed to investigate the anti-thrombotic potential of these agents

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and their interaction with the haemostatic mechanism. In particular, we investigated the effects of these agents on clotting tests, clotting factors, platelets and platelet derived microparticles.

Materials and Methods

Sample collection

Blood was collected from normal healthy volunteers following informed consent by clean venipuncture into one ninth its volume of 0.109 M trisodium citrate solution in plastic tubes. Platelet rich plasma (PRP) was harvested after centrifuging for 10 minutes at 150 g. Platelet poor plasma (PPP) was obtained by centrifugation for 20 minutes at 2,000 g. Samples were incubated with increasing sclerosant concentrations for 30 minutes at 20 °C.

Sclerosants

Sodium Tetradecyl Sulphate (STS) was obtained as FIBRO-VEIN 3% (Australian Medical & Scientific, Artarmon, NSW, Australia) and Polidocanol (POL) was obtained as AETHOXYSKLEROL 3% (Chemische Fabrik Kreussler & Co GmbH, Wiesbaden, Germany). These agents were slowly added to plasmas in plastic tubes or microwells using syringes or variable volume pipettors. Low concentrations of sclerosants used in this study are typical of those most commonly used in clinical practice to treat small vessels. We initially used much wider concentration ranges for the sclerosants but later only reported on the levels at which changes in clotting tests happened.

Clotting tests

Clotting tests were carried out on an ACL300 (IL/Beckman/Coulter, Milan, Italy) and a ST4 semi-automated machine (Diagnostica Stago, Asnieres, France). Platelet counts were determined with a Cell Dyn 4000 instrument (Abbott Diagnostics Division, Santa Clara, CA, USA).

Thrombin time (TT) tests were carried out by mixing 0.10 ml prewarmed plasma samples with 0.10 ml bovine thrombin (Dade-Behring, Marburg, Germany) at 2.5 NIH u/ml and timing to a clotting endpoint.

Prothrombin time (PT) tests were carried out using 0.05 ml test plasmas and 0.10 ml Thromborel S (Dade Behring, Marburg, Germany) in the ACL300.

Activated partial thromboplastin time (APTT) tests were carried out using 0.05 ml plasma samples preincubated with 0.05 ml of various APTT reagents for 5 minutes at 37 °C and then timed to a clotting endpoint after the addition of 0.05 ml 0.025 M calcium chloride.

Factor Xa activated clotting time (XACT) tests were carried out as previously described.[18] 0.05 ml plasma samples were mixed with 0.10 ml prewarmed XACT reagent (Haematex Research, Hornsby, Australia) and the time to a clotting endpoint at 37 °C was determined. In some cases the test samples were mixed 1:1 with a phospholipid deficient animal plasma to make the system more specific for procoagulant phospholipid. We have previously described the sensitivity of the XACT test for procoagulant phospholipid.[19]

Non-activated partial thromboplastin time (NAPTT) tests were carried out by prewarming 0.05 ml plasma samples for 2 minutes, then adding 0.1 ml of a 1:1 mix of procoagulant phospholipid reagent (Haematex Research) and 0.025 M calcium chloride and timing to a clotting endpoint at 37 °C. The NAPTT assay[19,20] is based upon a modification of the APTT test. Procoagulant phospholipids are retained in the test reagent, but contact activators are removed to allow the assay to be dependent upon contact activation.

Surface activated clotting time (SACT) is an APTT with contact activator but without phospholipid. The test method is very similar to the silica clotting time[21] and the kaolin clotting time[22] in that phospholipid is rate limiting. These tests are extremely sensitive to procoagulant phospholipid, usually derived from activated platelets. Thus SACT shortening is sensitive to procoagulant phospholipid. SACT tests were carried out by preincubating 0.05 ml plasma samples with 0.05 ml of a silicate-based contact factor activating reagent (SACT reagent, Haematex Research) at 37 °C for 5 minutes and then timing to a clotting endpoint after the addition of 0.05 ml of 0.025 M calcium chloride.

Clotting times were performed for a maximum of 60 seconds for TT, 120 seconds for PT, 300 seconds for APTT, 360 seconds for NAPTT, 240 seconds for SACT and 120 seconds for XACT.

A summary of the tests used and their specificities is shown in Table 1.

Clotting factor assays

Coagulation factor assays were carried out using standard one stage automated procedures on the ACL300. Thus test specimens were diluted 1/5, mixed with individual factor deficient plasmas and then tested by PT test for extrinsic factors and by APTT test for intrinsic factors. PT and APTT results were interpolated onto calibration plots generated with 1/5, 1/10, 1/20

Table 1. Specificity of the clotting tests used for various clotting factors and procoagulants

Clotting Test	Mainly dependent on
Thrombin Time (TT)	Fibrinogen and antithrombin
Prothrombin Time (PT)	Fibrinogen and factors VII, X, V, II
Activated Partial Thromboplastin Time (APTT)	Fibrinogen and factors XII, XI, PKK, HMWK, IX, VIII, X, V, II
Surface Activated Clotting Time (SACT)	Procoagulant phospholipid and all factors as for the APTT
Non Activated Partial Thromboplastin Time (NAPTT)	Activated clotting factors
Factor Xa Activated Clotting Time (XACT)	Procoagulant phospholipid, factors V and II.

and 1/40 dilutions of pooled normal plasma. The PT reagent used was Thromborel S (Dade-Behring) and the APTT reagent was Intrinsin LR (Haematex Research). Individual factor deficient plasmas were obtained from Dade Behring (Marburg, Germany).

Microparticle analysis

Flow cytometry antibodies, Annexin V and TRU-Count tubes were obtained from Becton Dickinson (USA). Microparticle counts were performed by adding 0.05 mL of test sample to 0.2 mL of 0.02 M HEPES pH 7.0 buffered 0.15 M sodium chloride solution. 0.01 mL of this mixture was added to antibody (CD41a-PerCP-Cy5.5), Annexin V-APC and HEPES buffered saline solution containing 2.5 mM $CaCl_2$. Control tubes contained 2.5 mM K_2EDTA. This was incubated for 30 minutes before the addition of 1 mL HEPES buffer containing 2.5 mM $CaCl_2$. Counting was performed relative to beads in TRU-Count tubes (Becton-Dickinson). Platelet-derived microparticles were defined as events less than 1.09 μm in diameter that bound Annexin V and CD41a-PerCP-Cy5.5.

Turbidity studies

Sample turbidity was measured in 0.2 ml volumes of plasma and sclerosant mixtures in 96 well flat-bottomed microplates. A Titertek Multiskan microplate reader (Thermo Lab Systems, Finland) was used with a 415 nm filter.

Protein analysis

This service was provided by the Australian Proteomic Analysis Facility (APAF) at Macquarie University (North Ryde). Proteins were digested with trypsin, peptides separated by high pressure liquid chromatography and then subjected to mass spectrometry using matrix assisted laser desorption ionisation (MALDI). Results were derived after computer analysis using standard libraries of known peptide fragments.

Results

Effect of STS and POL on clotting tests

Clotting tests were performed in PPP (Fig. 1) and PRP (Fig. 2) following incubation with varying concentrations of sclerosants for 30 minutes.

High concentrations of both sclerosants prolonged PT, APTT, NAPTT, XACT and SACT in both PPP and PRP. TT results were prolonged by increasing concentrations of STS, but not with POL.

A higher concentration of POL was required to achieve prolongation of most clotting tests when compared to STS. POL never achieved the same degree of prolongation when compared with STS.

In PRP, phospholipid sensitive XACT and SACT times were shortened at low concentrations of sclerosant (less than 0.2% for STS and 0.4% for POL), indicating the generation of procoagulant phospholipid.

Effect of time dependence

Both agents affected the XACT in a time dependant fashion in both PRP and PPP (Fig. 3a and 3b). The prolongation of XACT tests by both sclerosants in PPP increased with time. The shortening of XACT by both sclerosants in PRP was also time dependent. There was a general shift of the concentration curves to the left, so that very low concentrations demonstrating no significant change in the clotting time, exhibited shortening effects after 2.5 hours and low concentrations showing shortening of XACT, demonstrated prolongation after 2.5 hours of incubation. For example, 0.15% STS demonstrated an XACT time of 44 seconds at time 0 but that changed to 13.5 seconds after 2.5 hours. Conversely, when 0.3% STS was tested after 2.5 hours at 20 °C , the XACT prolonged from 22.4 seconds (time 0) to 73 seconds (time 2.5 h) indicating further destruction of platelets and microparticles and an increase in anticoagulant activity with increase in the exposure time. Similar results were obtained for POL. Results are shown in Fig. 3a and 3b.

Clotting factor assays

Certain clotting factor activities were reduced by STS, with the largest reduction in activity on Factors V, VII

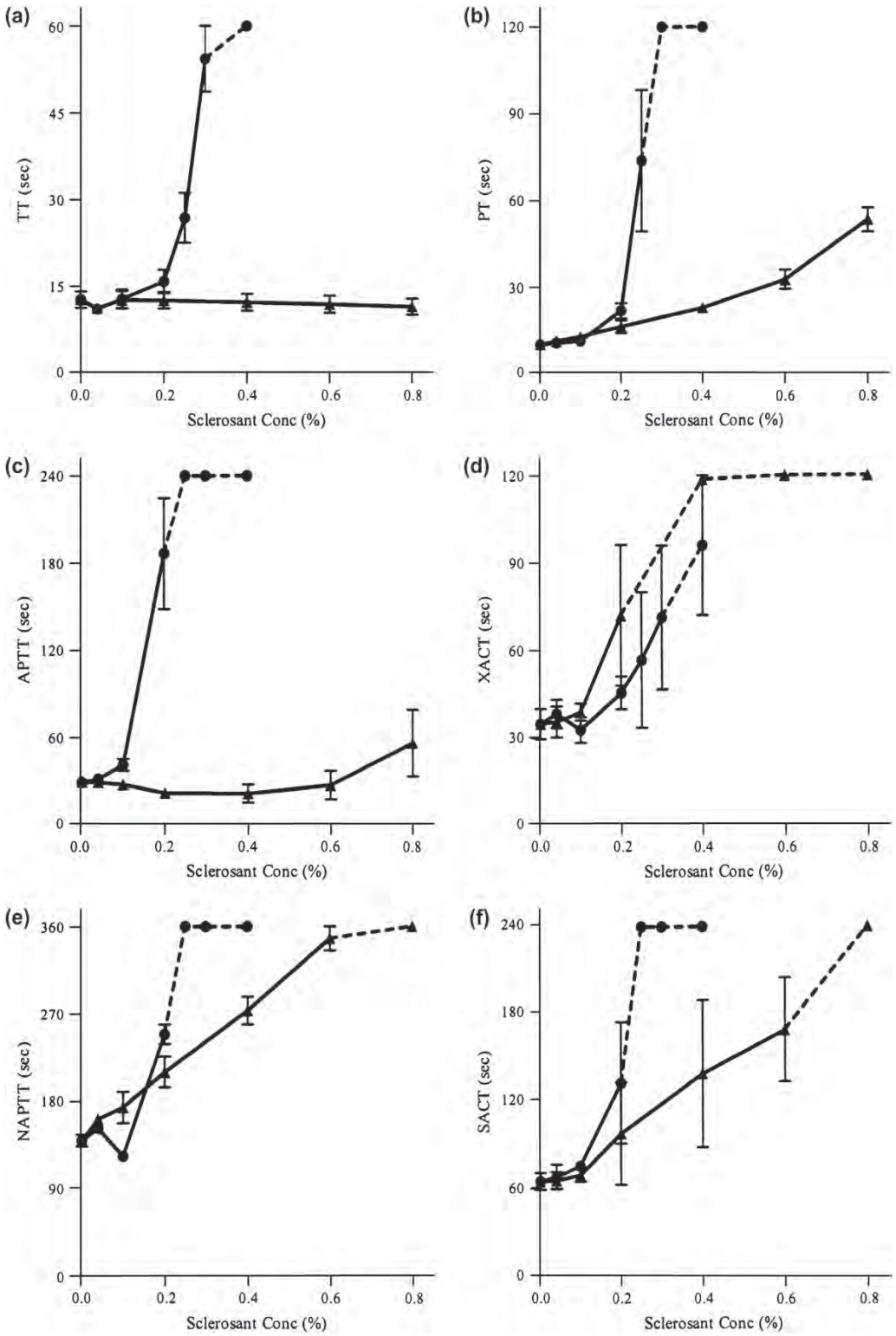

Fig. 1. The effect in PPP of STS (●) and POL (▲) on various clotting tests ($n = 3$). a) TT, b) PT, c) APTT (SynthaFax), d) XACT, e) NAPTT, f) SACT. Error bars represent the Standard Error of the Mean.

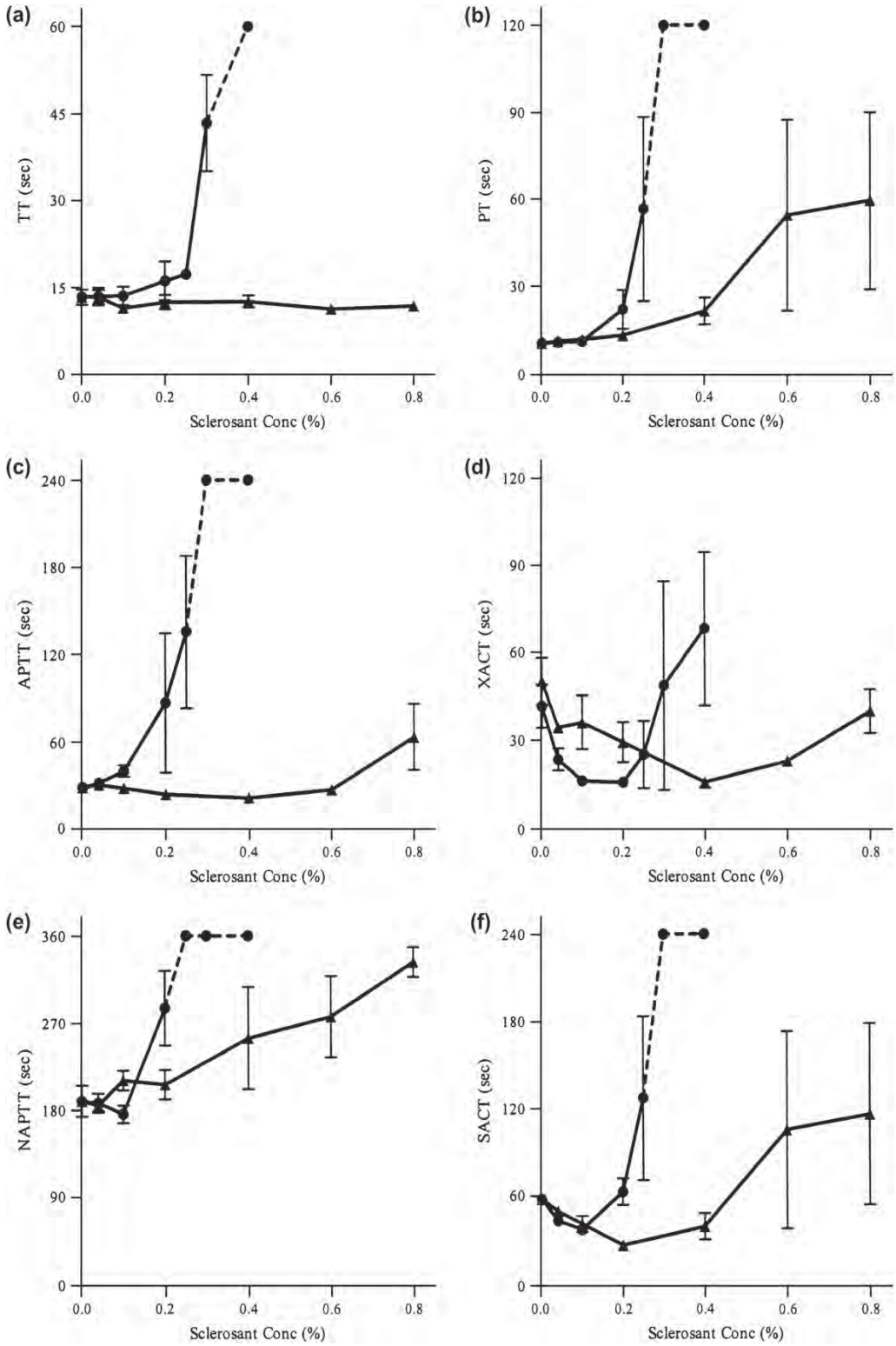

Fig. 2. Effect in PRP of STS (●) and POL (▲) on various clotting tests ($n = 3$). a) TT, b) PT, c) APTT (Synthafax), d) XACT, e) NAPTT, f) SACT. Error bars represent the Standard Error of the Mean.

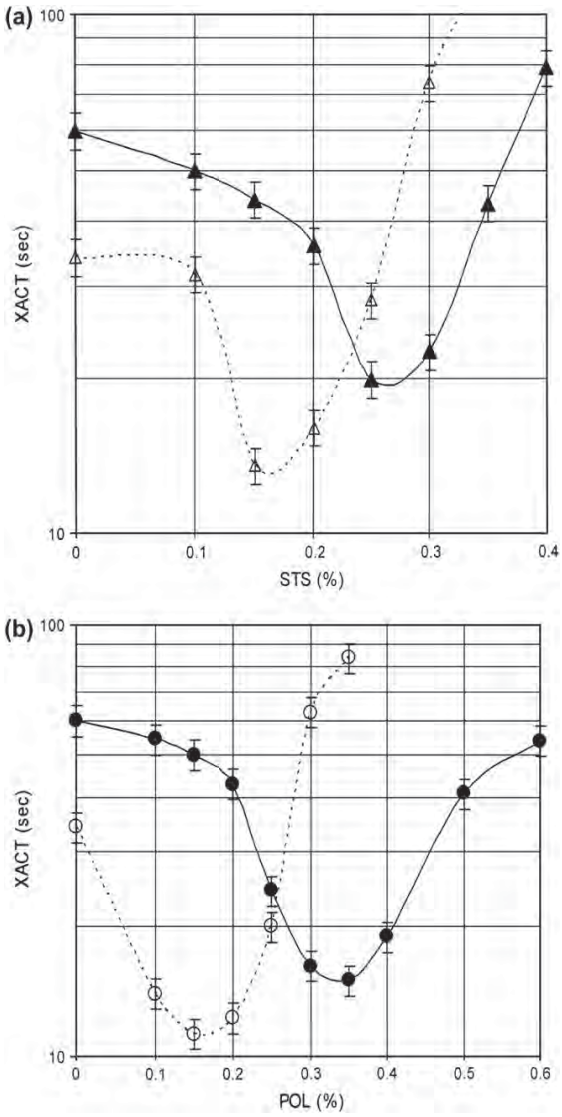

Fig. 3. (a). Effect of sodium tetradecyl sulphate (STS) in normal platelet rich plasma on XACT test initially (▲) and after 2.5 hours' incubation (**Δ**). (b). Effect of Polidocanol (POL) in platelet rich plasma on XACT test initially (●) and after 2.5 hours' incubation (○).

and X. The effect of STS on clotting factor activity was time dependent as clotting factor activity was markedly reduced in samples incubated for 30 minutes when compared with 5 minutes. Apparent activities of factors VIII, IX, XI and XII were slightly increased by POL at 5 and 30 minutes. Activities of factors V, X, and VII were slightly reduced by POL at 5 and 30 minutes. The reduction in clotting factor activity after 30 minutes was much more pronounced with STS when compared with POL.

To further investigate the changes in the activity of clotting factors, dilution experiments were performed. The tests were carried out on pooled normal plasma (PNP) preincubated with 0.3% STS or with 0.3% of POL for 5 minutes and 30 minutes at 20 °C (Table 2). Typical factor assay results for factor V are shown in Fig. 4a. The calibration curve obtained by plotting a clotting time (in this case PT) against concentration of factor V provided by appropriate dilutions of PNP mixed with factor V deficient plasma was used as a reference. Samples simply deficient in factor V usually yield long clotting times and lines parallel to the reference line, thus allowing simple interpolation to estimate factor V levels. However with sclerosants present in test samples the lines were not parallel as shown in Fig. 4b. In highly diluted samples, the residual sclerosant being also dilute had no effect on the clotting tests. However, in the more concentrated (less diluted, eg 1/5) samples, the residual sclerosant exerted a prolonging effect on the clotting times. These interfering effects were reduced as the sample were diluted and thus more valid results were obtained with more dilute samples.

It is clear that a true deficiency of factor V was induced by STS after 30 minutes incubation because the entire dilution line has moved to longer clotting times as shown in Fig. 4b. 1/5 dilutions showed non specific effects from higher levels of residual sclerosants. Thus assay results shown in Table 2 were derived from the linear portion of the graph, ie samples with 1/10 dilutions and higher. POL increased the apparent activity of intrinsic factors VIII, IX, XI and XII as it shortened APTT.

Macroscopic effects on platelets

Sample turbidity was decreased upon incubation with POL, indicating the lysis of platelets (Fig. 5). STS also

Table 2. Effect of Sclerosants on Clotting Factors. Factor assays carried out on pooled normal plasma (PNP = 100% activity all factors initially) mixed with 0.3% of either Polidocanol (POL) or sodium tetradecyl sulphate (STS) for 5 minutes or 30 minutes at 20°C

FACTOR	PNP with 0.3% POL		PNP with 0.3% STS	
	T = 5 min	T = 30 min	T = 5 min	T = 30 min
II	102%	97%	96%	92%
V	70	59	54	7
X	76	75	90	20
VII	82	79	72	5
VIII	117	106	97	61
IX	111	119	88	31
XI	152	126	90	42
XII	135	135	105	107

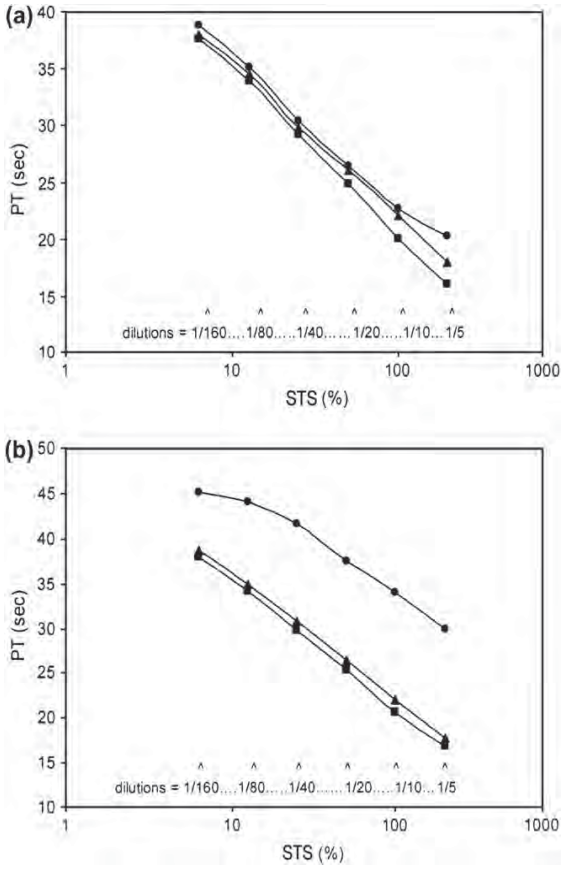

Fig. 4. (a). Results of Factor V assays based on the prothrombin time (PT) test. Figure shows calibration curve of PT plotted against factor V level in dilutions of pooled normal plasma (■). The other curves show PT plotted against dilution of PNP mixed with either 0.3% STS (●) or 0.3% POL (▲) for 5 min. (b). Results as described for Fig 4a but with samples aged at 20° C for 30 minutes.

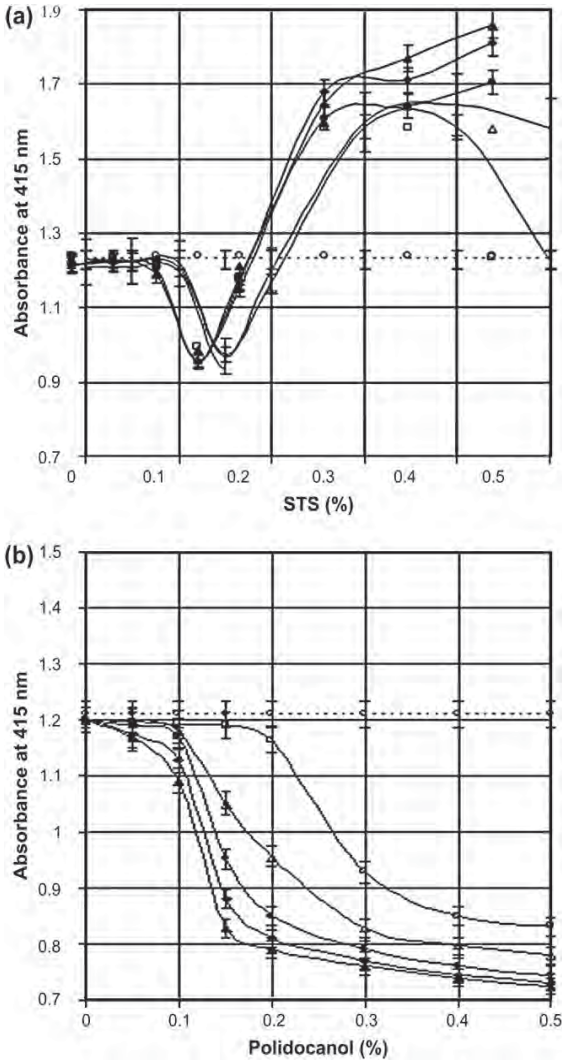

Fig. 5. (a) Sodium tetradecyl sulphate (STS) in platelet rich normal plasma (PRP). Showing absorbance at 415 nm due to platelets in PRP as a function of STS concentration after increasing time at 20° C (5 min □, 10 min △, 20 min ●, 40 min ◆ and 60 min ▲). (b) Polidocanol (POL) addition to platelet rich plasma (PRP). Showing decrease in turbidity (415 nm) due to platelet lysis as a function of POL concentration after increasing time at 20° C. (5 min □, 10 min △, 20 min ●, 40 min ◆ and 60 min ▲). Error bars represent the Standard Error of the Mean.

reduced the turbidity of PRP at low concentrations but then increased turbidity at higher concentrations. This increased turbidity with STS concentrations above 0.5% was apparent also in PPP (not shown).

The increase in sample turbidity was found to be due to precipitation of a complex of unknown plasma protein with or by STS. The material precipitated was insoluble in plasma, water and even 5% sodium dodecyl sulphate solution. Thus proteins contained within could not be analysed by SDS PAGE. It was sent to a facility for proteomic analysis where it was subjected to amino acid analysis and tryptic digestion. Mass spectrometric analysis using MALDI and computer analysis of the main fragments showed the main components to be apolipoprotein B and fibrin(ogen) (results not shown). As trypsin was used for solubilisation, differentiation between fibrin and fibrinogen could not be made.

Microparticle analysis

Increased microparticle counts were detected in PRP samples for sclerosant concentrations greater than

0.1% (Fig. 6). At concentrations above 0.3% microparticle counts began to decrease and this corresponded to similar decreases in procoagulant phospholipid activity, as assessed by the XACT method (Fig. 2 d). Both agents showed the same overlapping pattern for microparticles (Fig. 6), but their effects on clotting tests and in particular XACT test were quite different at high concentrations (Fig. 2). This is because STS at higher concentrations prolongs the XACT test due to its interference with the thrombin-fibrinogen interaction whereas POL does not. Thus STS and POL form microparticles similarly but STS has an additional inhibitory effect on XACT at concentrations above 0.3%.

Discussion

STS and POL are the two most commonly used sclerosants worldwide. The sclerosant concentration is chosen based upon the vessel diameter as well as the volume and speed of blood flow in the target vessel. Higher concentrations are required to occlude large vessels or to treat smaller vessels demonstrating high flow rates.

Ultrasound guidance monitors the delivery of the sclerosants into the target vessels. The movement of the sclerosant can be traced from the superficial veins (usual target vessels) to the deep veins via perforating veins and junctions. Despite this, deep vein thrombosis (DVT) remains a rare complication of UGS.[23,24] An interesting paradox appears with sporadic reports of DVT following direct vision sclerotherapy of reticular veins using low concentration agents.[25]

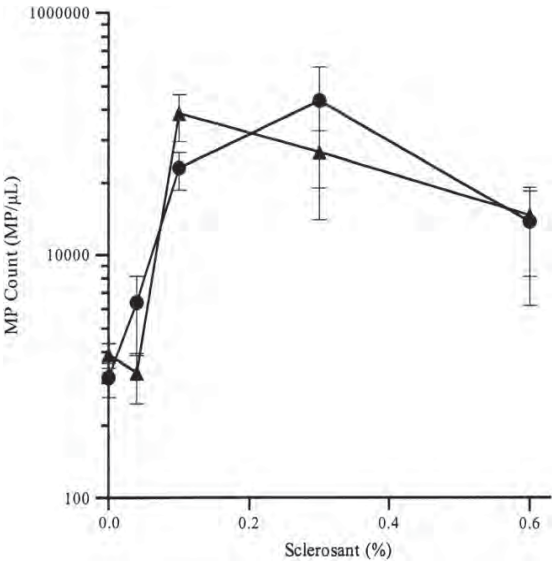

Fig. 6. Effect in PRP of STS (●) and POL (▲) on mean microparticle counts ($n = 3$). PRP was incubated with varying concentrations (0–0.6%) STS or POL for 30 mins before microparticle quantitation. Error bars represent the Standard Error of the Mean.

We investigated the thrombotic activity of both sclerosants. At higher concentrations, both agents demonstrated anticoagulant activity. STS demonstrated more anticoagulant activity than POL on conventional clotting tests. It prolonged all clotting tests at concentrations higher than 0.3% and dissolved and inactivated procoagulant phospholipids causing lysis of procoagulant microparticles.

TT results were prolonged by increasing concentrations of STS, but not with POL. TT measurement is dependant on thrombin and fibrinogen but not on phospholipids. Being a potent anionic detergent, STS affects proteins which can explain its prolongation of TT. Predictably, POL being a non-ionic detergent did not affect the TT as it primarily targets phospholipids.

POL has previously been reported to activate contact factors *in vitro*.[10] In our study POL did not shorten the NAPTT on either PPP or PRP and it seems unlikely to be activating contact factors *in vitro*. It is possible that denuded blood vessels may cause contact activation.

Both agents reduced sample turbidity indicating lysis of platelets. STS, however, increased the sample turbidity at concentrations above 0.5% due to formation of a precipitate of apolipoprotein B and fibrin (ogen). We can only postulate that such deposits can possibly occlude small vessels leading to arteriolar ischemia and possibly to skin necrosis associated with sclerotherapy.

At low concentrations, both sclerosants demonstrated some procoagulant activity in PRP. Both agents shortened phospholipid dependent clotting tests such as SACT and XACT at concentrations lower than 0.2–0.3%. Slightly shortened SACT and XACT results in PPP suggest that STS may be mimicking procoagulant phospholipid. We further explored this procoagulant activity by investigating the formation of platelet-derived microparticles. Microparticle counts reflected the change in procoagulant activity, with increasing microparticle counts occurring with low doses of sclerosants, and a subsequent decrease in microparticle formation at higher doses. To our knowledge this is the first report of the formation of microparticles by detergent sclerosants. Damage to the platelet membrane by low-doses of sclerosants induces platelet lysis and the subsequent release of microparticles, possessing procoagulant phospholipid activity. At higher sclerosant concentrations, both platelets and microparticles are destroyed, resulting

in decreased procoagulant phospholipid. In this study, the release of microparticles occurred at therapeutic concentrations of sclerosants and provides a novel mechanism by which platelet-derived microparticle formation may be induced in vivo. Microparticles also possess angiogenic properties[30,31] and may play a role in pathogenesis of post-sclerotherapy telangiectatic matting.

Sclerosants demonstrated interesting effects on the clotting factors. STS was found to significantly destroy mainly the clotting factors V, VII and X when incubated with pooled normal plasma at 0.3% for 30 minutes. At lower concentrations (0.15−0.2%), the reduction in clotting factor activity occurred immediately. This immediate prolongation of the clotting tests cannot be explained by an immediate destruction of clotting factors but rather to a non-specific inhibitory effect, probably on the procoagulant phospholipid necessary for these tests to work. This inhibitory activity was apparent in factor assays based on the PT, especially with the 1/5 dilutions.

In contrast to STS, 0.3% POL in normal plasma increased the apparent activity of intrinsic factors VIII, IX, XI and XII as measured by APTT suggesting a procoagulant activity. There are other agents reported to have a similar effect on clotting tests.[26−29] Lupus anticoagulants are typical agents which interfere with clotting factor assays in a similar way. POL in general demonstrates a procoagulant profile in concentration range of 0.3−0.6%. The Australian Polidocanol Study reported 3 cases of DVT when 0.5%−1% POL was used to treat telangiectasias (spider veins). Interestingly, the 3% concentration used to treat varicose veins did not produce a DVT.[25]

Immediate walking after sclerotherapy has been recommended in order to reduce the exposure of the deep veins to the sclerosants. Time dependence experiments were performed to investigate the relationship between the exposure time and procoagulant activity (Fig. 3a and 3b). Low concentration sclerosants (eg. 0.15% STS) with minimal effects on the clotting tests, given the increase exposure time achieve procoagulant activity by damaging the platelets and releasing microparticles. Given the dilution effects and plasma protein binding, a lower concentration of the sclerosants, as compared with what was initially injected, may reach the deep venous system with potential procoagulant activity. This may justify immediate walking after sclerotherapy to reduce the exposure of deep veins to low concentration sclerosants.

In summary, we have demonstrated the effect of the sclerosants on the coagulation mechanism to be concentration and time dependant. At lower concentrations both STS and POL exhibit procoagulant activity through stimulation of platelet lysis and the release of procoagulant platelet-derived microparticles. Also, low concentration POL increases the apparent activity of most clotting factors. Procoagulant activity increases with increased exposure time. At higher concentrations procoagulant activity is lost, possibly due to the additional lysis of the microparticles and in the case of STS, destruction of certain clotting factors. STS exhibits potent anticoagulant properties at concentrations higher than 0.3% *in vitro*. This may partly explain the low incidence of deep vein thrombosis when these agents are used in high concentrations clinically.

Both STS and POL are produced in liquid format but most clinicians prefer to use them to treat varicose veins in the foam format. Due to technical reasons, we only investigated the liquid sclerosants in this study. The effects of foam sclerosants on the coagulation system and the clinical relevance of the present study will be further investigated by the authors in *in vivo* studies.

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CHAPTER 3

The Lytic Effects of Detergent Sclerosants on Erythrocytes, Platelets, Endothelial Cells and Microparticles are Attenuated by Albumin and other Plasma Components *in vitro*

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I was the first author on this paper which was published in the European Journal of Vascular and Endovasular Surgery in 2008. This paper has been cited 9 times. I was the major (> 50%) contributor to this study and undertook experimental work under Dr Tom Exner's direct supervision. The data was analysed by myself, presented to the team and discussed in regular meetings. I wrote the manuscript and was responsible for the submission process and dealing with the reviewers comments. Dr David Connor performed the flow cytometry experiments for investigating the microparticles and Dr Andrea Herbert cultured the endothelial cells. Other co-authors contributed to the discussion and the manuscript. Andrea Herbert was a PhD student at the time but none of this work was included in her PhD theses. David Connor was a PhD student at the time and included the flow cytometry analysis of the microparticles, Figure 4, in his PhD thesis (PUBLISHED, IF= 3.007)

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The Lytic Effects of Detergent Sclerosants on Erythrocytes, Platelets, Endothelial Cells and Microparticles are Attenuated by Albumin and other Plasma Components *in Vitro*

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KEYWORDS
Sclerosants;
Haemolysis;
Platelets;
Endothelial cells;
Microparticles;
Albumin

Abstract *Objective:* To investigate the lytic effects of sodium tetradecyl sulphate (STS) and polidocanol (POL) on erythrocytes, platelets, endothelial cells and platelet-derived microparticle (PDMP) formation *in vitro* and the potential protective effects of serum albumin and agents such as procaine.
Materials and methods: The effects of sclerosants were studied in blood samples obtained from normal individuals. Absorbance densitometry was used to assess the lytic effects of sclerosants on blood cells and cultured human microvascular endothelial cells (HMEC) in plasma and in saline. PDMP were quantified by flow cytometry.
Results: Haemolysis occurred in whole blood at sclerosant concentrations greater than 0.25% for STS and above 0.45% for POL. Similar concentrations of both agents caused platelet and endothelial cell lysis. Both sclerosants released PDMP at low concentrations but destroyed PDMP at higher concentrations. Albumin significantly reduced the lytic effect of both sclerosants on all cells but had a greater inhibitory effect on POL. Protamine at 0.01% had a neutralising effect on STS, whereas procaine and lignocaine showed no such activity.
Conclusions: Sclerosants at therapeutic concentrations lyse blood cells and endothelial cells *in vitro*. This effect is strongly reduced by serum albumin possibly contributing towards the low incidence of thromboembolic complications of sclerotherapy.

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Introduction

Detergent sclerosants such as sodium tetradecyl sulphate (STS) and polidocanol (POL) are widely known to have lytic effects on endothelial cells.[1] The destruction of the endothelial lining of the vessel wall leads to exposure of the sub-endothelial collagen and a cascade of events that can either lead to endovascular sclerofibrosis, sclerothrombosis or even thrombophlebitis.[1]

Despite the acknowledged lytic effects that these drugs have on endothelial cells, little is known about their effects on circulating blood cells and platelets. The related literature remains contradictory with some authors reporting these sclerosants to be non-haemolytic *in vitro*[2] while others have reported them to cause haematuria[3] or haemoglobinuria[2,4] when used clinically in high volumes. In this study, we investigated the haemolytic effects of these agents *in vitro*.

We have previously reported on the effects of detergent sclerosants on clotting times, clotting factors, platelets and formation of platelet-derived microparticles (PDMP).[5] At low concentrations, both drugs shorten phospholipid sensitive clotting times and demonstrate procoagulant properties *in vitro*. This is achieved by damage to platelet membranes and the release of procoagulant PDMP. At high concentrations, STS destroys platelets and PDMP, prolongs the clotting times and demonstrates anticoagulant properties *in vitro*. By contrast, high concentration POL does not prolong the clotting times to the same extent.[5]

Clinically, the sclerosing effects of these drugs are thought to be limited to a short distance and usually more than a single injection is required to sclerose a vein segment which is more than a few centimetres long. The lack of distal sclerosing power has been presumed to be due to dilution of these agents in blood.[1,6] Similarly, the low thrombotic complication rate of sclerotherapy and the rarity of distal complications has been presumed to be due to dilution effects. Here, we investigated the possibility of a neutralising interaction between detergent sclerosants and albumin and human plasma.

Another rare but important complication of sclerotherapy is tissue necrosis and skin ulceration.[1,7] The local anaesthetic Procaine has been thought to bind and neutralise STS and is used clinically if impending STS induced tissue necrosis is suspected.[8] We also investigated the presumed protective benefit of Procaine and similar agents such as lignocaine hydrochloride and protamine sulphate in an *in vitro* setting.

Materials and Methods

Sample collection

Blood from normal healthy volunteer donors was obtained by clean venepuncture and collected in Vacutainer tubes (Becton Dickinson, USA) containing 0.109 M trisodium citrate.

Sclerosants and other agents

STS was obtained as FIBRO-VEIN 3% (Australian Medical and Scientific Limited, Chatswood, NSW, Australia) and POL as AETHOXYSKLEROL 3% (Chemische Fabrik Kreussler & Co, GMBH, Wiesbaden, Germany). Bovine serum albumin (BSA) was obtained from Bovogen (Melbourne, Vic, Australia). 20 g of BSA was dissolved in 100 ml of saline to produce a stock solution. A 4% physiologic concentration of albumin was used in these experiments. Procaine hydrochloride 2% was obtained as PROCAINE HYDROCHLORIDE INJECTION (DBL) from Mayne Pharma International (Melbourne, Vic, Australia). Lignocaine hydrochloride 2% was obtained as XYLOCAINE AMPOULES (PLAIN) INJECTION from AstraZeneca Pty Ltd (North Ryde, NSW, Australia). Protamine sulphate 1% was obtained as PROTAMINE SULPHATE INJECTION BP from Aventis Pharma Pty Limited Sanofi-Aventis Group (Macquarie Park, NSW, Australia).

Sample preparation

Preparation of Platelet Rich and Platelet Poor Plasma. Platelet rich plasma (PRP) was prepared by centrifugation of whole blood samples at 150 g for 10 minutes. Platelet poor plasma (PPP) was obtained by centrifugation of citrated blood at 1500 g for 20 minutes. Platelet counts were carried out with a Cell-Dyn 4000 (Abbott Diagnostics Division, Santa Clara, CA, USA).

Preparation of Washed Red Cells. Washed red cells were obtained by centrifugation of citrated whole blood. PPP was removed and the remaining red cells were mixed with a large volume of saline followed by recentrifugation at 2000 g for 15 minutes. The supernatant was then discarded and the washed red cells were resuspended in a volume of saline equal to the packed cell volume of 50%.

Preparation of Washed Platelets. PRP was centrifuged for 15 minutes at 3000 g to sediment platelets which were then pooled and washed in 20 ml total volume of 0.15 M NaCl, 0.01 M HEPES, 0.001 M EDTA pH 7.4 solution (diluting buffer). After centrifuging down again at 3000 g for 15 minutes and discarding the supernatant, the washed platelets were resuspended in 4.5 ml of HEPES-buffered saline. This was described as "10×" washed platelet suspension because it would have contained most of the original platelets in 1/10th the volume of the blood (45 ml).

Haemolysis studies

A) Detection of Haemoglobin Released From Lysed Red Blood Cells. The haemolytic effect of sclerosants was assessed by the addition of plasma containing various concentrations of sclerosants to 1/10th its volume of sedimented red blood cells that had been centrifuged down in microwells. Red blood cells were sedimented in microwells by centrifugation. The supernatant was removed and the pellet resuspended in plasma containing various concentrations of sclerosants. The sclerosants were dispersed into the plasma component before resuspending the red cells to avoid excessive localised lytic effects of high concentration sclerosants. After a ten minute incubation, the microplate was again centrifuged. The supernatant was liberated and transferred to another microplate. Free haemoglobin was then measured using a microplate reader (Titertek Multiskan MCC, Finland) at an absorbance of 520 nm.[9] Absorbances were converted to percent

haemolysis by interpolating onto a standard curve constructed from dilutions of red cells that had been fully haemolysed with excess STS.

B) Detection of Protective Effects of Albumin on Haemolysis. To examine the potentially protective effects of albumin, mixtures of sclerosants and bovine serum albumin (BSA) in saline were first prepared in round bottomed microwells. Next, small volumes of washed red cells were added and the mixtures were centrifuged at 200 g for 10 minutes. Unlysed red cells sedimented to form a compact spot in the centre of the wells. Absorbance measurements were then carried out at 405 nm.

Platelet lysis studies

Platelet lysis was assessed by means of changes in turbidity as previously described.[5] The inhibition of platelet lysis by the addition of bovine serum albumin (BSA) was assessed in mixtures of washed platelet suspensions and sclerosants. BSA was diluted to a concentration of 16% using diluting buffer. Serial dilutions were carried out down 2 columns of flat bottomed wells in a microplate with the bottom well containing 0% BSA. To each well, small volumes of the 10× washed platelet suspension were dispensed and absorbances were measured at 414 nm in a microplate reader (Titertek Multiskan MCC, Finland). The instrument was blanked with a column of buffer-containing wells before each reading. Small volumes of STS and POL were then added to the respective columns for a final sclerosant concentration of 0.015%. Absorbances were measured and then additional volumes of sclerosants were added stepwise to achieve a final concentration of 0.6%.

Effect of plasma on the release of platelet-derived microparticles

To examine the protective effects of plasma components, PDMP formation in response to STS or POL was assessed in both washed platelet samples and PRP. Samples (0.08 ml) were incubated with saline (0.02 ml) containing varying concentrations of sclerosant for 30 minutes. 0.05 ml of this mixture was then diluted using 0.2 ml of 0.02 M HEPES pH 7.0 buffered 0.15 M sodium chloride solution. 0.01 ml of this mixture was then added to CD41a-PerCP-Cy5.5 antibody (0.005 ml) and Annexin V-APC (0.002 ml) with the volume made up to 0.05 ml using HEPES-bufferred saline solution containing 2.5 mM $CaCl_2$. This was incubated for 30 minutes before the addition of 1 ml HEPES buffer containing 2.5 mM $CaCl_2$. Counting was performed using TRU-Count tubes. Platelet-derived microparticles (PDMP) were defined as events, less than 1.09 μm in diameter that bound Annexin V and CD41a-PerCP-Cy5.5.

Neutralisation of haemolytic effects of sclerosants by procaine, lignocaine and protamine

We investigated the potential protective benefits of these drugs in comparison with albumin. Microwells were filled with increasing concentrations of sclerosants in saline and then 1% washed red cells were added. After mixing and centrifuging, absorbances at 405 nm were determined in a microplate reader. These experiments were done with washed red cells in saline to avoid interference from plasma proteins.

Endothelial cell lysis

A human microvascular endothelial cell line (HMEC-1)[10] was cultured to confluence in endothelial growth medium: MCDB131 (JRH Biosciences, Lenexa, KS, USA) containing 10% Fetal Bovine Serum, Penicillin-Streptomycin and L-Glutamine (Gibco Invitrogen, Carlsbad, CA, USA) in 96 well microplates pre-coated with human fibronectin (Gibco Invitrogen). Mixtures of sclerosants and BSA or plasma dilutions were prepared in a separate microplate and added to the microwells after a single wash with saline. Following a 10 minute incubation at 20 °C, the solutions were gently removed, the residual cells washed twice with saline, dried and stained with Leishman's stain. Residual adherent material representing non-lysed cells was quantified by densitometry at 540 nm.

Results

Haemolysis studies

STS concentrations above 0.25% caused haemolysis in plasma whereas POL concentrations above 0.45% were necessary to induce haemolysis in this system (Fig. 1).

Fig. 2 shows the progressive inhibitory effects of albumin on the haemolytic activity of the sclerosants in saline. In the absence of albumin, much lower concentrations of both agents induced haemolysis. STS induced haemolysis at 0.02% and POL at 0.005%. Increasing concentrations

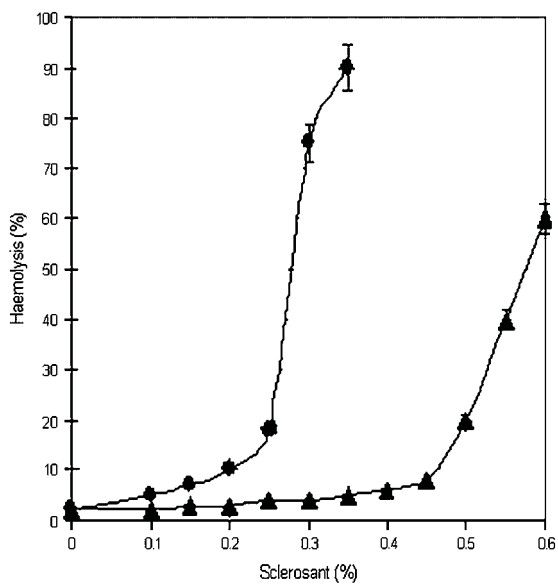

Figure 1 Haemolysis induced by sclerosants added to normal plasma containing 50% packed red cells. STS (●), POL (▲).

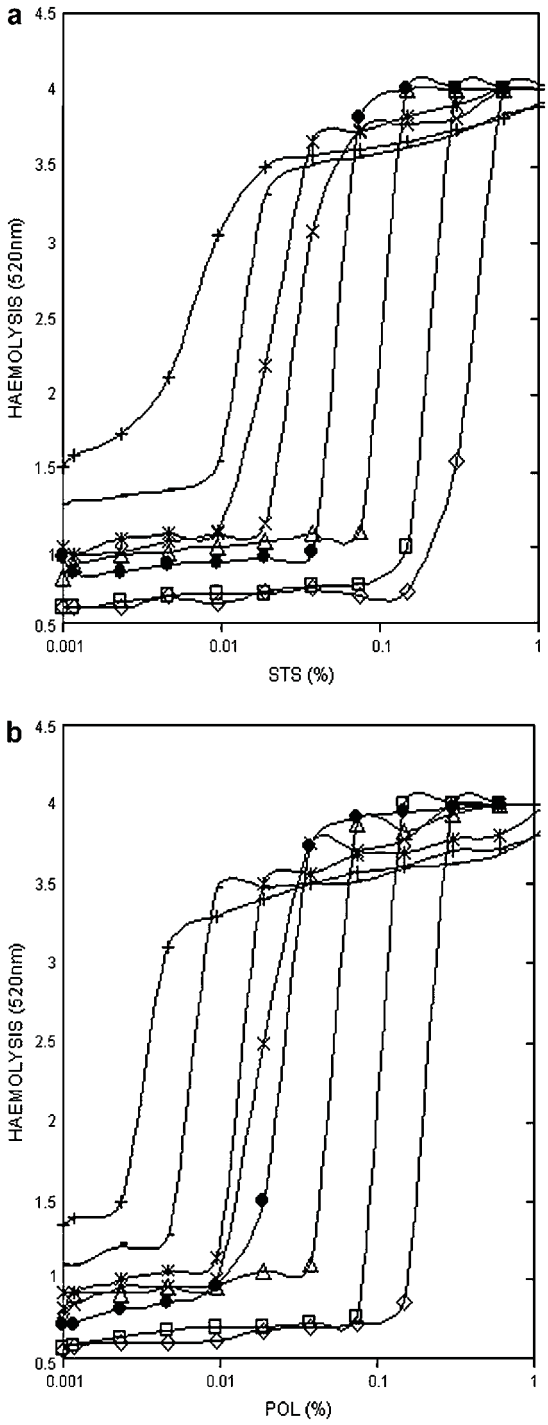

Figure 2 Haemolysis induced by sclerosants in the presence of varying concentrations of bovine serum albumin (BSA 8% ◇, 4% □, 2% △, 1% •, 0.5% ×, 0.25% *, 0.125% −, 0% +). (a) STS, (b) POL.

of albumin in saline required higher concentrations of sclerosants to induce haemolysis. STS was 50 fold and POL was 163 fold less haemolytic in blood than in saline (Table 1).

Table 1 Effects of sclerosants on red blood cells and platelets in saline, with 4% BSA present, and in whole blood

	STS (%)	POL (%)
	Red cells	
In saline alone	0.006 (1×)	0.0035 (1×)
In saline + 4% BSA	0.2 (33.3×)	0.1 (28.6×)
In whole blood	0.3 (50×)	0.57 (163×)
	Platelets	
In saline alone	0.03 (1×)	0.03 (1×)
In saline + 4% BSA	0.3 (10×)	0.3 (10×)
In whole blood	See below*	0.3 (10×)

Results extracted from 50% lysis points in Figs. 2a & b and 3a & b and Ref. 5. A 4% BSA concentration was chosen as the physiologic concentration.

* Due to a precipitate of apolipoprotein B and fibrinogen the turbidity rises after an initial drop with STS in whole blood and hence measurement of 50% platelet lysis with this method cannot be achieved.

Platelet lysis studies

In the absence of BSA, platelets were lysed by STS and POL concentrations above 0.02% (Fig. 3a and 3b). However, increasing concentrations of BSA interfered with the lytic activity of the sclerosants. At BSA concentrations above 4%, sclerosant concentrations greater than 0.2% were required to lyse platelets. POL did not dissolve platelets as completely as STS.

Effect of plasma on the release of platelet-derived microparticles

We have previously shown that low concentrations of sclerosants release PDMP.[5] Here we investigated the potential inhibitory effects of plasma on the release of PDMP. Microparticle formation was induced by STS or POL concentrations of 0.01% in washed platelet samples, whereas higher concentrations (0.15%) were required to achieve the same in PRP samples. This suggests that plasma confers a degree of lytic protection to the platelets. Both agents at higher concentrations (>0.2%) in saline reduced the PDMP count (Fig. 4b).

Neutralisation of haemolytic effects of sclerosants by procaine, lignocaine and protamine

Results obtained are shown in Tables 2 and 3. It is apparent that the haemolytic activity of STS which emerged at a concentration of 0.25% was not impeded by 0.1% procaine or lignocaine. Indeed these drugs slightly increased the haemolytic activity of STS. The best neutralising agent for STS was BSA which at a low concentration of 0.5%, completely blocked haemolysis at STS concentrations of up to 0.05%. Protamine sulphate had a mild neutralising effect on STS. As noted earlier, POL was more haemolytic at lower concentrations when compared with STS in saline. The haemolytic activity of POL was inhibited only by BSA and not procaine, lignocaine or protamine at the concentrations examined.

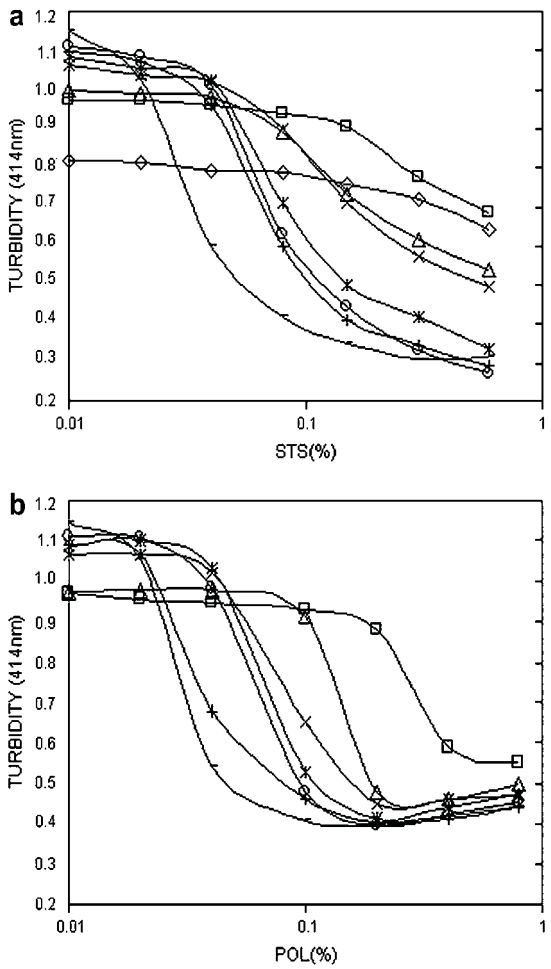

Figure 3 Turbidity studies performed to determine the effects of detergent sclerosants on washed platelets in the presence of varying concentrations of bovine serum albumin (BSA 4% □, 2% △, 1% ×, 0.5% *, 0.25% ○, 0.125 +, 0% −). Baseline zero STS or POL is shown as ''0.001%'' so as to be accommodated on a logarithmic scale. (a) STS, (b) POL.

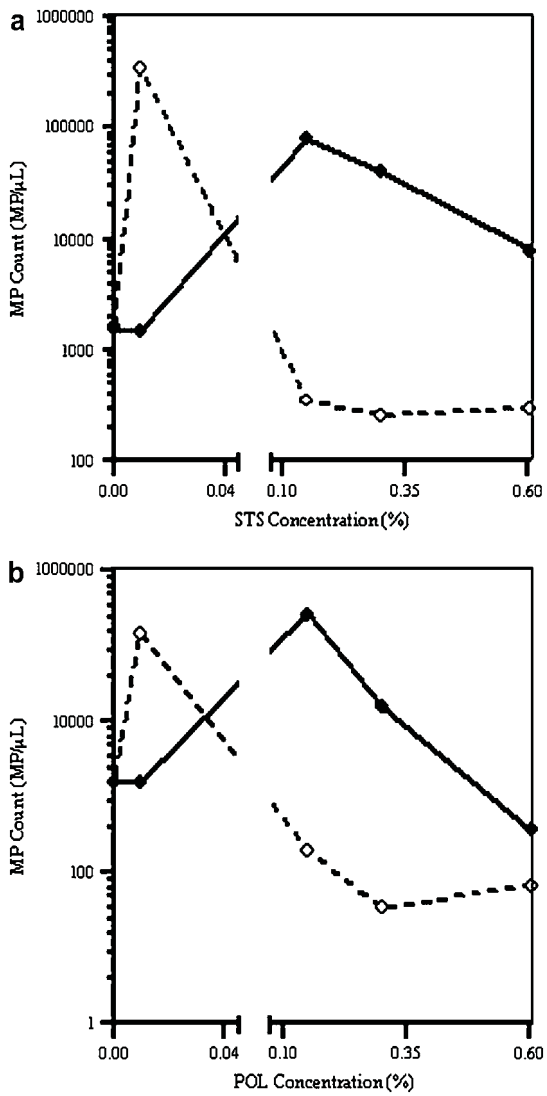

Figure 4 Effect of plasma proteins on sclerosant induced platele-derived microparticle (PDMP) formation. PDMP counts in platelet rich plasma (●) and a washed platelet suspension (○) incubated with varying concentrations of (a) STS and (b) POL.

Effect of sclerosants on endothelial cells

Results obtained are shown in Fig. 5a and b. STS reduced the amount of stainable material in the microwells in a concentration-dependent manner. POL had less lytic effects on endothelial cells (results not shown). Both plasma and BSA reduced the lytic effects of STS on the endothelial cells (Fig. 5a and b).

Discussion

It is well known that detergents including STS and POL disrupt lipid and phospholipid membranes.[1] Detergents are widely used to permeabilise cells allowing the ingress of various dyes and markers. For instance, sodium dodecyl sulphate (SDS), an anionic detergent with a similar chemical structure to STS, readily dissolves most cells even at relatively low concentrations.[11] Fegan noted that red cell lysis was caused by STS concentrations of 0.125% or greater in normal saline added to heparinised blood.[12]

In this study, both detergent sclerosants were shown to cause haemolysis, platelet lysis and endothelial cell lysis. Both drugs released PDMP in low concentrations but destroyed them at higher concentrations. Platelets appeared to be more resistant to lysis by sclerosants when compared with red blood cells (Table 1). This is possibly due to the differences in the composition of the cell membranes and the internal structure of these cells. Previous studies have reported platelet rich clots occluding femoral arterial eversion grafts to be much more resistant to lysis than erythrocyte rich whole blood clots.[13]

Table 2 Haemolysis of washed red cells in saline by STS and the effect of various therapeutic agent additives. No haemolysis is indicated by ''O'', partial haemolysis by ''+'', and complete haemolysis by ''++''. Haemolysis defined as lysis of more than 50% of red blood cells

STS (%)	No additive	Lignocaine 0.1%	Procaine 0.1%	Protamine 0.01%	BSA 0.5%
0	O	O	O	O	O
0.010	O	O	O	O	O
0.020	O	O	O	O	O
0.025	O	++	+	O	O
0.030	+	++	++	O	O
0.040	++	++	++	O	O
0.050	++	++	++	+	O

We demonstrated for the first time that plasma components appeared to neutralise the lytic effects of sclerosants in an *in vitro* setting. STS was 50 fold and POL was 163 fold less haemolytic in blood than in saline (Table 1). These findings highlight the importance of plasma components and in particular albumin in protecting against the lytic effects of detergent sclerosants. It can be concluded that lower concentrations of sclerosants can achieve the same lytic effects if blood is removed from the target vessels. In a clinical setting, this can be partially achieved by an empty vein technique or if blood is displaced by foam, diluted by an infusion of saline, or forced out by perivenous infiltration of saline or tumescent anaesthesia. The displacement of blood from the vessel lumen decreases the exposure of the sclerosant to plasma components and increases the exposure of the endothelial lining of the vessel lumen to the active agent. This concept may partly explain why foam sclerosants are found to be more effective when compared with the liquid counterparts.

Compared with the 3 other drugs tested (procaine, lignocaine and protamine), albumin proved to be the best neutralising drug with the potential to completely block sclerosant-dependent cell lysis. Albumin is a transport protein with the potential to bind to a number of drugs via two major and three minor binding sites.[14] The binding strength of a drug to serum albumin is the main factor that

Table 3 Haemolysis of washed red cells in saline by POL and the effect of various therapeutic agent additives. No haemolysis is indicated by ''O'', partial haemolysis by ''+'' and complete haemolysis by ''++''

POL (%)	No additive	Lignocaine 0.1%	Procaine 0.1%	Protamine 0.01%	BSA 0.5%
0	O	O	O	O	O
0.008	O	O	O	O	O
0.015	++	++	++	++	O
0.02	++	++	++	++	O
0.03	++	++	++	++	O
0.035	++	++	++	++	O
0.05	++	++	++	++	+

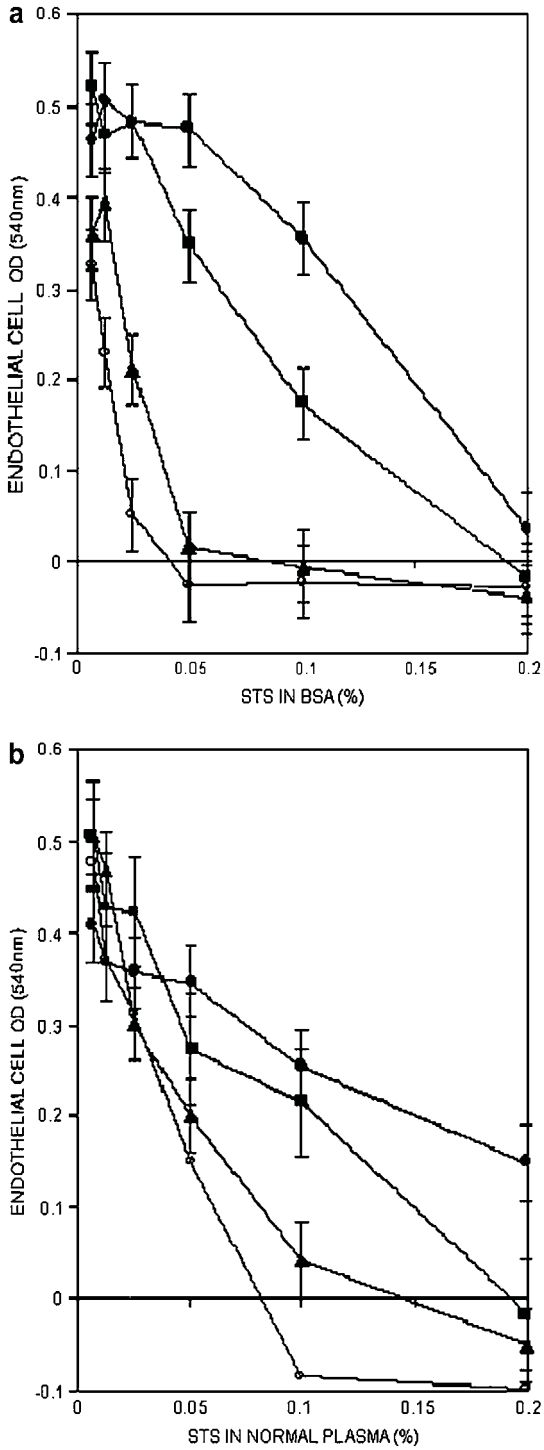

Figure 5 Effect of STS on cultured endothelial cells in the presence of varying concentrations of (a) bovine serum albumin (BSA 1.5% ●, 0.5% ■, 0.17% ▲, 0% ○) and (b) normal plasma (NP/2 ●, NP/6 ■, NP/18 ▲, NP/54 ○).

determines the amount of free agent in the circulation available to reach the target tissue. STS has been shown to bind to 10-11 sites on BSA with Kd (M) of 6.7×10^{-7}.[15]

Apart from albumin, other plasma proteins and possibly lipoproteins may also play a role in neutralisation of detergent sclerosants. We have previously isolated a precipitate induced by STS which was found to be mainly apolipoprotein B and fibrin(ogen).[5] STS can also form complexes with other proteins such as gammaglobulin.[16] Association of detergents with lipid-related proteins is probably not unexpected and we have also noticed an insoluble STS-red cell complex form when washed red cells are lysed by STS. It is also conceivable that POL is binding to plasma components other than albumin. We observed POL to be more active than STS in both saline and with 4% BSA present (Table 1). 0.0035% POL was required to achieve 50% haemolysis in saline, whereas 0.1% (28.6×) was required in the presence of 4% BSA and 0.57% (163×) was required in whole blood (Table 1). These findings imply that other plasma components play an important role in neutralisation of POL. These plasma components and their interaction with POL have not been characterised.

Sclerosants have been observed to enter the deep venous system during sclerotherapy. It is likely that the concentration of the active drug entering the deep veins is quite low due to neutralisation and dilution effects. However, we have previously demonstrated low concentrations of sclerosants to possess procoagulant properties[5] and deep vein thrombosis has been described in association with the clinical use of low concentration sclerosants.[17] Therefore, infusion of large volumes of detergent sclerosants from a single access point should be avoided as an initial high concentration will undergo a neutralisation and dilution process reaching the deep veins in a low concentration 'pro-coagulant window' which may lead to deep vein thrombosis (DVT). The extent of the neutralisation process depends on the volume of blood with which the sclerosant is mixed at the time of injection. Larger blood volumes will dilute and neutralise the sclerosants more significantly. Based on our present findings, 1 ml of blood will contain enough plasma protein to neutralise 0.2 ml of STS and 0.4 ml of POL at the therapeutic concentration of 3%. The quantity of the intravascular blood volume depends on the diameter of the target vessel and the length of the target segment. The sympathetic tone is responsible for the vessel diameter at any time. Anxiety, pain, cold temperature, vasoactive medications and other factors can induce vasoconstriction reducing the volume of the intravascular blood in the target segment. Leg elevation can help with emptying the vessel and reducing the intravascular blood. Needle penetration can also induce vasospasm and further reduce the vessel diameter. Furthermore, bubbles generated from foam sclerosants have been observed at distant sites such as the right atrium.[18,19] Sample collection from the right atrium is an invasive procedure and to our knowledge has not been attempted as yet. Given our present findings however, these bubbles are highly unlikely to have any sclerosing activity due to the neutralisation and dilution process.

We compared the protective effects of three other agents with albumin. Procaine is an anaesthetic agent and its role in preventing STS induced tissue necrosis is not well understood. Procaine has been presumed to bind and inactivate STS.[20,21] In this study, procaine and lignocaine slightly increased the haemolytic activity of STS and showed no neutralising properties. Veno-arteriolar axonal reflex has been previously proposed by one of the authors to be one of the pathogenic mechanisms of post-sclerotherapy ischemic ulcers.[7] Procaine used as a local anaesthetic possibly impedes this axonal reflex, thus exerting a protective effect. The clinical utility of Procaine in prevention of STS induced tissue necrosis requires further evaluation.

In this study, Protamine was found to mildly neutralize STS. A slight turbidity was apparent after this interaction. This is possibly because the positively charged protamine ionically binds to the negatively charged STS forming an insoluble complex.[22] Protamine is well known to neutralize other negatively charged polymeric molecules such as heparin.[22,23] POL is an anionic agent and predictably, Protamine did not inhibit the lytic effects of POL. None of the other agents, except for BSA, inhibited the lytic effects of POL.

In summary, both STS and POL at therapeutic concentrations have lytic effects on red cells, platelets, and endothelial cells and release procoagulant platelet-derived microparticles. STS causes significant haemolysis at concentrations higher than 0.3% in whole blood. Serum albumin neutralizes the lytic effects of both sclerosants. Lower sclerosant concentrations are required to achieve equivalent lytic effects in the absence of plasma components. Foam sclerosants are more effective than liquids in sclerosing blood vessels possibly due to displacement of blood and a reduced exposure to serum albumin and other plasma components. Neutralisation of sclerosants by albumin possibly plays a role in the low incidence of post-sclerotherapy deep vein thrombosis and low incidence of distal effects. Finally, procaine and lignocaine have no protective effect on the lytic properties of detergent sclerosants while protamine confers a mild degree of protection.

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CHAPTER 4

In vitro Effects of Detergent Sclerosants on Antithrombotic Mechanisms

Parsi K, Exner T, Low J, Ma DDF, Joseph JE.

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I was the first author on this paper which was published in the European Journal of Vascular and Endovasular Surgery. This paper has been cited three times since its publication in 2009. I was the major (>50%) contributor to this study and conducted experimental work under Dr Tom Exner's supervision. The data was analysed by myself, presented to the team and discussed in regular meetings. I wrote the manuscript and was responsible for the submission process and dealing with the reviewers comments. Joyce Low performed the protein C and S measurements. Other co-authors contributed to discussion and the manuscript. No other authors were students. (PUBLISHED, IF= 3.007)

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In vitro Effects of Detergent Sclerosants on Antithrombotic Mechanisms

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KEYWORDS
Detergent sclerosants;
Protein C;
Protein S;
Activated protein C;
Antithrombin

Abstract *Objectives:* To investigate the *in vitro* effects of detergent sclerosants on antithrombotic pathways.
Materials and methods: Proteins C, S and antithrombin (AT) were assayed in normal plasma treated with increasing concentrations of sodium tetradecyl sulphate (STS) and polidocanol (POL). Activated protein C (APC) was investigated by mixing normal plasmas with sclerosants and testing with the activated partial thromboplastin time (APTT) and dilute Russell's viper venom time in the presence and absence of APC. The effect on factor Xa (FXa), heparin and enoxaparin was investigated using chromogenic anti-FXa and APTT methods.
Results: High concentration (>0.6%) STS significantly destroyed proteins C, S and AT whereas POL only caused a mild reduction in PC and AT and a moderate (60%) reduction in PS levels. STS potentiated the anticoagulant effect of APC while POL increased APC resistance. STS mimicked AT and demonstrated significant anti-Xa and anti-IIa activity. STS demonstrated a similar anticoagulant profile to heparin but was 1000× weaker. It also significantly potentiated the anticoagulant effect of heparin while POL had less effect.
Conclusion: STS and POL demonstrated quite distinct and sometimes opposite effects on the antithrombotic mechanisms assayed. These effects were concentration-dependent and in general, STS had the greatest effect on antithrombotic proteins.

Introduction

Detergent sclerosants such as sodium tetradecyl sulphate (STS) and polidocanol (POL) function by destroying the endothelial lining of the target vessels and inducing endovascular fibrosis and occlusion. Under normal physiological conditions, damage to endothelium leads to a sequence of events that triggers the formation of a fibrin clot. Plasma

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coagulation inhibitors such as protein C (PC), protein S (PS), antithrombin (AT) and a number of endothelial surface membrane components such as heparinoids play a critical role in limiting the process of thrombus formation.[1] These antithrombotic proteins are dependent on the integrity of the endothelium surrounding the focus of injury.[2,3]

By inducing vessel wall injury, detergent sclerosants might be expected to initiate the same coagulation pathways that ultimately lead to the generation of thrombin and formation of a fibrin clot. Given the detergent nature of these agents, they might also be expected to destroy the endothelial-dependent antithrombotic proteins and induce an overall prothrombotic state. However, as previously demonstrated by the authors, the interaction of these agents with blood components is quite complex and STS in particular destroys a number of clotting factors and demonstrates anticoagulant activity at high concentrations.[4,5]

In this study, we investigated the effect of detergent sclerosants on plasma coagulation inhibitors PC, PS and AT and studied their interaction with activated protein C (APC), heparin, enoxaparin and factor Xa (FXa).

Materials and Methods

Sample collection

Fresh frozen normal plasma used for these studies was derived from donor blood deemed unsuitable for clinical indications. This was obtained from the Australian Red Cross Blood Transfusion Service, Sydney.

Sclerosants and other compounds

STS was obtained as Fibro-Vein 3% from Australian Medical and Scientific Limited (Chatswood, NSW, Australia). Fibro-Vein excipients include benzyl alcohol, dibasic sodium phosphate, monobasic potassium phosphate, sodium hydroxide and water for injection. POL was obtained as Aethoxysklerol 3% from Chemische Fabrik Kreussler & Co (GMBH, Wiesbaden, Germany). Aethoxysklerol excipients include ethanol, sodium phosphate-dibasic dihydrate, potassium phosphate monobasic and water for injection. Equivalent products in other countries include Sotradecol (Bionichepharma, USA; sodium tetradecyl sulphate, excipients include benzyl alcohol, dibasic sodium phosphate, monobasic sodium phosphate and/or sodium hydroxide and water for injection) and Sclerovein (Resinag AG, Switzerland; polidocanol, excipients include ethanol and chlorobutanolumhemihydricum). Although these products and their excipients are quite similar, the results of this study are only applicable to the drugs tested and other products should be assessed independently.

Bovine serum albumin (BSA) was obtained from Bovogen (Melbourne, Victoria, Australia). Unfractionated heparin was obtained from Astra Zeneca (1000 IU/ml clinical grade, North Ryde, NSW, Australia). Clexane (enoxaparin) was obtained from Sanofi-Aventis (Macquarie Park, NSW, Australia). Human factor Xa was obtained from Enzyme Research Lab (IN, USA) and chromogenic substrate for FXa (acetyl-D-CHA-GLY-ARG-pNA) was obtained from Pentapharm (Basel, Switzerland). Platelin, Intrinsin LS, Intrinsin LR and dRVVT-LR reagents were donated by Haematex Research Laboratory (Hornsby, NSW, Australia). Human activated protein C (APC) was acquired from Hyphen BioMed (Neuville sur Oise, France). Clotting tests were carried out on an ACL300R (Instrumentation Laboratory, Italy). The microplate reader used was manufactured by Titertek MCC, Finland.

Sample preparations

Freeze dried samples were prepared by using normal plasma spiked with STS and POL in siliconised glass vials, frozen quickly at −50 °C and then freeze dried under vacuum over a period of 24 h. Prior to testing, each vial was reconstituted with 2 ml of water. The APC resistant plasma was prepared using 3% alumina adsorbed porcine plasma (Haematex Research Laboratory) and 97% human plasma. Samples were processed immediately after preparation and reconstitution to minimise time-dependent variations in results.

Effect on protein C

PC was measured by the Berichrom® protein C assay (Dade Behring, Marburg, Germany) which measures functionally active protein C, using a chromogenic substrate.

Effect on protein S

The functional (clotting) assays of PS measure the activity of free PS, while the antigenic assays can measure free PS, bound PS or total PS.[6] We measured the antigenic levels of free PS by the STA-Liatest® free protein S assay (Diagnostica Stago, Asnieres, France); an immuno-turbidimetric method utilising latex microparticles coated with a monoclonal antibody specific for free PS.

Effect on antithrombin

AT was measured by the STA-Stachrome® ATIII assay (Diagnostica Stago) which measures functionally active AT, using a chromogenic substrate in freeze dried samples containing increasing concentrations of sclerosants. This method involves mixing the sample with excess thrombin (AT inactivates thrombin) in a heparin buffer and then measuring the residual thrombin activity.

To investigate the apparent increase in AT activity induced by high concentration STS, freeze dried samples containing 1.5% STS and 10% BSA (containing no plasma), 1.5% STS and hydrolysed gelatine (containing no plasma) and 1.5% STS in normal plasma were compared for AT activity. BSA is known to completely neutralise STS[5] and hence any apparent AT activity due to STS should be inhibited by albumin whereas any apparent AT activity in samples containing STS and gelatine but no plasma, would be due to the direct effect of STS on the thrombin used in this assay. These were compared with control plasma containing 1.5% STS.

Effects on activated protein C (APC) and APC resistance

APTT-based assays

To determine the effects of sclerosants on APC, normal plasma containing increasing levels of both sclerosants was

tested with and without APC present in calcium chloride and APTT was measured. A concentration of 0.05 μg/ml of APC was found to be necessary to prolong the APTT of normal plasma significantly, thus demonstrating APC responsiveness. The APTT reagent used was mainly Intrinsin LS.

Dilute Russell's viper venom (dRVVT)-based assays
To evaluate the effect of sclerosants on APC resistance (APCR) in normal and abnormal plasma more specifically, APC resistant plasma was prepared by using 3% porcine plasma/97% human plasma. Porcine factor V (FV) is resistant to cleavage by human APC. Normal and APC resistant plasmas containing 0.15—0.25% sclerosants were premixed with APC for 3 min, and then mixed with dRVVT-LR reagent and timed to clotting endpoints. Ratios of clotting times with APC to those without APC were derived. A ratio of 1.6 or less was defined as abnormal.

Comparison with anticoagulant activity of heparin

This study was done to compare the anticoagulant activity and dose response curve of heparin with detergent sclerosants. Small volumes of heparin (10 u/ml stock solution) or sclerosants were added to 0.3 ml volumes of normal citrated plasma and tested for APTT at 20 °C.

Interaction with heparin

To assess the effect of sclerosants on the anticoagulant properties of heparin, both drugs at various dilutions were added to normal plasma with or without heparin and APTT was measured. The APTT reagent used was Platelin. A range of heparin concentrations was selected to derive the heparin sensitivity curve.[7,8]

Effects on factor Xa activity

Normal plasma samples containing varying concentrations of STS or POL and none or 0.2 IU/ml of unfractionated heparin or enoxaparin (concentrations similar to those used therapeutically) were incubated with an equal volume (0.05 ml) of factor Xa (FXa) and then tested with chromogenic substrate for FXa activity. Residual FXa activity was assessed from absorbance readings at 414 nm on a microplate reader. As previously reported by the authors, there is an increase in turbidity with concentrations of STS above 0.3% due to precipitation of complexes between apolipoprotein B and fibrinogen.[4] The artefactual effect of turbidity on the optical density was partially reduced by measuring the optical density at a number of time points and subtracting the artefact.

Results

All figures contain representative results and the relative errors in quantity and times values are all within the 10-15% range based on the number of experiments.

Effect on protein C

STS caused a moderate reduction in protein C level at a concentration of 0.3%. However at concentrations higher than 0.6%, STS reduced PC levels down to 10%. POL only caused a mild reduction in PC levels (Fig. 1).

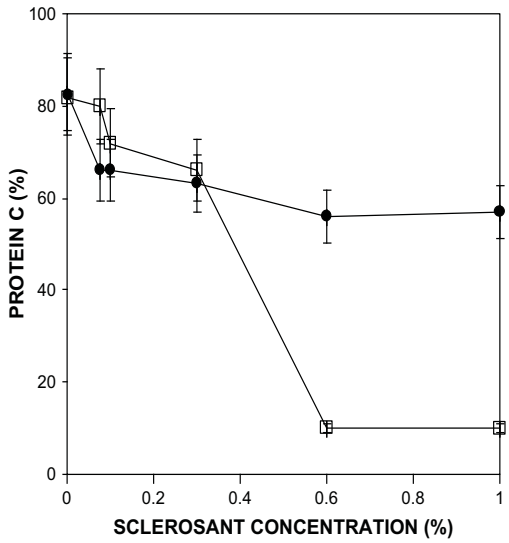

Figure 1 Effect of detergent sclerosants on protein C (PC). STS at concentrations above 0.6% significantly destroyed PC whereas POL only caused a 20% reduction in plasma levels ($n = 2$; □, STS; •, POL).

Effect on protein S

STS at concentrations of 0.6% and above completely destroyed free PS whereas POL reduced PS levels by 60% (Fig. 2).

Effects on AT

STS significantly destroyed AT at concentrations above 0.3% whereas high concentration POL only reduced AT levels

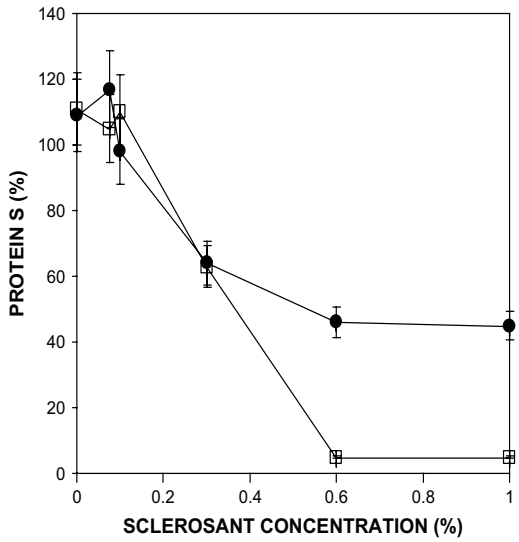

Figure 2 Effect of detergent sclerosants on protein S. STS at concentrations above 0.6% completely destroyed PS whereas POL reduced PS levels by 60%. ($n = 2$; □, STS; •, POL).

down to 60%. AT levels appeared to rise at STS concentrations above 0.6% (Fig. 3). To investigate this apparent rise, samples containing 1.5% STS and BSA or hydrolysed gelatine (but no plasma) were tested in this system. Sample containing BSA displayed no AT activity due to neutralisation of STS by BSA. Samples containing hydrolysed gelatine and STS produced further AT activity indicating this activity to be due to the direct effect of STS on thrombin.

Effects on APC and APCR

STS prolonged APTT potentiating the anticoagulant activity of APC, whereas POL had the opposite effect (Fig. 4a,b). POL at concentrations of 0.1% and above increased APCR (i.e. reduced APCR ratio) whereas STS reduced APCR (i.e. increased APCR ratio) (Fig. 5).

Comparison with anticoagulant activity of heparin

Both STS and heparin prolonged APTT however heparin gave a more linear response plot and was 1000× more potent than STS. POL had much less effect on APTT (Fig. 6).

Interaction with heparin

STS prolonged APTT and enhanced the anticoagulant effect of heparin whereas POL had less effect (results not shown).

Effects on factor Xa activity, heparin and enoxaparin

In the absence of STS, heparin and enoxaparin significantly reduced the enzymatic activity of FXa (results not shown). Low concentrations of STS slightly increased the apparent enzymatic activity of FXa. However, STS at concentrations above 0.1% rapidly decreased FXa activity to low levels similar to those achieved by heparin. High concentration STS paradoxically demonstrated an increase in optical density in this system when measuring FXa chromogenic activity. This is due to precipitation of apolipoprotein B and STS complexes which generates turbidity.[4] STS destroyed AT at concentrations above 0.3% hence the lack of difference between samples with and without heparin or enoxaparin.

Increasing levels of POL reduced the activity of FXa slightly in normal plasma and plasmas containing heparin or enoxaparin (results not shown). However the difference between the normal plasma and the heparin-containing plasmas was maintained even at high POL levels indicating no interference by POL in heparin-mediated inhibition of FXa.

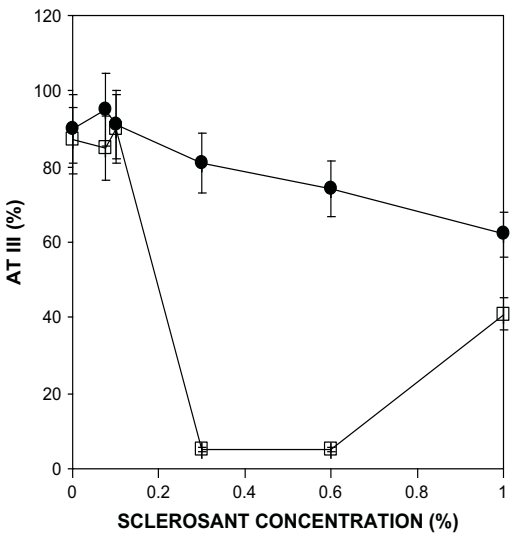

Figure 3 Effect of detergent sclerosants on antithrombin (AT). STS at concentrations above 0.3% destroyed AT but the AT activity returned at concentrations above 0.6%. This is due to the direct effect of STS on thrombin mimicking the action of AT. POL caused a 20% reduction in AT activity ($n = 3$; □, STS; •, POL).

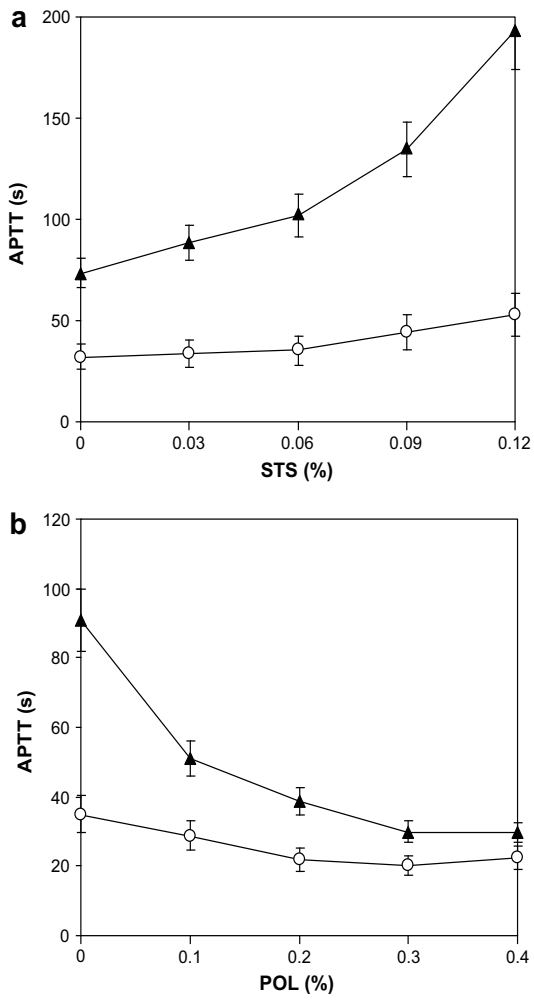

Figure 4 Interaction of detergent sclerosants with APC. STS potentiated the anticoagulant activity of APC whereas POL reduced this activity. (a) STS; (b) POL ($n = 4$; ▲, APC and normal plasma; ○, normal plasma).

Discussion

Antithrombotic mechanisms including the protein C anticoagulant pathway, thrombin—antithrombin complex and

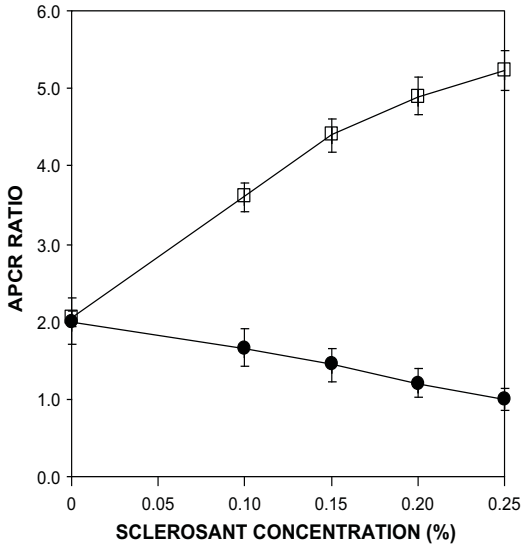

Figure 5 Effect of detergent sclerosants on APC resistance derived from dRVVT results. POL decreases the ratio, hence increasing APC resistance while STS increased the ratio. Ratios equal or less than 1.6 were defined as abnormal ($n = 2$; □, STS; •, POL).

endothelial surface membrane components such as heparinoids operate to maintain the patency of blood vessels and the fluidity of the circulating blood. The ultimate aim of sclerotherapy is to cause sclerosis of incompetent superficial veins and perforators while having a negligible effect on the underlying deep veins. Hence the ideal sclerosant should have minimal disruptive effects on the antithrombotic mechanisms that maintain the patency of deep veins. The aim of this study was to investigate the *in vitro* effects of the commonly used detergent sclerosants, STS and POL, on plasma coagulation inhibitors PC, PS and AT. We also examined the interaction of these drugs with therapeutic anticoagulants heparin and enoxaparin. Clinicians should note that the sclerosant concentrations reported here are the final active concentrations. Once injected, the sclerosant undergoes dilution and neutralisation by blood components and the final intravascular concentration will not necessarily be the same as the initial concentration.[5]

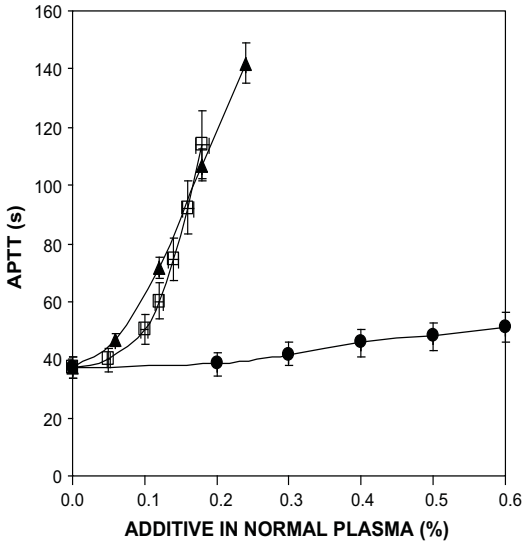

Figure 6 Comparison of anticoagulant activity of detergent sclerosants with that of heparin showing STS to have a similar anticoagulant profile but 1000 times weaker. One milligram of heparin is roughly equivalent to 100 IU ($n = 4$; ▲, Hep × 1000; □, STS; •, POL).

In this study, STS destroyed most antithrombotic proteins at high concentrations while POL had less destructive effects (Table 1, Fig. 7a,b). This is possibly due to denaturation and unfolding of the protein molecules as expected of potent detergents such as STS.[9] STS at concentrations higher than 0.6% significantly destroyed PC whereas POL caused a 20% reduction. Both agents reduced PS levels quite significantly. Only 40% of PS circulating in plasma is free and the rest is bound to C4b binding protein (C4BP) with no APC cofactor activity.[6,10] High concentration POL caused a 60% reduction in free PS levels while STS completely destroyed this protein. PS has lipid and steroid binding domains and enhances the binding of APC to phospholipid membranes.[11–13] The destructive effect of detergent sclerosants on PS could be due to their interaction with the lipid binding domain of PS, release of proteases from activated platelets[14] or direct denaturation of the protein molecule.

Sclerotherapy with both STS and POL is associated with necrosis presenting with a stellate or reticulate pattern.[15] Venoarteriolar reflex (VAR) vasospasm of accompanying arterioles,[16] and entry of sludge[5] or direct entry of sclerosants from the injected veins into these arterial vessels via open AV shunts,[17,18] have been proposed as mechanisms underlying this complication. VAR sympathetic reflex due to rapid dilation of the target vein can lead to vasospasm of the associated arterioles. High pressure, high volume and rapid injection of the sclerosant can trigger this sympathetic reflex. VAR vasospasm followed by a local deficiency of proteins C and S induced by both STS and POL may contribute to the thrombotic occlusion of these arterial vessels leading to skin and tissue necrosis. Two other conditions with a similar pattern of ulceration, warfarin necrosis and calciphylaxis, also present with underlying PC or S deficiency.[19,20]

In this study, STS destroyed AT almost completely at concentrations above 0.3% but the apparent activity of AT was increased by STS concentrations above 0.6%. We postulated this to be due to the inhibitory effect of STS on thrombin as seen by prolongation of thrombin time.[4] The apparent rise in AT activity at higher STS levels was completely reversed by adding albumin (which binds STS) but not by hydrolysed gelatine (which does not bind STS). Hence, high concentration STS mimicked the antithrombotic activity of AT by direct inhibition of thrombin. POL only caused a mild reduction in AT levels.

STS demonstrated antithrombotic activity by significantly increasing the sensitivity of FVa to the proteolytic effect of APC. High concentration STS induces a true deficiency of FV, which is a precursor to FVa, the main target of APC.[4] Also rapid inactivation of FVa by APC requires

Table 1 Effect of STS and POL on clotting factors, clotting tests and antithrombotic mechanisms derived from our present and previous studies

Effect	STS	POL
Clotting factors[4]	Destroys factor V and VII. Reduces factor X	Increases the apparent activity of factors VIII, IX, XI and XII
Clotting tests[4]	Prolongation of PT, APTT, TT, XACT, NAPTT and SACT in PRP at >0.3% Shortens XACT, SACT, NAPTT in PRP at 0.1—0.3%	No significant prolongation. Mild prolongation of PT at 0.8% Shortens XACT, SACT, NAPTT in PRP at 0.2—0.4%
Protein C	Destroyed at >0.6%	20% Reduction at >0.6%
Protein S	Destroyed at >0.6%	60% Reduction at >0.6%
AT activity	Reduced at 0.3—0.6%. Increased at >0.6% (direct anti-IIa activity)	20% Reduction at >0.6%
APC anticoagulant activity	Increased	Reduced
APC resistance	Reduced	Increased
Comparison with heparin	Similar anticoagulant profile, 1000× weaker	No similarity
Potentiation of heparin anticoagulant activity	Moderately potentiated	Not significant
Factor Xa activity	Significantly reduced	Minimally reduced

PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; XACT, factor Xa clotting time; NAPTT, non-activated partial thromboplastin time; SACT, surface activated clotting time; PRP, platelet rich plasma; AT, antithrombin; APC, activated protein C.

negatively charged phospholipid membrane surfaces containing phosphatidyl serine.[21] This phospholipid surface is normally provided by activated platelets, platelet derived microparticles (PDMP) and damaged endothelial cells.[22] Low concentration STS activates platelets and releases PDMP while higher concentrations would destroy platelets, PDMP, endothelial cells and other cell membranes providing the required phospholipid surfaces.[4,5]

POL by contrast demonstrated prothrombotic activity by increasing APC resistance, as evident by both APTT and dRVVT assays. This effect was concentration-dependent and higher concentrations of POL showed further inhibition of APC activity. POL, a non-ionic detergent, possibly binds to the phospholipid binding site of FVa which would reduce the sensitivity of FVa to APC.

We compared the anticoagulant effect of the detergent sclerosants with that of heparin. Similar to heparin, STS inhibited both thrombin and FXa while POL did not demonstrate a significant anticoagulant profile. STS was about 1000× weaker than heparin on a direct concentration basis but produced a similar dose response curve to heparin although less linear. Although heparin and STS appear to have similar anticoagulant characteristics, they achieve this activity through different mechanisms. Heparin requires AT to function whereas the anticoagulant activity of STS is due to direct destruction of clotting factors, platelets and PDMP and is independent of AT. In a later experiment, STS potentiated the anticoagulant effect of heparin whereas POL had a mild effect. STS works synergistically with heparin due to its direct inhibition of thrombin and FXa.

We have previously shown a reduction in FX levels by STS.[4] Here we investigated the effect of STS on FXa, the active form of FX. STS reduced FXa activity consistent with the anticoagulant effect of STS on clotting tests.[4] By contrast, POL only slightly reduced FXa activity. FXa usually occurs as part of the prothrombinase complex with FVa. This complex assembles on negatively charged phospholipid membranes in the presence of calcium ions and catalyses the conversion of prothrombin to thrombin. Based on our present and previous studies, STS interferes with thrombin generation by its direct inhibition of FXa and FVa.[4] The anti-Xa activity of STS is not due to potentiation of AT as in this study STS at high concentrations destroyed AT.

Our present finding that high concentration STS destroyed PC, PS and AT may appear contradictory to our previous reports of the anticoagulant activity of this agent in the same concentration range.[4] In this study, STS in the active concentration range of 0.1—0.3% showed very little destructive effect on natural antithrombotic proteins but these low concentrations activate the coagulation cascade as previously shown by the authors.[4] By contrast, if the final active concentration of STS, after mixing with blood and neutralisation, stays at 0.6% or higher, the agent will effectively destroy key clotting factors, platelets and PDMP[4] and, as demonstrated in this study, most natural antithrombotic proteins. Although these pro- and antithrombotic effects may appear to neutralise each other, high concentration STS in this study achieved a net anticoagulant profile, independent of natural antithrombotic pathways, as evident by its anti-Xa, anti-IIa, and anti-Va activity. These findings further confirm our previous report of significant prolongation of all clotting times by high concentration STS.[4]

Similarly, POL in the active concentration range of 0.2—0.4% demonstrates procoagulant activity as evident by platelet activation, release of PDMP and shortening of clotting times.[4] High concentration POL (>0.6%) does not significantly prolong most clotting times and in this study even demonstrated some prothrombotic activity by

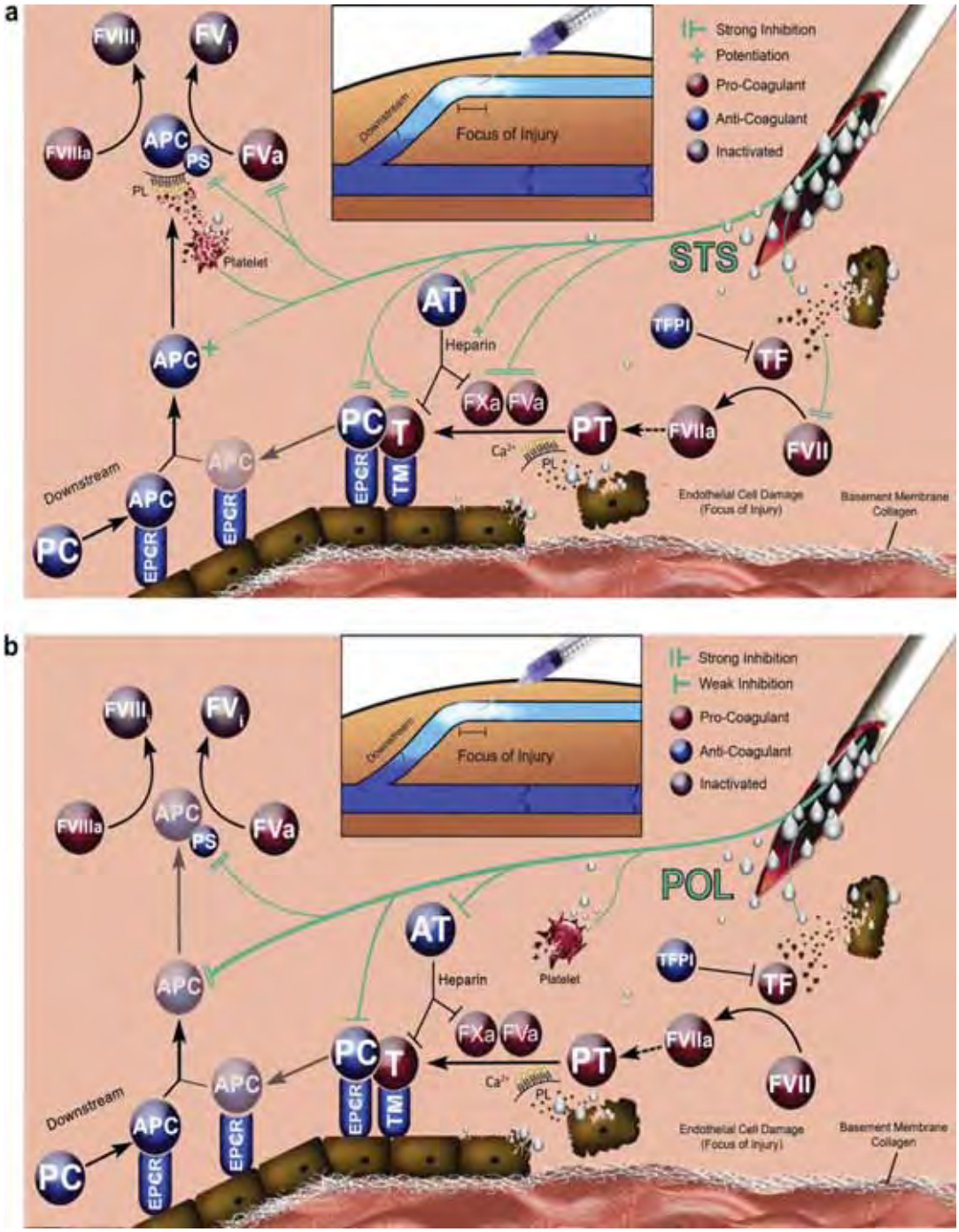

Figure 7 Cartoons demonstrating the effect of high concentration STS (a) and POL (b) on clotting factors and antithrombotic mechanisms. The effect demonstrated is at the point of entry of the sclerosant into the blood vessel and the immediate adjacent area. High concentration STS (a) destroys platelets and the key clotting factors and hence aborts the coagulation cascade. Thrombin normally activates the antithrombotic pathway, but by destroying thrombin, PC, PS and AT, high concentration STS also effectively interrupts the natural antithrombotic pathways. High concentration STS demonstrates potent anti-Xa, anti-IIa and anti-Va activity, potentiating the anticoagulant effect of APC. POL (b) has a less disruptive effect on natural antithrombotic proteins but increases APC resistance (TF, tissue factor; TFPI, tissue factor pathway inhibitor; PL, phospholipid; FVII, factor VII; PT, prothrombin; FXa, factor Xa; T, thrombin; TM, thrombomodulin; PC, protein C; EPCR, endothelial protein C receptor; APC, activated protein C; PS, protein S; FVa, activated factor V; FVi, inactivated factor V; FVIII, factor VIII; FVIIIi, inactivated factor VIII).

increasing APC resistance, moderate reductions in PS levels and mild reductions in PC and AT.

The clinical relevance of these findings requires further investigation in the context of the sclerosant's concentration and its site of action. PC, PS, AT and most other antithrombotic proteins are dependent on the integrity of the endothelial lining away from the primary focus of injury (Fig. 7a,b). Activation of PC by thrombin—thrombomodulin complex and the binding of PC to the endothelial cell PC receptor (EPCR) require an intact endothelial cell membrane.[23,24] The final effect of the sclerosant on the target vessel and its antithrombotic mechanisms will depend on the degree of damage to the endothelium, the subsequent vasospasm and vessel wall approximation and the final active intravascular concentration of the sclerosant. Clinically, sclerosants are observed to enter the deep veins via perforators or junctions. For high concentration STS to exert an antithrombotic effect on the deep veins, it will have to avoid sclerosing the exposed segment which means avoiding contact with the vascular wall and its endothelial lining. This is unlikely, however, as it requires small volumes of the high concentration liquid agent to enter the deep veins via laminar flow. Immediate vessel wall approximation due to vasospasm, turbulent flow due to fast delivery, rapid entry of large volumes of the liquid agent as well as the use of the foam format of the sclerosant will increase the exposure of the detergent to the vascular wall causing endothelial cell lysis which may lead to sclerotic occlusion of the exposed segment of the deep vein. Consistently, deep vein occlusion has been reported to be more common in association with foam sclerosants than liquid especially when high volumes of foam have been used.[25] Therefore, the antithrombotic activity of high concentration STS may not be clinically useful as the same high concentrations, given adequate vessel wall approximation, would cause partial or full sclerosis of the exposed vein segment.

This study had a number of limitations. Firstly, due to its *in vitro* nature, its clinical relevance needs to be established by *in vivo* studies. Although STS demonstrated more anticoagulant properties *in vitro*, it does not necessarily make this drug safer than POL and the comparative incidence of post-treatment DVT needs to be derived from clinical studies. Also, although we studied a number of key antithrombotic proteins, further work is required to investigate the interaction of these agents with other members of the antithrombotic pathway including tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), EPCR, prostacyclin and heparinoids. Finally, due to technical reasons, we only investigated the liquid sclerosants in this study. The interaction of foam sclerosants with the antithrombotic system is the subject of the authors' *in vivo* studies.

In summary, STS and POL demonstrated quite distinct and sometimes opposite effects on the antithrombotic mechanisms *in vitro*. High concentration STS demonstrated significant anti-Xa, anti-IIa and anti-Va activity but also had the greatest destructive effect on antithrombotic proteins. The clinical relevance of these findings is the subject of further investigations by the authors.

Conflict of Interest

None.

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Graphical art by David Du, Research Scientist, Phlebology Research Lab.

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CHAPTER 5

In vitro Effects of Detergent Sclerosants on Fibrinolytic Enzymes and Inhibitors

Parsi K, Exner T, Ma DDF, Joseph JE.

Thrombosis Research 2010 ;126:328-36.

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I was the first author on this paper which was published in Thrombosis Research in 2010. I was the major (more than 50%) contributor to this study. The data was analysed by myself, presented to the team and discussed in regular meetings. I wrote the manuscript and was responsible for the submission process and dealing with the reviewers comments. Dr Tom Exner performed the experiments on plasminogen. Other co-authors contributed to discussion and the manuscript. No other authors were students. (PUBLISHED, IF=2.449)

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Regular Article

In vitro effects of detergent sclerosants on fibrinolytic enzymes and inhibitors

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ABSTRACT

Objective: To investigate the effects of Sodium Tetradecyl Sulphate (STS) and Polidocanol (POL) on fibrinolytic mechanisms.
Materials and methods: Measurements were done with serial dilutions of sclerosants in whole blood (WB), platelet rich (PRP) and platelet poor plasma (PPP). Control experiments were done in 5% bovine serum albumin (BSA), spiked with the enzyme/inhibitor. Plasminogen was measured with a chromogenic assay. Alpha-2-antiplasmin (AP) activity, plasmin-alpha-2-antiplasmin (PAP) complexes, plasminogen activator inhibitor-1 (PAI-1) activity, tissue plasminogen activator (t-PA) total antigen, t-PA activity, t-PA/PAI-1 complexes, thrombin activatable fibrinolysis inhibitor (TAFI) antigen and activated TAFI (TAFIa) were measured by ELISA.
Results: At high concentrations (>0.3%), STS destroyed plasminogen, PAI-1, t-PA/PAI-1 complexes and total t-PA antigen but increased t-PA activity. At low concentrations (<0.3%), both agents reduced PAP complexes while increasing AP activity. Low concentration STS increased PAI-1 activity, t-PA/PAI-1 complexes, TAFI and TAFIa. Low concentration POL mildly increased the total t-PA antigen and TAFI.
Conclusion: At low concentrations, both agents demonstrated a prothrombotic, antifibrinolytic (increase in PAI-1, total t-PA antigen, AP, TAFI and TAFIa) activity. At high concentrations, STS demonstrated non-prothrombotic (destruction of PAI-1, t-PA/PAI-1 complexes), antifibrinolytic (destruction of plasminogen, increase in AP) activity while POL had minimal effect.

Introduction

Detergent sclerosants, sodium tetradecyl sulphate (STS) and polidocanol (POL), are the most commonly used agents to treat varicose veins and venous malformations. Serious adverse events including deep vein thrombosis, pulmonary embolism and rare cases of strokes have been reported following sclerotherapy [1,2]. As previously demonstrated by this group, these agents interfere with blood cells, clotting factors and antithrombotic mechanisms [3–5]. High concentration STS prolongs most clotting tests and demonstrates significant anti-Xa, anti-IIa and anti-Va activity but also has the greatest destructive effect on antithrombotic proteins [4]. Both STS and POL at therapeutic concentrations have lytic effects on red cells, platelets, and endothelial cells [5] and at low concentrations release procoagulant platelet-derived microparticles (PMP) [3,4]. Both agents cause significant haemolysis at high concentrations (STS>0.3%, POL>0.6%). Albumin and other plasma components neutralise the lytic effects of sclerosants in an *in vitro* setting with STS being 50 fold and POL 163 fold less haemolytic in blood than in saline [5].

Occlusion of target vessels is the ultimate aim of sclerotherapy and recanalisation constitutes treatment failure. Conversely, occlusion of deep veins exposed to the sclerosants is an unwanted complication of sclerotherapy and their recanalisation and restored competence are the desired outcomes. Fibrinolytic mechanisms are responsible for recanalisation of thrombosed vessels and restoration of blood flow through the occluded segments. The interaction of sclerosants with the fibrinolytic system may be crucial in determining the ultimate fate of the intentionally treated or unintentionally occluded vessels and may play a role in the pathogenesis of serious thromboembolic adverse events.

The fibrinolytic system has been recently reviewed (Fig. 1) [6]. The key fibrinolytic enzyme, plasmin, is a serine protease that degrades fibrin and also activates matrix metalloproteinases (MMPs) and in particular collagenases responsible for degradation of collagen and the extra-cellular matrix (ECM). Fibrin deposition occurs in thrombosed vessels while deposition of collagen and ECM results in endovascular fibrosis, as expected with successful sclerotherapy. Hence plasmin possibly plays a role in post-sclerotherapy re-canalisation of treated or unintentionally occluded vessels.

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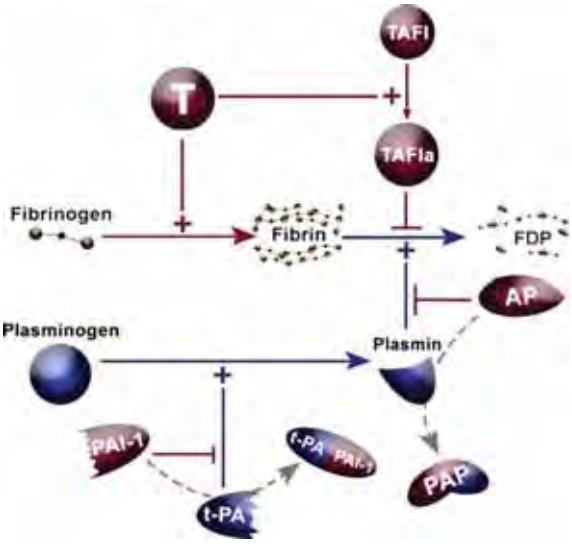


Fig. 1. Fibrinolysis results in the generation of plasmin which degrades fibrin into fibrin degradation products (FDP). Activation (blue) and inhibition (red) of fibrinolysis occurs at multiple levels. AP, alpha-2 antiplasmin; PAI-1, plasminogen activator inhibitor-1; PAP, plasmin-antiplasmin complex; T, thrombin; TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa, activated TAFI; t-PA, tissue plasminogen activator.

The main inhibitor of plasmin is alpha-2-antiplasmin (AP), a serine protease inhibitor (SERPIN) that binds the lysine binding sites on plasminogen thus competitively inhibiting the binding of plasminogen to fibrin. In blood, AP protects the plasma proteins from the proteolytic activity of plasmin by forming a stable plasmin-antiplasmin (PAP) complex. In clots, AP is cross-linked to fibrin by activated factor XIII which provides an additional measure of protection to the fibrin clot from proteolysis by plasmin.

Plasmin is generated from plasminogen by the action of tissue-type plasminogen activator (t-PA) in blood and urokinase (u-PA) in tissue. Clotting and fibrinolysis are initiated simultaneously [7] and plasma t-PA is synthesized and released by endothelial cells when stimulated by clotting factors thrombin and activated factor X [8]. Only 5% of plasma t-PA is free and active and the remaining 95% is rapidly captured by inhibitors and loses activity (Fig. 2) [9].

Inhibitors of t-PA include plasminogen activator inhibitor-1 (PAI-1), AP and C-1 inhibitor. PAI-1 is expressed by activated platelets, [10] platelet derived microparticles (PMP), [11] and other sources [12]. Endothelial cells secrete PAI-1 *in vivo* only during the acute-phase response [13,14]. 95% of the circulating PAI-1 is stored in platelets and only 5% is found in plasma. Platelets retain mRNA and can synthesize PAI-1 in its latent (inactive) form [10,15]. Following platelet activation, the latent PAI-1 undergoes a conformational change and is released into the circulation as active PAI-1. In plasma, 60% of PAI-1 remains active while the remaining 40% is inactivated after binding to t-PA [16] or reversion to its latent form [17]. The binding results in the formation of stoichiometric 1:1 t-PA/PAI-1 complexes (Fig. 2) [18,19].

Thrombin activatable fibrinolysis inhibitor (TAFI) acts as a link between the coagulation and fibrinolytic systems [20]. This zymogen is activated by thrombin, thrombin-thrombomodulin complexes and plasmin. Thrombin alone is a weak activator of TAFI, but in complex with thrombomodulin, has a 1250-fold higher catalytic activity [20]. Activated TAFI (TAFIa) cleaves the C-terminal lysine and arginine residues from partially degraded fibrin. This results in a decrease in the binding of plasminogen to fibrin and its conversion to plasmin. TAFIa is a heat labile protein and is converted into an inactivated form (TAFIai) by conformational change. Excess of TAFIa has been associated with an increased risk of thrombosis [21].

In this study, we investigated the interaction of detergent sclerosants, STS and POL, with key fibrinolytic enzymes and inhibitors *in vitro*.

Materials and Methods

Sclerosants and Reagents

STS (FIBRO-VEIN 3%) was obtained from Australian Medical and Scientific Limited, Chatswood, NSW; POL (AETHOXYSKLEROL 3%) from Chemische Fabrik Kreussler & Co, GMBH, Wiesbaden, Germany and Streptokinase (STEPTASE) from Calbiochem Behring, Kingsgrove, NSW. Bovine serum albumin (BSA) was obtained from Bovogen, Melbourne, Victoria and 5% BSA was prepared by dilution in water. Normal plasma (NP) was obtained as fresh frozen plasma from Hyphen Biomed, Neuville-sur-Oise, France.

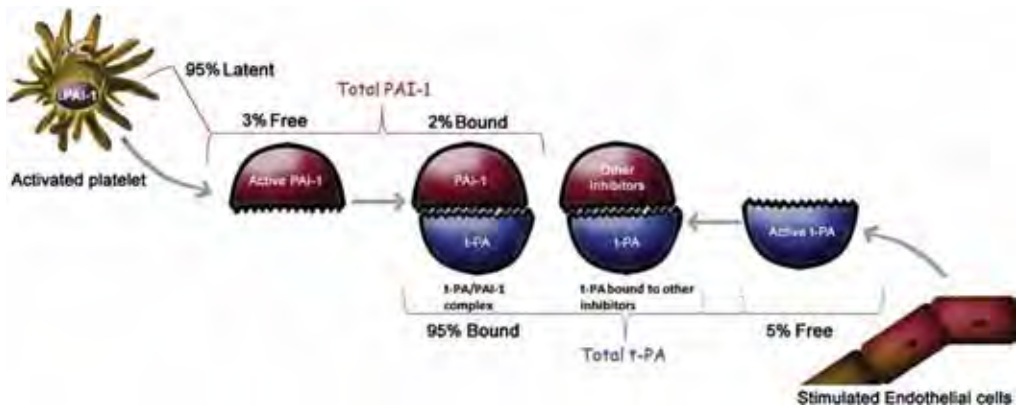


Fig. 2. Distribution of active and bound t-PA and PAI-1. 95% of plasma PAI-1 is found within platelets in a latent form, 3% is found free in plasma as active PAI-1 and the remaining 2% binds t-PA to form t-PA/PAI-1 complexes. t-PA is released by stimulated endothelial cells. 5% of plasma t-PA is active while the remaining 95% is bound to inhibitors including PAI-1, alpha-2 antiplasmin, and C-1 inhibitor.

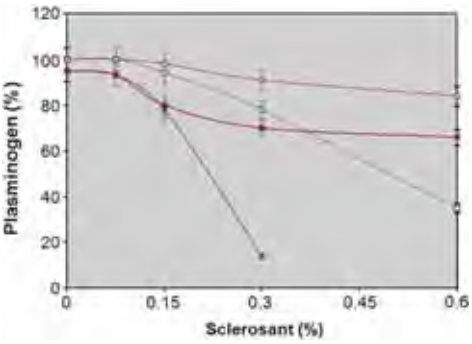


Fig. 3. Residual plasminogen plotted against sclerosant added to normal plasma initially and after 90 minutes at 37 °C. (n = 2, □ STS t = 0 min, ■ STS t = 90 min; ○ POL t = 0 min; ● POL t = 90 min).

Sample Collection

Having obtained informed consent, fresh donor whole blood (WB) was collected in citrate tubes (BD Vacutainer, 0.105 M) from healthy volunteers taking no medications or supplements.

Sample Preparation

Platelet Rich Plasma (PRP) was generated by centrifugation of WB for 10 min at 150 g. Platelet Poor Plasma (PPP) was generated by centrifugation of WB for 30 min at 1700 g.

ELISA Assays

WB, PRP and PPP samples containing serial dilutions of sclerosants (1.2%, 0.6%, 0.3%, 0.15%, 0.075%, 0%) were tested in ELISA assays that utilise horseradish peroxidase (HRP) conjugated secondary antibodies to detect antigens on proteins or complexes bound to primary

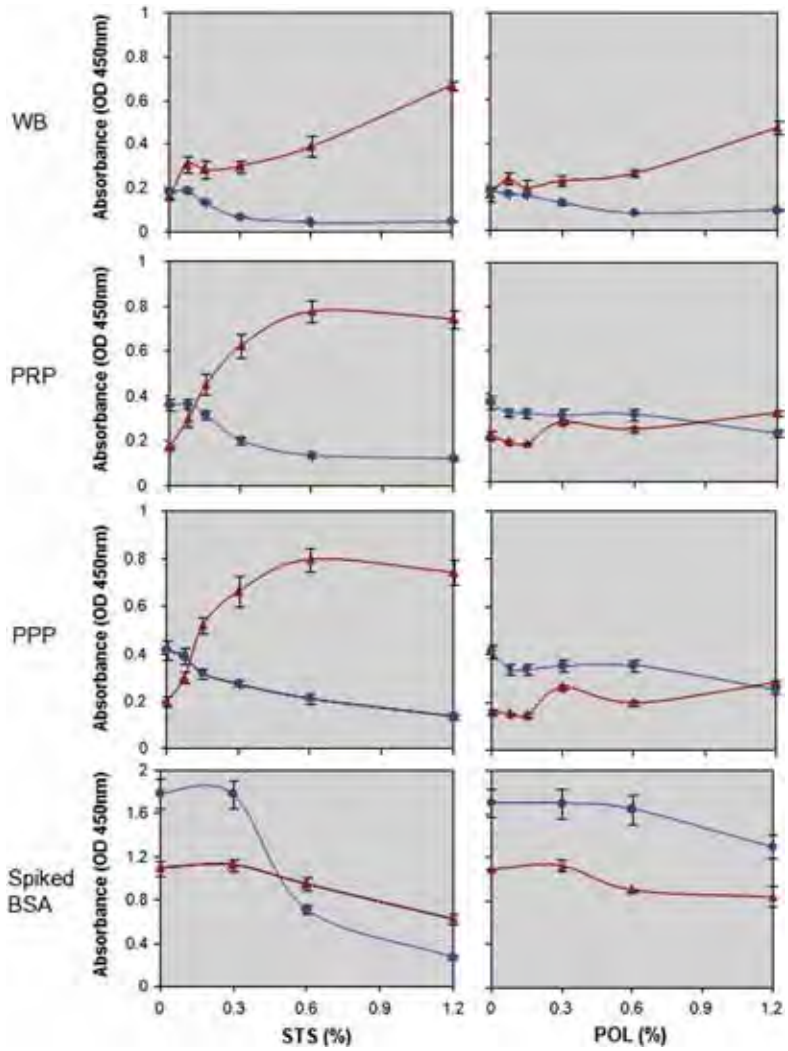


Fig. 4. Effect of sclerosants on AP (n = 2, —▲—) and PAP complexes (n = 2, —●—) in whole blood (WB), platelet rich plasma (PRP), platelet poor plasma (PPP) and bovine serum albumin (BSA) spiked with AP or PAP complexes.

antibodies on the wells. Tetramethylbenzidine (TMB) chromogenic substrate for HRP was used to generate a colour that was then detected by a plate reader (RT-6100, RAYTO Life and Analytical Sciences, China) at 450 nm. All ELISA plates used in these experiments were coated by passive adsorption (personal communication with the manufacturers).

Control experiments were performed to exclude interference of sclerosants with ELISA assays. Serial dilutions of sclerosants were prepared in 5% BSA spiked with enzyme/inhibitor (10 ng of standard human AP plasma; 5 ng of PAP complexes; 10 ng of total t-PA, t-PA/PAI-1 complexes, PAI-1 or active t-PA; and 6.5 ng of standard human TAFI plasma or TAFIa/ai) and tested in each assay.

Further control experiments were performed to investigate the capacity of STS to destroy the solid phase of ELISA assays especially at high concentrations. We identified three ELISA assays where high concentration STS caused a significant reduction in the target protein in all media tested. These included assays for total t-PA antigen, PAI-1 activity and t-PA/PAI-1 complexes, all coated by passive adsorption. WB, PRP, PPP, 5% BSA and normal saline (NS) samples were spiked with serial dilutions of STS (1.2%, 0.6%, 0.3%, 0.15%, 0.075%, 0%) and incubated in all three ELISA assays at room temperature for one hour. After five washes, 200 μl of the supplied calibrator of each kit at the concentration provided (3.4 ng/mL, total t-PA; 2.5 ng/mL, PAI-1 activity and 2.5 ng/mL, t-PA/PAI-1 complexes) was added to all wells and incubated for one hour. The normal protocol for each ELISA assay per kit was then followed. Any interference by STS would affect the concentration of the calibrator in the affected wells.

All assays were done in duplicates and data presented are the average of at least two determinations.

Effect on Plasminogen

Functional plasminogen levels were determined using a chromogenic method [22]. NP containing serial dilutions of sclerosants was pre-incubated briefly with streptokinase (10,000 U/ml). The

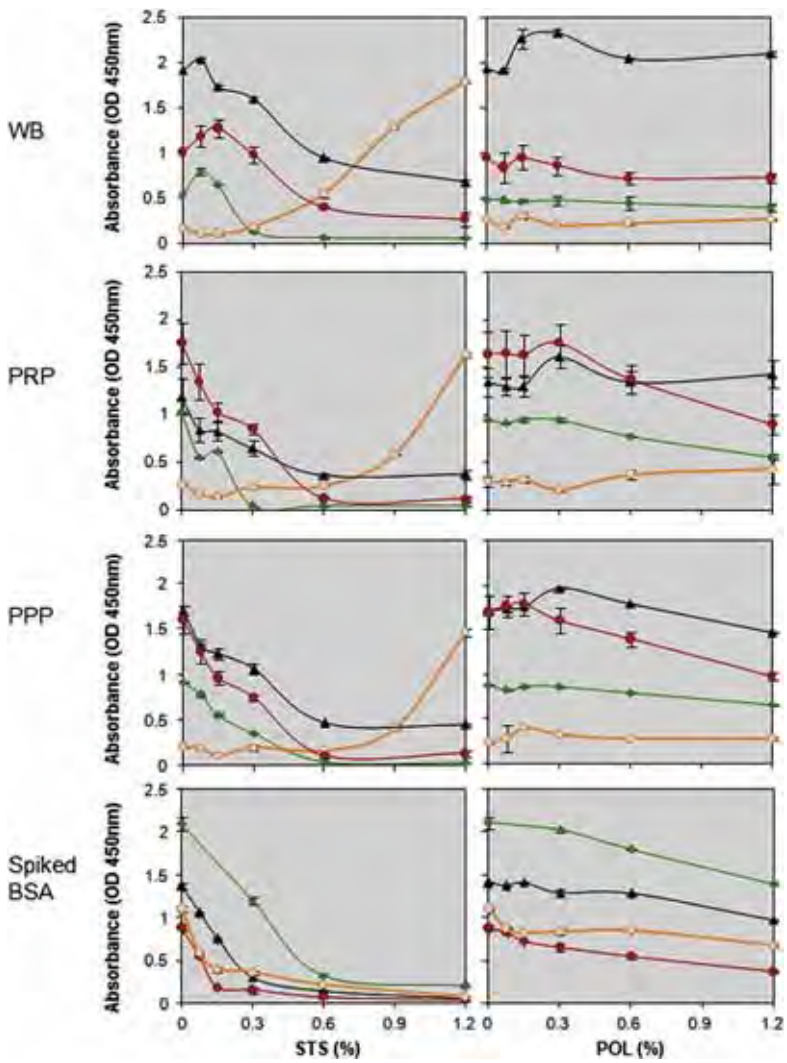


Fig. 5. Effect of sclerosants on total t-PA (n = 8, —▲—), active PAI 1 (n = 8, —●—), t-PA/PAI-1 complexes (n = 2, —◆—), and active t-PA (n = 2, —○—) in whole blood (WB), platelet rich plasma (PRP), platelet poor plasma (PPP) and bovine serum albumin (BSA) spiked with the enzyme/inibitor or complexes.

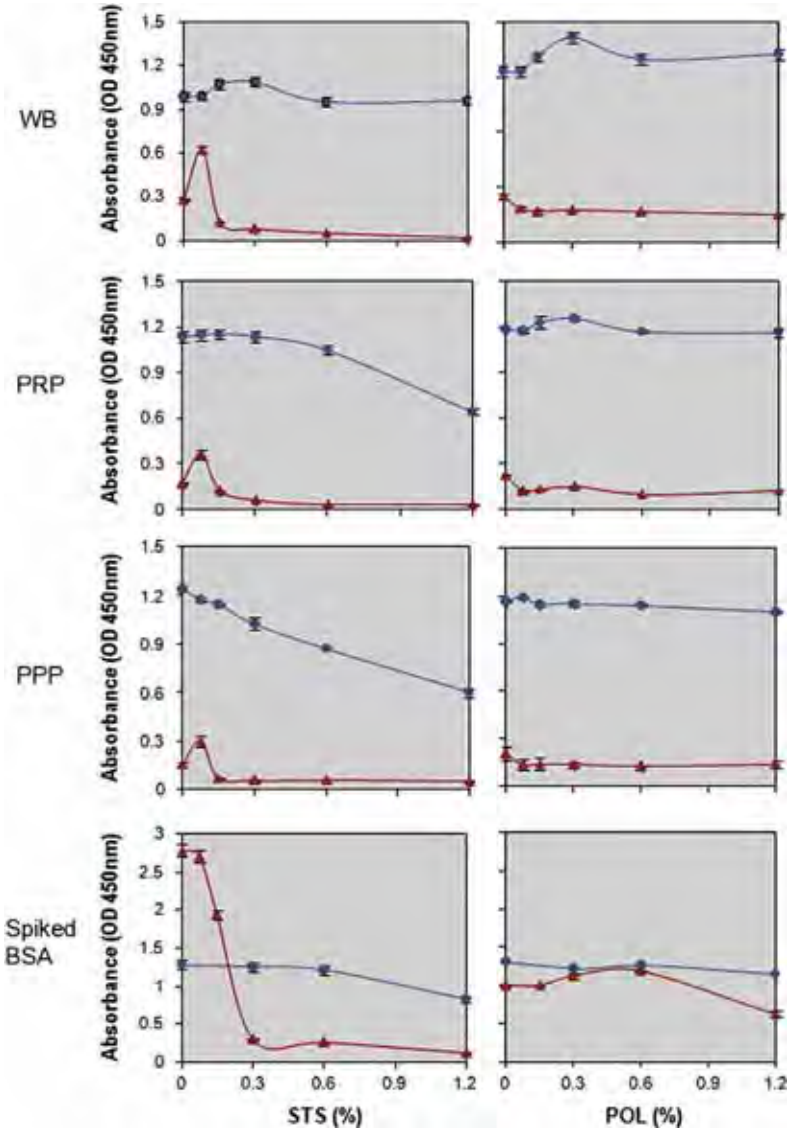


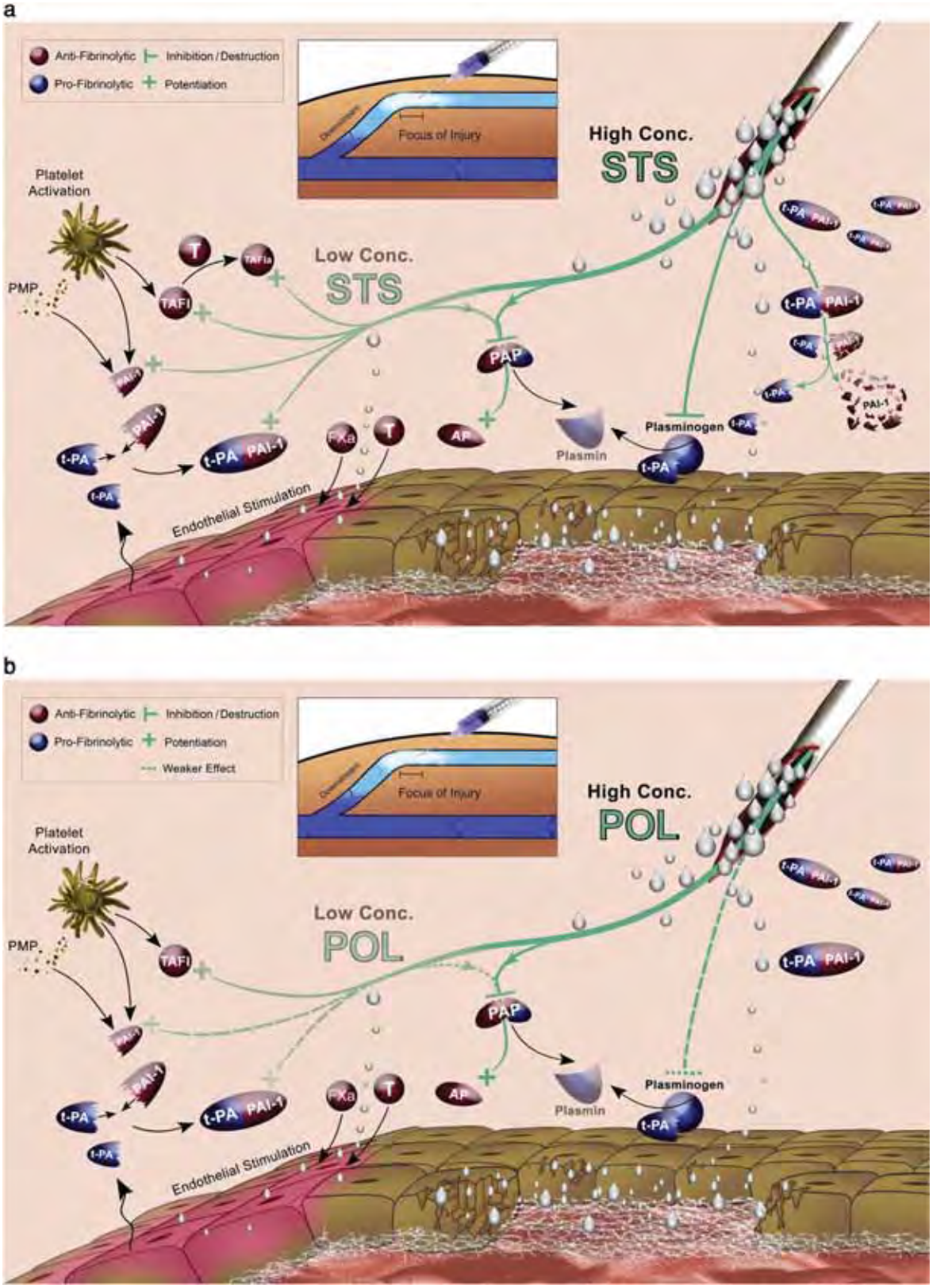
Fig. 6. Effect of sclerosants on TAFI (n = 2, —●—) and TAFIa/ai (n = 2, —▲—) in whole blood (WB), platelet rich plasma (PRP), platelet poor plasma (PPP) and bovine serum albumin (BSA) spiked with TAFI or TAFIa/ai.

chromogenic substrate for plasmin (Chromozym PL, Boehringer Mannheim, Germany) was added and absorbances were read at 414 nm. Plasminogen levels were derived from the chromogenic activity measurement of plasmin. Calibration curves were set up using varying dilutions of NP from 1/10 through 7 serial dilutions in microwells. The calibration curve obtained at 15 minutes was used to interpolate absorbance results. Plasminogen results were obtained initially after mixing (t = 0) and after 90 minutes (t = 90) at 37 °C.

Effect on AP activity and PAP Complexes

AP activity was detected by ELISA (IHA2APKT, Innovative Research, Mi, USA). Active AP in the sample binds to the plasmin

Fig. 7. Cartoons demonstrating the observed effects of STS (a) and POL (b) on fibrinolytic enzymes and inhibitors. The effect demonstrated is at the point of entry of sclerosants into the target vessel where the sclerosant concentration is at its highest and downstream where the concentration drops. High concentration STS appeared to dissociate t-PA/PAI-1 complexes, destroyed PAI-1 and released active t-PA. It also destroyed plasminogen, the main substrate for t-PA but increased alpha-2 antiplasmin (AP), demonstrating an overall pro-occlusive activity which was non-prothrombotic. High concentration POL demonstrated a limited antifibrinolytic activity by increasing AP levels. Both agents at low concentrations increased active PAI-1 possibly due to activation of platelets and release of platelet derived microparticles (PMP). They also elevated t-PA/PAI-1 complexes, AP and thrombin activatable fibrinolysis inhibitor (TAFI). Low concentration STS also caused an increase in activated TAFI (TAFIa) possibly due to generation of Thrombin (T). Overall, low concentration sclerosants exhibited a prothrombotic antifibrinolytic effect.



coated plate and is then detected by anti-AP antibodies. PAP Complexes were detected by ELISA (EIA3763, DRG Diagnostics, Marburg, Germany). Monoclonal antibodies (PAP-6) coating the plate bind with PAP complexes in the sample which are then recognised by HRP-conjugated anti-plasminogen antibodies.

Effect on PAI-1 activity, t-pA/PAI-1 complexes, total t-PA antigen, and t-PA activity

PAI-1 activity ELISA (RK019A, Hyphen Biomed) utilises a t-PA coated plate which captures free PAI-1 in the sample. Captured PAI-1 is then recognised by HRP-conjugated anti-PAI-1 antibodies.

t-PA/PAI-1 complexes ELISA (RK017A, Hyphen Biomed) uses a monoclonal anti-t-PA antibody coated plate which captures t-PA/PAI-1 complexes through the t-PA moiety. HRP-conjugated anti-PAI-1 monoclonal antibodies then detect the PAI-1 moiety in t-PA/PAI-1 complexes.

Total t-PA antigen ELISA (RK011A, Hyphen Biomed) uses a monoclonal anti-t-PA antibody coated plate which captures any form of t-PA. HRP-conjugated anti-t-PA antibodies then bind to a different epitope on the t-PA molecule. This method recognizes both free and bound forms of t-PA antigen.

t-PA activity ELISA (HTPAKT, Molecular Innovations, Mi, USA) utilises biotinylated human PAI-1 bound to avidin on the microwells to form complexes with active t-PA in the sample. Polyclonal anti-t-PA antibodies are then used to bind to the captured t-PA. HRP-conjugated secondary antibodies would then detect the primary antibodies. This method recognizes active t-PA only.

Effect on TAFI and TAFIa/ai

TAFI ELISA (RK008A, Hyphen Biomed) utilises an anti-TAFI antibody coated plate to capture TAFI. HRP-conjugated polyclonal anti-TAFI antibodies then bind to the free epitopes of the captured TAFI. TAFIa/TAFIai ELISA (Asserachrome, Diagnostica Stago) uses a monoclonal antibody coated plate to capture TAFIa/ai. An HRP-conjugated secondary antibody then binds to the remaining antigenic determinants of the bound TAFIa/ai.

Results

Effect on Plasminogen

STS at 0.6% reduced plasminogen to 40% of its initial value (Fig. 3). This effect was time dependent as 0.3% STS reduced plasminogen to 80% of initial level on contact but to less than 20% after 90 minutes of exposure. POL had much less effect on plasminogen than STS and even after 90 minutes it only reduced plasminogen to approximately 70% of its initial level.

Effect on AP activity and PAP Complexes

STS at concentrations above 0.15% increased AP levels in WB, PRP and PPP (Fig. 4). This effect was concentration dependant with STS at 1.2% causing a three-fold increase in AP levels in WB. 1.2% POL caused a two-fold increase in AP levels in WB. STS at concentrations above 0.15% reduced PAP complexes in WB, PRP and PPP. POL at 0.3% and higher, reduced PAP complexes in WB but not in PRP or PPP. Both agents at high concentrations destroyed AP and PAP in spiked BSA control samples.

Effect on PAI-1 activity, t-pA/PAI-1 complexes, total t-PA antigen, and t-PA activity

In WB, 0.15% STS increased PAI-1 activity and t-PA/PAI-1 complexes while POL only elevated total t-PA antigen (Fig. 5). In PRP and PPP, low concentration POL (but not STS) elevated PAI- 1 activity and total t-PA antigen levels. No elevations were observed in control experiments.

In WB, PRP and PPP, high concentration STS caused a 50-100% reduction in PAI-1 activity, t-PA/PAI-1 complexes and total t-PA antigen while inducing a 6-9 fold increase in t-PA activity. This significant elevation in t-PA activity was not observed in control experiments and to the contrary active t-PA levels were reduced by high concentration STS in spiked BSA samples. High concentration POL had little effect on all the measurements in WB and only mildly reduced PAI-1 activity in PRP and PPP.

In control experiments, STS showed no effect on the solid phase of the ELISA assays in all media tested (results not shown).

Effect on TAFI and TAFIa

Both sclerosants at low concentrations elevated TAFI in WB (Fig. 6). High concentration STS (but not POL) reduced TAFI in PRP and PPP but not in WB.

STS at low concentrations increased TAFIa by two-fold in all sample types while at high concentration destroyed this enzyme. POL had minimal effect on TAFIa.

Discussion

Detergent sclerosants have biological activity and interfere with coagulation [3] and antithrombotic mechanisms [4] and at low concentrations release PMP [3]. Here we report the interference of both agents with fibrinolytic enzymes and inhibitors in a concentration dependent manner (Fig. 7).

STS at high concentrations demonstrated antifibrinolytic activity by significantly destroying plasminogen while POL only caused a mild reduction. The inactivation of plasminogen is possibly due to denaturation of the protein molecule as expected of strong detergents such as STS. The effect of STS on plasminogen was time dependant and with more exposure time, even lower concentrations of STS achieved significant destruction of plasminogen.

In this study, AP levels were increased by POL in WB and by STS in WB, PRP and PPP. AP is produced in the liver and kidneys and transported by platelets [23]. Release of AP from platelets could partly explain its rise at low sclerosant concentrations in WB and PRP but not in PPP. We postulated this rise to be due to dissociation of PAP complexes and found a concurrent decrease in PAP complexes. Other mechanisms may be involved in the rise of AP and further studies may be required to fully explain this finding.

High concentration STS significantly destroyed PAI-1 while POL only caused a mild reduction. A similar anionic detergent, SDS, induces oxidative inactivation of PAI-1 by causing a conformational change in the protein structure [24] and the resulting SDS/PAI-1 complexes lack activity [25]. Furthermore, PAI-1 is structurally related to the super-family of SERPINS [26]. STS destroys antithrombin, another SERPIN, as previously reported by the authors [4].

The fall in PAI-1 was paralleled closely by a reduction in t-PA/PAI-1 complexes and the total t-PA antigen. In contrast, t-PA activity was significantly elevated by high concentration STS in all sample types. Multiple previous studies have also reported a positive correlation between PAI-1, total t-PA antigen and t-PA/PAI-1 complexes [13,14,27,28]. The observed rise in t-PA activity was most likely due to dissociation of t-PA/PAI-1 complexes and a subsequent release of active t-PA by high concentration STS, an effect also induced by SDS (Fig. 7) [18,19].

High concentration STS tipped the balance between t-PA and PAI-1 in favour of active t-PA, an effect which may appear to be pro-fibrinolytic. However, t-PA is a specific enzyme and given the concurrent destruction of its substrate, plasminogen, and interference with the generation of its co-factor, fibrin, the rise in t-PA activity would not lead to plasmin generation. Furthermore, the half life of t-PA is less than 5 minutes [29] and hence STS-induced rise in t-PA activity would be transient.

In this study, STS at lower concentrations caused a rise in PAI-1 activity, t-PA/PAI-1 complexes and the total t-PA antigen levels in WB.

POL caused a rise in PAI-1 and total t-PA in PRP and PPP but had less effect in WB possibly due to its extensive neutralisation by plasma components as previously reported by the authors [5]. Low concentration sclerosants activate platelets and release PMP [3], both of which are known to express PAI-1[10,11] . The active conformation of latent PAI-1 can be restored by negatively charged phospholipids [18,19], [30] which are released by both sclerosants from cell membranes [3]. Consistently, sodium dodecyl sulphate (SDS), a similar anionic detergent to STS, enhances the activity of PAI-1 [19,31] and activates a latent form of this inhibitor [32,33].

The rise in active PAI-1 was followed closely by a rise in t-PA/PAI-1 complexes and in total t-PA antigen levels. PAI-1 is an important regulator of the concentration and half-life of t-PA [27]. 95% of t-PA is bound to inhibitors including PAI-1 and elevated total t-PA antigen is associated with hypofibrinolysis and shows a negative correlation with t-PA activity [34]. Therefore, total t-PA antigen is a strong predictor of myocardial infarction and cerebrovascular thrombotic events [35,36]. Elevated plasma PAI-1 levels have been associated with venous [37] and arterial thrombosis [38] and significantly increase the risk of strokes and acute coronary occlusion [39,40]. Release of PAI-1 may play a role in both local and distant thrombo-occlusive complications of sclerotherapy and needs to be further examined in clinical studies.

Both sclerosants at low concentrations elevated the total TAFI antigen levels in WB while POL also caused a rise in PRP. TAFI is produced by the liver and released by activated platelets [41] and monocytes [42] which may explain its rise in WB and PRP. STS at 0.075% caused a rise in TAFIa while POL had no significant effect. This effect is possibly due to generation of thrombin and the subsequent activation of TAFI or may be due to a direct effect of STS on TAFI molecule.

Clinically, recanalisation is observed at the junction of treated segments with untreated patent vessels (such as tributaries or perforators) that contain running bloodflow [43,44]. This is due to plasminogen reaching the area via running blood, its conversion to plasmin by locally generated t-PA and the subsequent fibrinolytic activity of plasmin. Recanalisation is more likely to occur downstream from the point of entry of sclerosants where the active concentration of the sclerosant drops below 0.3% due to dilution and neutralisation [5]. These low concentration sclerosants can induce a low grade thrombotic process[1] which leads to the formation of FXa and thrombin and the subsequent stimulation of endothelial cells to release t-PA. Hence, a 'sclerothrombus' formed by low concentration sclerosants would be a suitable target for degradation by plasmin and the subsequent recanalisation.

Vessels exposed to high concentration STS, by contrast, may be less likely to recanalise due to local destruction of plasminogen and generation of AP. Furthermore, by destroying fibrinogen and other clotting factors, high concentration STS effectively prevents the generation of fibrin. Fibrin potentiates the effect of plasmin by a factor of up to 1000 times [45]. Therefore, in the absence of a thrombotic process, plasmin would not be very effective in recanalising a truly fibrosed (sclerosed) vessel. Under these circumstances, plasmin's role would be limited to activation of MMPs which may play an indirect and limited role in recanalisation of the occluded vessel.

This study had a number of limitations. Other inhibitors of fibrinolysis including C1 inhibitor and alpha 2–macroglobulin could be incorporated in future studies. The *in vivo* effects of sclerosants and their direct fibrinolytic and thrombolytic activity are being investigated by this group in other studies.

In summary, the net *in vitro* effect of high concentration STS can be summarised as pro-occlusive, (destruction of plasminogen, rise in AP activity) while non-prothrombotic (destruction of PAI-1, t-PA/PAI-1 complexes and reduction in total t-PA antigen). High concentration POL demonstrated a limited antifibrinolytic effect due to its elevation of AP levels. Both sclerosants at low concentrations potentiated all inhibitors of fibrinolysis demonstrating a prothrombotic antifibrinolytic profile.

Conflict of Interest

None.

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CHAPTER 6

In vitro Effects of Detergent Sclerosants on Clot Formation and Fibrinolysis

Parsi K, Exner T, Low J, Ma DDF, Joseph JE.

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I was the first author on this paper published in the European Journal of Vascular Endovascular Surgery early this year. I was the major (> 50%) contributor to this study and conducted the experimental work under Dr Tom Exner's supervision. The data was analysed by myself, presented to the team and discussed in regular meetings. I wrote the manuscript and was responsible for the submission process and dealing with the reviewers comments. Dr Tom Exner performed the fibrin gel experiments and Dr Joyce Low performed the measurements on D-dimer. Other co-authors contributed to discussion and the manuscript. No other authors were students. (PUBLISHED, IF=2.919)

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In Vitro Effects of Detergent Sclerosants on Clot Formation and Fibrinolysis

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KEYWORDS
Detergent sclerosants;
Fibrinogen;
Factor XIII;
Clot formation;
Fibrinolysis;
D-dimer

Abstract *Objective:* To investigate the *in vitro* effects of detergent sclerosants sodium tetradecyl sulphate (STS) and polidocanol (POL) on clot formation and lysis.
Materials and methods: clot kinetics were assessed in whole blood by thromboelastography (TEG®) and rotational thromboelastometry (ROTEM®). Fibrinogen was measured by the Clauss method in plasma and factor XIII (FXIII) by enzyme-linked immunosorbent assay (ELISA). Turbidity measurements were used to assess clot lysis in plasma, and fibrinolysis in non-cross-linked and cross-linked fibrin. D-dimer was measured by VIDAS®, STA®Liatest® and AxSYM® assays.
Results: Strong clots were formed at low sclerosant concentrations (0.075–0.1%). At midrange concentrations (0.15% STS, 0.15–0.3% POL), both agents inhibited the contribution of platelets to clot firmness and formed weak clots prone to lysis. At higher concentrations (STS $\geq$0.3% and POL $\geq$0.6%), clot formation was inhibited. STS destroyed FXIII at $\geq$0.15% and fibrinogen at $\geq$0.6%. Neither sclerosant had a significant effect on cross-linked fibrin, but STS had a lytic effect on non-cross-linked fibrin. STS caused an artefactual elevation of D-dimer in the VIDAS® assay when fibrinogen was present.
Conclusion: Detergent sclerosants demonstrated a trimodal effect on clot formation, initiating strong clots at low concentrations, weak clots at midrange concentrations and preventing clot formation at higher concentrations. Neither agent had fibrinolytic activity.

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Introduction

Detergent sclerosants, sodium tetradecyl sulphate (STS) and polidocanol (POL), function by lysing the intimal lining of blood vessels, exposing the underlying collagen with the ultimate aim of inducing endovascular fibrosis. These agents were historically assumed to achieve this by forming a clot within the target vessel, the organisation of which could lead to vessel fibrosis and the lysis of which would lead to vessel recanalisation and treatment failure.[1] Our group has previously shown that these detergents are biologically active and interfere with the coagulation, anti-thrombotic and fibrinolytic mechanisms.[2–4] We have also shown that these agents have a concentration-dependent effect on the coagulation system *in vitro* with STS exhibiting anticoagulant activity at >0.3%, while both agents demonstrate procoagulant activity at lower concentrations.[2–4] Nonetheless, the direct effect of sclerosants on clot formation and lysis was not previously investigated and was the subject of the present study.

The ultimate step in the formation of a fibrin clot is the conversion of soluble fibrinogen to insoluble fibrin. This process is mediated by thrombin, which also activates Factor XIII (FXIII). Fibrin monomers are first assembled in a non-covalent fashion and then covalently cross-linked by activated FXIII (FXIIIa). Cross-linking enhances the mechanical strength of the fibrin polymer, and leads to increased clot stability, stiffness and resistance to fibrinolysis and deformation.[5]

Fibrin is degraded by plasmin generated from plasminogen by the action of tissue plasminogen activator (t-PA) in blood and urokinase in tissues. The enzymatic degradation of fibrin leads to the formation of fibrin degradation products and, in particular, D-dimer. As shown by the authors, both sclerosants interfere with fibrinolytic enzymes and inhibitors and demonstrate antifibrinolytic activity.[4] Here, we studied the direct lytic effects of sclerosants on FXIII, fibrinogen and fibrin and their potential for generation of D-dimer.

Materials and Methods

Materials

The following were used in this study: STS (FIBRO-VEIN 3%; Australian Medical and Scientific, NSW, Australia); POL (AETHOXYSKLEROL 3%; Chemische Fabrik Kreussler, Wiesbaden, Germany); TEG® reagents (Haemoscope, IL, USA); ROTEM® reagents (Pentapharm, Munich, Germany); *Echis carinatus* venom (ECV; RUDINtest, Haematex Research, NSW); urea, low-melting-point agarose (type VII), *Agkistrodon rhodostoma* venom (ARV) and hydrolysed gelatin (Sigma Chemical, MO, USA); human thrombin (Enzyme Research Laboratories, IN, USA); recombinant human t-PA (Haematologic Technologies, Vermont, USA) and bovine serum albumin (BSA; Bovogen, Victoria, Australia).

Sample collection for fresh-frozen plasma (FFP)

Normal plasma (NP) was collected as FFP derived from donor blood unsuitable for clinical indications and obtained from the Australian Red Cross Blood Transfusion Service, Sydney. Samples were collected in acid citrate dextrose (ACD) venous blood vacuum collection tubes.

Preparation of platelet-rich (PRP) and platelet-poor plasma (PPP)

Centrifugation of whole blood (WB) for 10 min at 150 *g* was performed to generate platelet-rich plasma (PRP) and for 30 min at 1700 *g* to generate platelet-poor plasma (PPP).

Preparation of freeze-dried plasma

Freeze-dried samples were prepared using NP (2.0 ml, 0.5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) dispensed into siliconised vials spiked with sclerosants to a final stated concentration (0%, 0.075%, 0.1%, 0.3%, 0.6% and 1%). The vials were frozen at −50 °C and vacuum dried for 2 days. Prior to testing, each vial was reconstituted with 2 ml of water.

Preparation of fibrin agarose gel

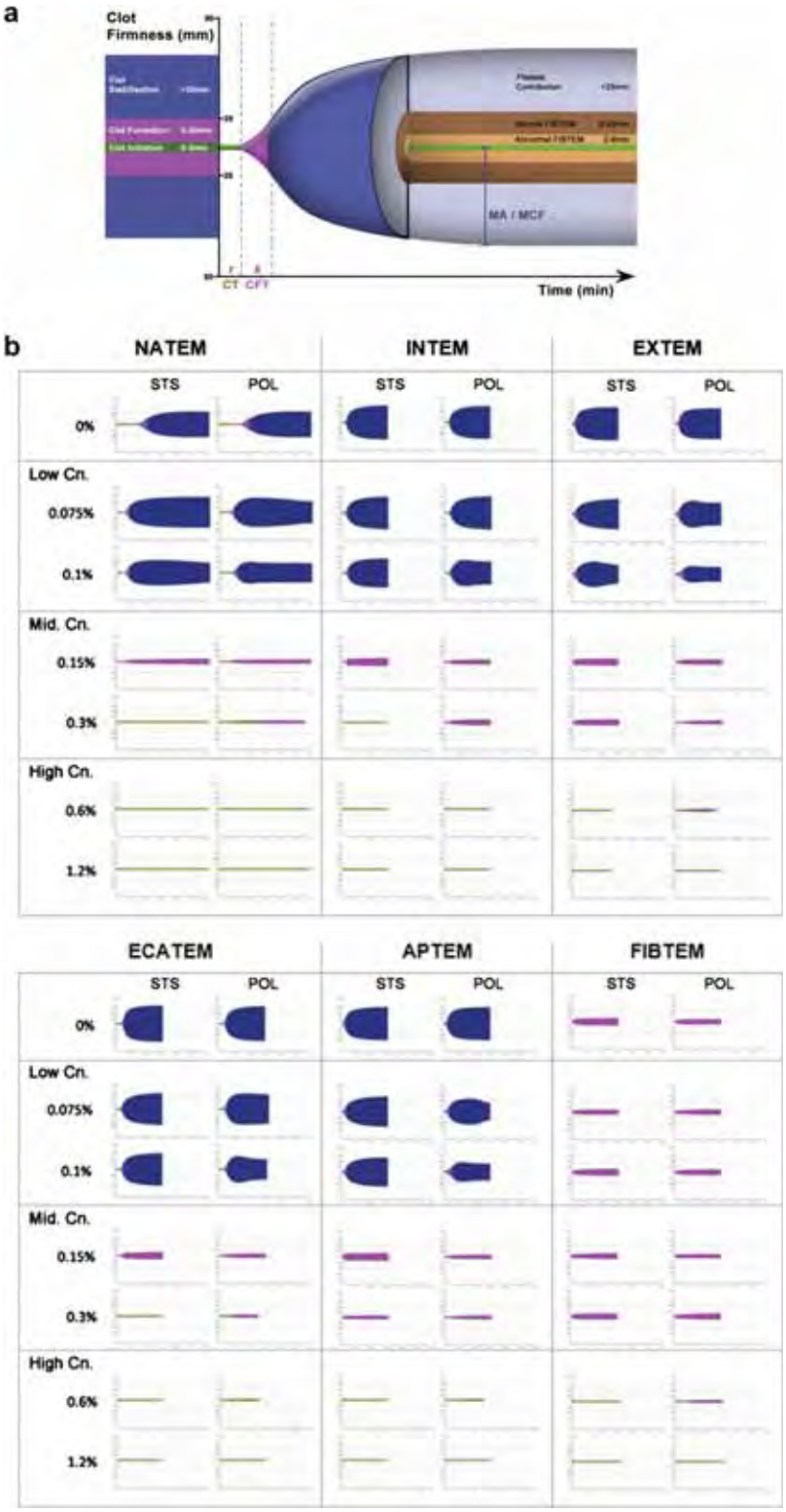
Fibrinogen was isolated from NP by precipitation with 20% ammonium sulphate. Fibrinogen solution and low-melting point agarose (2%w/v in 0.1 M sodium chloride (NaCl), 0.02 M HEPES pH 7.0, 37 °C) were mixed, 0.1 U ml^{-1} thrombin and 0.01 M calcium chloride ($CaCl_2$) were added, and 0.1-ml volumes were dispensed into pre-warmed microwells. For non-cross-linked fibrin, thrombin was replaced with 0.0001% ARV (containing the thrombin-like enzyme Ancrod) without $CaCl_2$. The agarose set on cooling and the fibrinogen within each well was slowly converted with time to non-cross-linked fibrin (with ARV) and to cross-linked fibrin (with thrombin that activates FXIII). Urea (6M), known to dissolve non-cross-linked fibrin but not cross-linked fibrin, was used as a control to confirm the cross-linking status of the two types of fibrin agarose gels.[6]

Clot kinetics

Clot viscoelastic properties were assessed by thromboelastography (TEG®, Haemoscope) and rotational thromboelastometry (ROTEM®, Pentapharm).[7] Both systems use a vertical pin within a cup that contains the WB sample. In TEG®, the cup oscillates and the pin is stationary whereas in ROTEM®, the cup is stationary while the pin oscillates. As a clot forms between the cup and the pin, the reduction in transmitted rotation from the cup to the pin (TEG®) or impedance to the oscillatory movement of the pin (ROTEM®) is detected and a trace is generated (Fig. 1(a)).

WB was collected from consenting healthy volunteers on no medications or supplements into vacutainer tubes containing 0.105 M buffered sodium citrate (BD Diagnostics, Basel, Switzerland) and spiked with increasing concentrations of sclerosants. Parameters measured are detailed in Table 1 and assays performed are summarised in Table 2.

For TEG®, 1 ml volumes of WB-spiked samples were pipetted into Kaolin vials. A total of 20 μl of 0.2 M $CaCl_2$ was placed in each cup followed by 300 μl of the Kaolin-spiked samples.



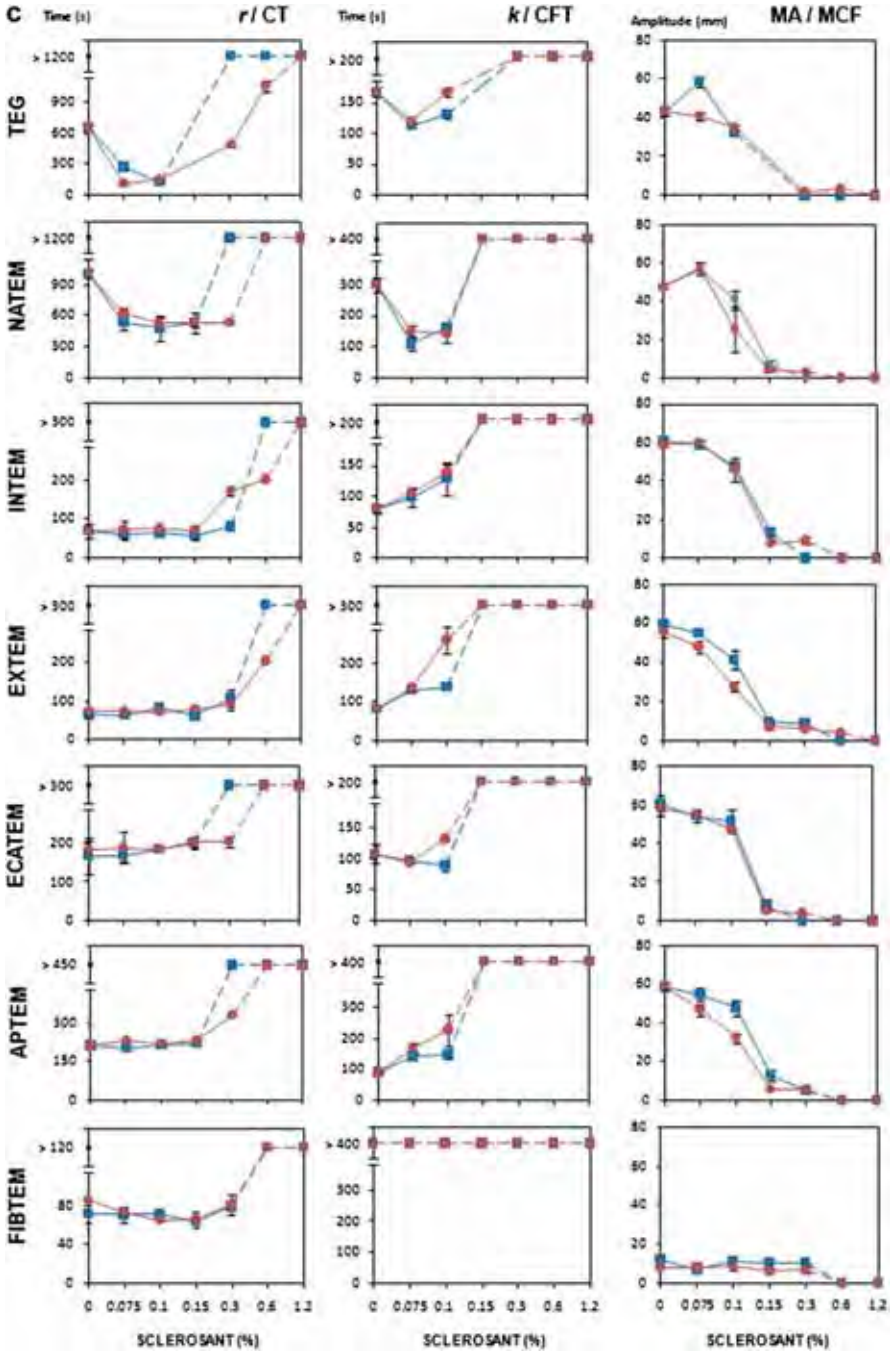


Figure 1 (a) Diagrammatic representation of a typical TEG®/ROTEM® trace indicating the commonly reported variables. *r*, reaction time; CT, clotting time; *k*, coagulation time; CFT, clot formation time; *MA*, maximum amplitude; MCF, maximum clot formation. (b) Representative sclerosant clot kinetics as analysed by ROTEM®. Clot firmness <20 mm is marked with pink, and ≥20 mm with blue. Green line indicates no clot formation. Cn, concentration; Mid., midrange. (c) TEG®/ROTEM® parameters measured in various assays. In FIBTEM, CFT is indefinitely prolonged as by definition, no strong clots with amplitudes >20 mm (which requires a contribution from platelets) can be achieved. This is a normal finding for FIBTEM (NATEM, $n = 5$; other assays, $n = 2$; ■ STS and ● POL).

Table 1 TEG® and ROTEM® parameters measured in this study.

TEG®	ROTEM®	Description
Reaction time (*r*)	Clotting Time (CT)	The time from beginning of measurement until clot formation at 2 mm amplitude. This value represents the initiation of clotting and formation of fibrin.
Coagulation time (*k*)	Clot Formation Time (CFT)	The time from initial clot formation at 2 mm until a firm clot of 20 mm amplitude is formed. It represents fibrin polymerization and cross-linking.
—	Clot Formation Rate (CFR)	The rate of clot formation based on α, the angle between the baseline and a tangent to the curve running through the 2 mm amplitude.
Maximum Amplitude (MA)	Maximum Clot Firmness (MCF)	Maximum amplitude of the trace representing maximum clot firmness. It represents the increasing stabilisation of the clot by the polymerised fibrin, platelets and FXIII.
Coagulation Index (CI)	—	This value is derived from *r*, *k*, α and MA and functions as an overall assessment of coagulation, with values less than −3.0 signifying a hypocoagulable state and values over +3.0 indicating hypercoagulability.[28]

In all ROTEM® assays (except ECATEM), 20 μl of the STARTEM reagent (0.2 M $CaCl_2$ in HEPES pH 7.4 buffer) was placed in each cup followed by 20 μl of the relevant reagent per assay (EXTEM, INTEM, APTEM or FIBTEM) or 20 μl of RUDINtest (ECATEM) or no other reagent (NATEM). A total of 300 μl volumes of WB-spiked samples were then added.

All TEG® and ROTEM® assays were performed at 37 °C and run for 30 min except NATEM, which was run for 60 min due to the prolonged initial clotting time.

Clot lysis time (CLT)

NP (FFP, 1 ml) was briefly mixed with small volumes of t-PA stock solution to a final concentration of 25 ng l^{-1}. Volumes (0.1 ml) were distributed into microwells and mixed with increasing concentrations of sclerosants. Volumes (0.1 ml) of thrombin (2U ml^{-1}) were then added and the mixtures containing approximately 50% diluted NP were allowed to clot. Turbidities were determined periodically at 414 nm.

Interaction with exogenous t-PA

A total of 1.0 μg l^{-1} of t-PA in 0.2% hydrolysed gelatine (protein-poor) and 8.0 μg l^{-1} in NP (protein rich) was pre-incubated briefly with serial dilutions of sclerosants in NaCl pH 7.0 buffer. 0.02 ml volumes were applied to fibrin

Table 2 TEG® and ROTEM® assays used in this study and reagents used in each assay.

Assays	Reagent	Interpretation
TEG	Calcium Chloride ($CaCl_2$) + Kaolin	Thromboelastography (TEG) was used to assess the global effects of sclerosants in the assay, the clotting cascade was activated with kaolin.
NATEM	'STARTEM' ($CaCl_2$)	Non-activated (NA) Thromboelastometry (TEM), NATEM, is sensitive towards any kind of coagulation activation or inhibition in the sample and provides a general overview of the haemostatic process.
INTEM	$CaCl_2$ + Ellagic acid + Phospholipids	Intrinsic pathway TEM, INTEM, is an assay that initiates a controlled activation of the contact system using ellagic acid and phospholipids to evaluate the intrinsic pathway.
EXTEM	$CaCl_2$ + Tissue factor (TF)	Extrinsic pathway TEM, EXTEM, uses a stabilised preparation of TF for a mild but consistent activation of coagulation to examine the extrinsic pathway.
ECATEM	Echis Carinatus Venom (ECV)	Ecarin TEM, ECATEM, uses ECV to mediate the conversion of prothrombin to thrombin. ECATEM evaluates the final two steps of the coagulation cascade i.e. the conversion of prothrombin to thrombin and the subsequent generation of fibrin from fibrinogen. The trace generated is independent of phospholipids and other plasma clotting factors.
APTEM	$CaCl_2$ + TF + Aprotinin	Aprotinin TEM, APTEM, incorporates a plasmin inhibitor (aprotinin) in the ap-TEM® reagent together with ex-TEM® activation. The trace generated by this assay is compared with EXTEM. In presence of fibrinolysis, aprotinin will inhibit fibrinolysis and the abnormal EXTEM trace will be corrected to a normal trace in APTEM.
FIBTEM	$CaCl_2$ + TF + Cytochalasin D	Fibrinogen TEM, FIBTEM, incorporates Cytochalasin D, an inhibitor of platelet cytoskeletal re-organisation. The trace generated would represent the contribution of clotting factors and in particular fibrinogen to clot strength and is compared with EXTEM to assess the contribution of platelets to clot formation. The FIBTEM maximum clot firmness (MCF) normal range is much lower (9–25 mm) than the EXTEM (50–72 mm).

agarose wells and incubated at 37 °C overnight. Turbidities were determined periodically at 414 nm.

Fibrinogen
Fibrinogen levels were measured in freeze-dried plasma samples by the Clauss method,[8] using a Thrombin reagent (Dade Behring, Marburg, Germany) on an STA-R analyzer (Diagnostica Stago, Asniere, France).

FXIII
Fresh donor WB, PRP and PPP samples containing serial dilutions of sclerosants were prepared on non-coated plates and tested following the standard procedure in the FXIII ELISA kit (EF 1013-1, Assaypro, MI, USA). In control experiments, serial dilutions of sclerosants in 5% BSA were spiked with 12 ng of FXIII and tested in the assay system (inter-assay coefficient of variation: 6.8%). All assays were done in duplicate.

Fibrinolytic activity
Serial dilutions of sclerosants and t-PA (0.02 ml, 25 ng ml^{-1}) in 0.1% BSA or hydrolysed gelatin buffer were applied to cross-linked and non-cross-linked fibrin agarose gels (0.1 ml) and incubated overnight at 37 °C.

0.1 ml volumes of 2% cross-linked fibrin powder[9] suspended in HEPES-buffered saline were dispensed into microwells, mixed with 0.1 ml of sclerosant dilutions and incubated for 4 h at 37 °C.

For both methods, turbidities were measured periodically at 414 nm following the incubation.

D-dimer
D-dimer was measured in freeze-dried samples by the VIDAS® D-dimer Exclusion assay™ (bioMérieux®sa, Marcy-l'Étoile, France) on the Mini-VIDAS® instrument (bioMérieux Vitek, MI, USA), AxSYM® D-dimer (Abbott Laboratories, IL, USA) and STA®Liatest® D-dimer (Diagnostica Stago).

To investigate the observed elevation of D-dimer by STS (see Results 7), the D-dimer levels were re-measured in the following samples using the VIDAS® assay:

1 Original plasma samples following removal of a previously reported STS-induced precipitate[2];
2 Serial dilutions of STS in saline or in VIDAS® kit diluent;
3 1% STS in heat-defibrinated pooled NP (PNP, 30 min, 56 °C) neat and after centrifugation (10 min at 16,100 *g*); and
4 Purified fibrinogen (Fibrinogen Fraction 1, type I Sigma Cat F3879) added to the VIDAS® kit diluent to a concentration of 2.3 *g* l^{-1} and then spiked with STS.

Presentation of Sampling Fluctuations
Sampling fluctuations are reflected by the standard errors (standard deviation divided by the square root of the number of sample) for Figures 1, 5 and 6 and within the 10-15 % range for the remaining Figures.

Results

Clot kinetics

Low concentrations (0.075–0.1%)
Strong clots (amplitude >20 mm) were formed in TEG® and all ROTEM® assays (Fig. 1(b)). The reaction (*r*)/clotting time (CT) and coagulation time (*k*)/clot formation time (CFT) were reduced in TEG® and NATEM (Fig. 1(c)) while the clot formation rate (CFR) was increased (results not shown). TEG® Coagulation Index showed a hypercoagulable state (CI > +3.0).

Midrange concentrations (0.15% STS, 0.15–0.3% POL)
Weak clots (amplitude <20 mm) formed in all assays except in FIBTEM where the amplitude remained within the normal range of 9–25 mm (Fig. 1(b) and (c)). This indicated an inhibition of platelet contribution to clot firmness. In tissue factor (TF)-based assays (EXTEM, APTEM and FIBTEM), weak clots formed at 0.3% STS but clot formation was inhibited at higher concentrations.

High concentrations (≥0.3% STS, ≥0.6% POL)
Both agents inhibited clot formation and indefinitely prolonged *r*/CT and *k*/CFT (Fig. 1(b) and (c)). CFR was reduced to zero. No value for TEG® CI could be obtained (CI < −3.0), indicating a hypocoagulable state.

Other findings
Addition of aprotinin (APTEM) did not influence the maximum amplitude as compared with EXTEM and, hence, no fibrinolysis was detected. Direct prothrombin activation (ECATEM) yielded results similar to other non-TF-based assays.

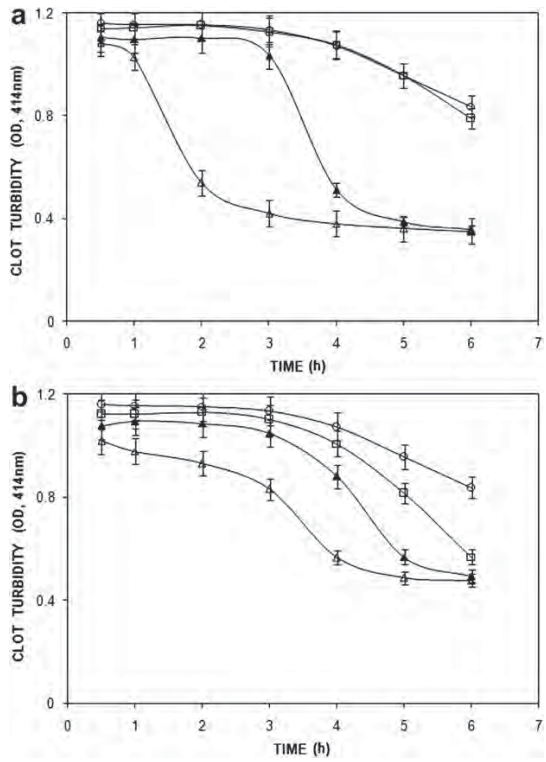


Figure 2 Clot lysis times with increasing concentrations of STS (2a) and POL (2b) ($n = 2$, ○ 0%, □ 0.05%, ▲ 0.1%, Δ 0.15%).

Clot lysis time (CLT)

t-PA at 25 ng ml^{-1} within a 50% diluted plasma clot induced clot lysis within 6 h. STS at 0.1% shortened CLT to 4 h and at 0.15% to <2 h (Fig. 2). POL had less effect.

Interaction with exogenous t-PA

t-PA was inactivated by STS at ≥0.4% in plasma while POL had less effect (Fig. 3). STS interfered more with t-PA activity in protein-poor buffer, whereas, in plasma (which is protein rich), 10× higher levels of STS were required due to protein binding.

Fibrinogen

STS at 0.3% reduced fibrinogen levels in plasma by 20%, and at ≥0.6% destroyed this protein completely (Fig. 4). POL had minimal effect.

FXIII

STS at 0.075% reduced FXIII to less than 50%, and at 0.15% completely destroyed this protein in WB (Fig. 5). POL had minimal effect. Similar results were obtained in PRP and PPP samples (results not shown). Control experiments showed no interaction with the assay system (results not shown).

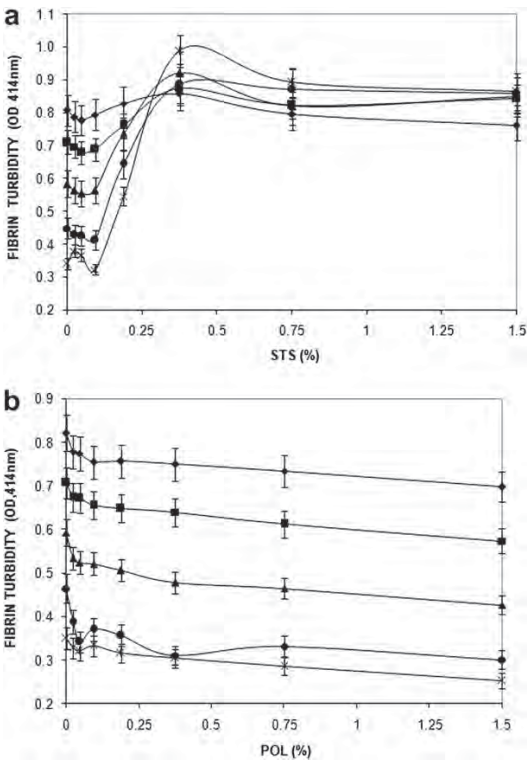


Figure 3 Effect of STS (3a) and POL (3b) on t-PA induced fibrinolysis ($n = 2$, STS: ◆ $t = 0$, ■ $t = 1$hr, ▲ $t = 2$hr, • $t = 3$hr, $_*t = 4$hr) (POL: ◆ $t = 0$; ■ $t = 1$hr, ▲ $t = 2$hr, • $t = 3$hr, $_*t = 4$hr).

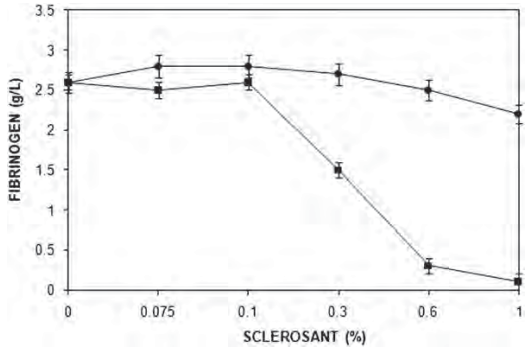


Figure 4 Effect of increasing concentrations of sclerosants on fibrinogen levels. ($n = 2$, ■ STS and • POL).

Fibrinolytic activity

STS at >0.4% solubilised non-cross-linked fibrin, but at 3% demonstrated a significant lytic effect (Fig. 6(a)). STS had a similar effect to t-PA but was 100,000 times weaker on a direct concentration basis. POL had little effect on non-cross-linked fibrin. Both agents showed no significant effect on cross-linked fibrin agarose gels (Fig. 6(b)) or cross-linked fibrin powder (results not shown).

D-dimer

In VIDAS® assay, STS at ≥0.6% significantly elevated the D-dimer levels to 2.5 mg l^{-1} (normal range 0–0.5 mg l^{-1}) while POL had no effect (results not shown). This elevation was not detected by AxSYM® or STA®Liatest®.

Removal of a previously reported STS-induced precipitate[2] did not influence the D-dimer elevation. STS dilutions in saline, in VIDAS® kit diluent (∼0.6 mg l^{-1}) and in heat-defibrinated PNP showed no measurable D-dimer levels. However, STS spiking of the VIDAS® kit diluent to which purified fibrinogen was added caused elevations of D-dimer (up to 7.4 mg l^{-1} with 1% STS). Therefore, fibrinogen was required for the STS-induced elevation of D-dimer, as measured by the VIDAS® assay.

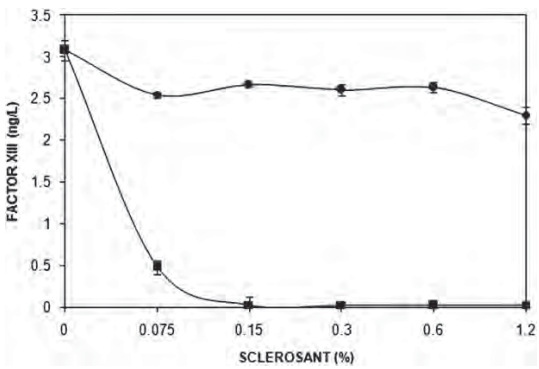


Figure 5 Effect of increasing concentrations (0%, 0.075%, 0.15%, 0.3%, 0.6%, 1.2%) of sclerosants on factor XIII levels in whole blood. ($n = 4$, ■ STS and • POL).

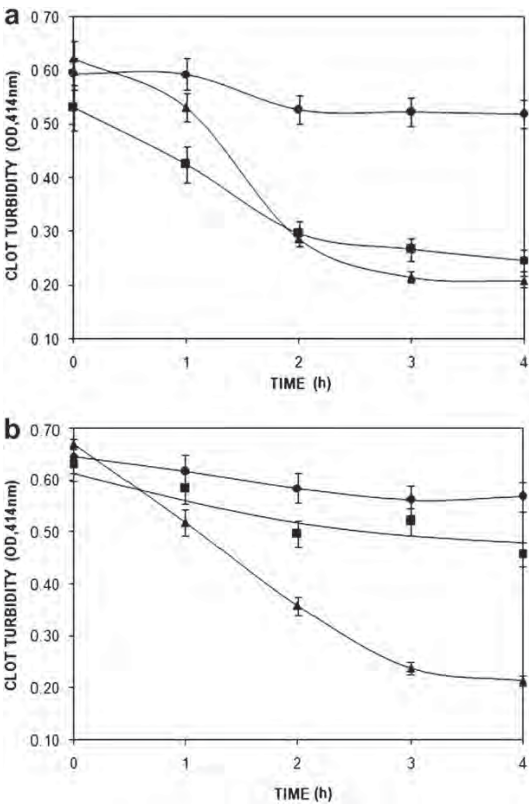


Figure 6 Comparison of fibrinolytic activity of detergent sclerosants with that of t-PA on cross-linked (6a) and non-cross-linked (6b) fibrin. Only results obtained with 3% sclerosants are shown as they are typical. ($n = 3$, ■STS $\times 10^5$, • POL $\times 10^5$ and ▲t-PA).

Discussion

Detergent sclerosants are used clinically to occlude vessels. The sequence of events leading to vessel occlusion has been unknown and whether clot formation plays a part in this process has been debated. In this study, both agents demonstrated a trimodal effect on clot formation and firmness (Fig. 7). At low concentrations (0.075—0.1%), clot formation was enhanced by both agents as manifested by the formation of strong clots (>20 mm amplitude) in all assays. In addition, strong clots were initiated by both agents in the non-activated NATEM assay, in the absence of any activators, and where the only reagent added was $CaCl_2$ for re-calcification purposes. The clotting and clot formation times were shortened in NATEM and TEG® and the Coagulation Index showed a hypercoagulable state. This procoagulant activity is consistent with the release of platelet-derived microparticles (PMPs) and shortening of phospholipid-dependent Xa and surface-activated clotting times (XACT and SACT) previously reported by this group.[2,10] At midrange concentrations (0.15% STS, 0.15—0.3% POL), both agents induced weak clots (<20 mm amplitude). There was a reduction of amplitude in EXTEM while the amplitude in FIBTEM remained within normal limits, indicating a lack of contribution from platelets to clot formation and firmness. At higher concentrations (0.6—1.2%), no clots were formed, indicating clotting factor and, in particular, fibrinogen deficiency. This confirms our previous reports that both agents at high concentrations bind phospholipids, destroy a number of clotting factors and inhibit clotting interactions.[2,3]

We investigated whether clots formed in presence of sclerosants were more prone to lysis. Although both agents increased the clot lysis rates at midrange concentrations, there was no synergistic (or inhibitory) effect between the detergents and t-PA. At higher concentrations, STS (but not POL) had an inhibitory effect on t-PA-induced fibrinolysis most likely due to denaturation of the t-PA protein structure. Plasminogen, the main t-PA substrate, is also destroyed by STS at such high concentrations.[4] Hence, the faster clot lysis rates were not due to a synergistic interaction with t-PA but due to formation of weak clots susceptible to lysis as confirmed by thromboelastometry.

Clot strength is affected by fibrinogen,[11] FXIII[12] and platelets.[13] We found all three to be affected by midrange concentrations of STS while POL had no effect on fibrinogen or FXIII. High fibrinogen concentrations are required for increased clot rigidity[14] and density.[11] In this study, fibrinogen levels were reduced at midrange concentrations of STS and, at ≥0.6%, this protein was completely destroyed. Consistently, the FIBTEM signal was lost at 0.6% STS. This is most likely due to denaturation and unfolding of the fibrinogen protein structure.[15] Furthermore, both cationic and anionic detergents precipitate fibrinogen by forming fibrinogen—detergent complexes.[2,16]

In this study, FXIII was destroyed by STS at ≥0.15%. FXIIIa covalently cross-links fibrin monomers to form cross-linked fibrin. This enhances clot stability and increases resistance to fibrinolysis and deformation.[5] Apart from its cross-linking function, FXIIIa covalently binds inhibitors of fibrinolysis, such as antiplasmin, to the fibrin clot. This is possibly the main mechanism by which FXIIIa increases the resistance of the clot to lysis.[17] Destruction of FXIII prevents cross-linking, reduces clot firmness and leads to formation of weak and unstable clots susceptible to lysis.[12]

Platelet contribution to clot firmness plays an important role in clot amplitude in thromboelastometry.[18] Comparing the clot firmness in FIBTEM (where platelets are blocked) with EXTEM (includes platelet contribution), midrange concentrations of both sclerosants showed a lack of contribution from platelets to clot firmness. This is consistent with our earlier reports that both agents at similar concentrations (≥0.15%) induce platelet lysis.[2,10]

We investigated the fibrinolytic activity of sclerosants. Both agents had no destructive effect on cross-linked fibrin and, consistently, no fibrinolysis was detected in APTEM. However, STS destroyed non-cross-linked fibrin and produced a similar dose response curve to t-PA although approximately 100,000 times weaker. This is possibly due to depolymerisation of the non-cross-linked fibrin polymer by STS. Similarly, plasmin has less effect on cross-linked fibrin[19] and the presence of FXIIIa slows down the process of clot lysis induced by t-PA.[20] A similar anionic detergent, sodium dodecyl sulphate (SDS), also exhibits some fibrinolytic activity.[21] The process of cross-linking happens very quickly and non-cross-linked fibrin is normally not present

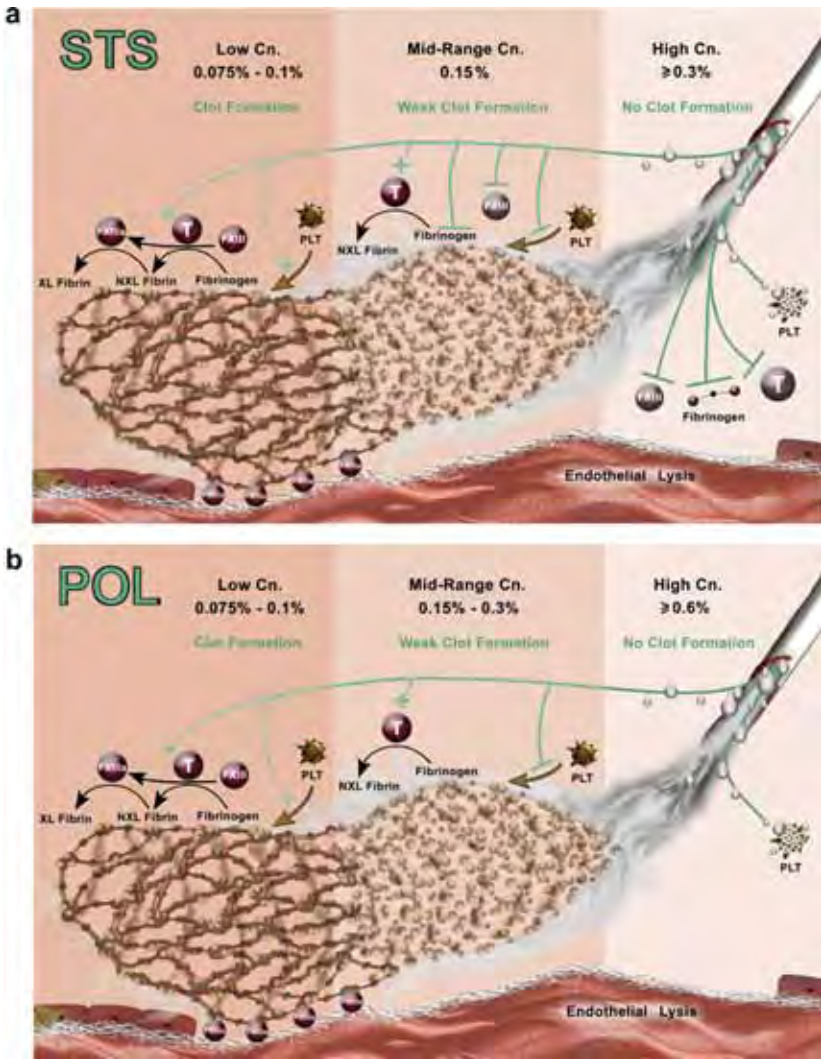


Figure 7 Cartoons demonstrating the observed effects of STS (a) and POL (b) on clot formation. The effect demonstrated is at the point of entry of sclerosants into the target vessel where the sclerosant concentration is at its highest and downstream where the concentration drops. High concentration (>0.3%) STS destroys fibrinogen, factor XIII (FXIII), thrombin (T)[2] and platelets (PLT) and a number of other clotting factors[2] and in this study inhibited clot formation. High concentration (>0.6%) POL destroys platelets and a number of clotting factors[2] and in this study also inhibited clot formation. At midrange concentrations (0.15% STS, 0.15—0.3% POL), both agents inhibited the contribution of platelets to clot firmness and generated weak clots. 0.15% STS destroyed FXIII and reduced fibrinogen. Both agents at low concentrations (0.075% and 0.1%) initiated and enhanced strong clot formation. Cn., concentration; FXIIIa, activated FXIII; XL, cross-linked; NXL, non-cross-linked.

in a thrombus.[22] Hence, exposure of STS to a fully formed clot would not lead to detectable lysis. POL, a non-ionic detergent, demonstrated no fibrinolytic activity. Non-ionic and zwitterionic detergents are reported to increase the apparent activity of t-PA, although they have little net thrombolytic[23] or fibrinolytic activity.[21]

In this study, high-concentration STS caused an artefactual elevation of D-dimer levels when measured in the VIDAS® ELISA assay. This only occurred when fibrinogen was present in the sample. One possible mechanism is that STS caused a conformational change in fibrinogen, exposing an epitope recognised by monoclonal antibodies used in the VIDAS® assay, but not in the other two commercial assays tested. Fibrinogen can form flexible polymer chains similar to polymeric fibrin by binding end-to-end in D-dimer configuration where the neighbouring D domains form 'DD' regions.[24] D-dimer specific antibodies have been previously reported to react with such soluble fibrinogen aggregates.[25]

Based on our present findings, high-concentration sclerosants inhibit clot formation and hence, their modus operandi can potentially by-pass thrombosis to achieve fibrosis. However, given that these agents act rapidly,

prolonged high intravascular concentrations are unlikely. In addition, although relatively high concentrations can be achieved at the point of entry, much lower final concentrations are generated when these agents are mixed with large volumes of blood, diluted and neutralised.[10]

Foam sclerosants are more effective than liquid agents, possibly due to displacement of blood and a reduced exposure to serum albumin and other plasma components. Clinical recurrence, despite the use of high concentrations of sclerosants, is most likely secondary to inadequate vascular injury, formation of intra-vascular thrombus and subsequent recanalisation.[26] Treatment modifications aiming at minimising the intra-vascular blood content may achieve higher final concentrations and a lower recanalisation rate.

Clinically, sclerotherapy has been associated with thrombo-embolic complications, including paradoxical clot embolism and stroke, following the use of low- to midrange concentrations of sclerosants.[27] Clot formation normally occurs due to interaction of running blood from the adjoining patent vessels reaching the site of vascular injury. The subsequent activation of platelets and the coagulation system would result in clot formation. Formation of such clots is initiated by vascular injury. Based on our present findings, clots can also be initiated directly by low- to midrange concentrations of both sclerosants upon contact with blood and in the absence of vascular damage. The formation of such clots and their viscoelastic properties would depend on the final intra-vascular concentrations achieved. A weak clot deficient in FXIII, as that formed by midrange concentrations of sclerosants, would have the potential for detachment from the vessel wall and embolisation, as well as lysis and clinical recanalisation.

This study was not without limitations. We did not use the euglobulin clot lysis time method as it remains a complex and time-consuming procedure. The *in vivo* effects of sclerosants on clot formation and lysis, including the effects of foam sclerosants, are currently being investigated by this group in other studies. We report that low-concentration sclerosants initiate a platelet-dependent clot formation process. Platelets play a small role in venous thrombosis and subsequently there have been no systematic studies on the effects of sclerosants on platelets and the influence of platelet inhibitors on sclerotherapy treatment outcomes. Given our current findings, these topics may require further evaluation.

Summary

Sclerosants demonstrated a trimodal effect on clot formation whereby, at low concentrations (0.075—0.1%), they enhanced strong clot formation, at midrange concentrations (0.15% STS, 0.15—0.3% POL) induced weak clots susceptible to lysis and, at higher concentrations, prevented clot formation. Neither agent had a lytic effect on cross-linked fibrin but high-concentration STS destroyed non-cross-linked fibrin. STS caused an artefactual elevation of D-dimer in the VIDAS® assay.

Conflict of Interest

None.

Funding

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CHAPTER 7

Low Concentration Detergent Sclerosants Induce Platelet Activation but Inhibit Aggregation *in vitro*

Parsi K, Connor DE, Pilotelle A, Low J, Ma DDF, Joseph JE.

Parsi K, Connor DE, Pilotelle A, Low J, Ma DDF, Joseph JE. Low Concentration Detergent Sclerosants Induce Platelet Activation but Inhibit Aggregation *in vitro*

I was the first author of this paper submitted to Thrombosis and Haemostasis. I was the major (>50%) contributor to this study. The data was analysed by myself, presented to the team and discussed in regular meetings. I wrote the manuscript and was responsible for the submission process and dealing with the reviewers comments. This project evolved to become fairly extensive and required collaboration with other centres and colleagues. Royal Prince Alfred Hospital staff kindly performed the platelet counts in whole blood using the automated cell counter, Dr Anne Pilotelle assisted with platelet activation studies, Dr David Connor performed the flow cytometry analysis of microparticles and Dr Joyce Low performed the light transmission aggregometry. Other co-authors contributed to discussion and the manuscript. No other authors were students.

Low Concentration Detergent Sclerosants Induce Platelet Activation but Inhibit Aggregation in vitro

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SUMMARY

Background: Sclerotherapy to treat varicose veins is associated with thromboembolic and ischemic neurological adverse events, but the role of platelets in the pathogenesis of these complications is unknown.

Objectives: To investigate the *in vitro* effects of detergent sclerosants Sodium Tetradecyl Sulphate (STS) and Polidocanol (POL) on platelet activation and aggregation.

Methods: Whole blood (WB) and platelet rich plasma (PRP) samples were incubated with STS or POL. Platelet and platelet microparticle (PMP) counts were measured by flow cytometry using CD41a, annexin V and lactadherin. Platelet activation was examined by ELISA for soluble (s) factors (sP-selectin, von Willebrand factor, sCD40L and serotonin) and by flow cytometry for membrane-bound markers (CD62p, CD63) and cytoplasmic calcium. Platelet aggregation was assessed by PFA-100®, light transmission and impedance (Multiplate®) aggregometry, and by flow cytometry for platelet glycoprotein (GP) IIb/IIIa subunit and heterodimer expression and activation (PAC-1 binding).

Results: Platelets and PMP were lysed at higher concentrations of both sclerosants. At lower concentrations (≤0.1%), both agents activated platelets in the absence of other agonists as evident by a rise in the soluble and membrane-bound markers, cytoplasmic calcium and release of phosphatidylserine+ PMP. Agonist-stimulated platelet aggregation was inhibited by both sclerosants. In the absence of agonists, 0.1% STS induced a spontaneous reversible platelet aggregation. Membrane expression of GPIIb/IIIa heterodimer or the individual subunits was not affected by sclerosants but the activation of this receptor was suppressed.

Conclusion: Low concentration sclerosants activated platelets and released microparticles but inhibited platelet aggregation due to inhibition of GPIIb/IIIa activation.

Keywords

Detergent Sclerosants; Platelets; Platelet activation; Platelet aggregation; Platelet microparticles; Platelet glycoprotein IIb/IIIa complex.

INTRODUCTION

Detergent sclerosants, sodium tetradecyl sulphate (STS) and polidocanol (POL) are commonly used to treat varicose veins, vascular malformations and oesophageal varices. Sclerotherapy of varicose veins is complicated by venous thromboembolic events in up to 1-3% of patients.[1, 2] Neurological adverse events including rare cases of transient ischemic attacks and ischemic stroke have been reported in patients undergoing sclerotherapy who were later diagnosed with a patent foramen ovale (PFO).[3] It is unknown whether activated platelets and platelet microparticles (PMP) contribute to post-sclerotherapy ischemic, thromboembolic or inflammatory complications.

The present paper follows a series of publications by this group investigating the effects of detergent sclerosants on coagulation, antithrombotic and fibrinolytic mechanisms.[4-8] We had preliminary *in vitro* evidence that platelets may play a role in the procoagulant activity exhibited by sclerosants.[4, 5] At low concentrations (0.075%-0.1%), both agents shorten phospholipid-dependent clotting times [5], initiate strong clots[6], and stimulate the release of PMP [4, 5]. At mid-range concentrations (0.15%-0.3%), both inhibit the contribution of platelets to clot strength and initiate weak clots prone to lysis.[6] At higher concentrations, both detergents induce platelet lysis and inhibit clot formation.[5, 6] Prior to the present study, the effects of sclerosants on platelets were not examined in great detail.

In this study, we investigated the *in vitro* effects of STS and POL on platelet integrity, PMP phenotype and platelet activation and aggregation. We also investigated the direct effect of these agents on platelet glycoprotein (GP) IIb/IIIa receptor structure and activation.

MATERIALS AND METHODS

Sclerosants

STS was obtained as FIBRO-VEIN 3%(w/v), 47.4mM (Australian Medical and Scientific, NSW, Australia) and POL as AETHOXYSKLEROL 3%(w/v), 51.5mM (Kreussler, Wiesbaden, Germany). All stated concentrations refer to the final concentrations.

Flow Cytometry Materials

The following were obtained from Becton-Dickinson (New Jersey, USA): flow cytometry tubes, annexin V-PE, flow cytometry antibodies to CD41a-FITC, CD42b-PE, CD42b-PE-Cy5, CD61-FITC, CD41a-PerCP-Cy5.5, CD62p-APC, CD63-PE, activated GPIIb/IIIa (PAC-1-FITC) and their respective isotype controls. Other materials included: lactadherin-FITC (Haematologic Technologies, Vermont, USA), GPIIb/IIIa heterodimer antibody (Abnova, Taipei, Taiwan), anti-mouse IgG-PE (Dako, Glostrup, Denmark), phosphate-buffered saline (PBS) and FLUO-3AM (Invitrogen, CA, USA), paraformaldehyde (ProSciTech, QLD,

Australia), collagen-related peptide (CRP, Department of Biochemistry, University of Cambridge, UK), thrombin receptor-activating peptide (TRAP), adenosine diphosphate (ADP) and latex size beads (Sigma-Aldrich, MO, USA).

Sample Collection and Processing

Whole blood (WB) was collected following informed consent from healthy volunteers taking no medications or supplements. In all experiments, WB was collected into citrate tubes (BD Vacutainer, 0.105M) except for Multiplate® where WB was collected in 3mL hirudin (25μg/mL) tubes (Dynabyte Medical, Munich, Germany). Platelet rich plasma (PRP) was obtained by centrifugation of WB at 150g for 10 minutes. Platelet poor plasma (PPP) was obtained by centrifugation of WB at 1700g for 30 minutes. All experiments were performed under non-shear conditions, with the exception of platelet function analyzer (PFA-100).

1. Platelet and Platelet Microparticle Count

Platelet and PMP counts were performed by flow cytometry using CD41a and annexin V as previously described.[9] PRP samples were incubated with dilutions of both sclerosants for 15 minutes. 50μL of test sample was added to 200μL of HEPES-buffered saline. 10μL of this mixture was then added to 5μL CD41a-PerCP-Cy5.5 antibody, 2μL annexin V-PE and 33μL HEPES-buffered saline containing $CaCl_2$ (2.5mM). This was incubated for 30 minutes before the addition of 1mL HEPES-buffered saline containing 2.5mM $CaCl_2$.

We detected a reduction in annexin V binding at higher concentrations of STS (see Results) and further assessed phosphatidylserine expression using lactadherin. Here, annexin V was replaced with 5μL of lactadherin-FITC and incubated in HEPES-buffered saline (without $CaCl_2$) for 30 minutes before the addition of 1mL of HEPES-buffered saline.

Counting was performed using TRU-Count tubes. Platelet-derived events were detected using CD41a. Platelets and PMP were discriminated using the forward scatter parameter and annexin V binding. The mean forward scatter of latex beads of mean diameters 0.82μm and 1.09μm was used to standardize the lower and upper size limits of platelets and PMP respectively. Flow cytometry was performed using an LSR-II flow cytometer (Becton-Dickinson) and FACSDiva software. Results were analyzed using FlowJo software (v8.7.1, Tree Star Inc, Oregon, USA).

2. Platelet Activation

The effect of sclerosants on platelet activation was assessed using ELISA and flow cytometry. In ELISA experiments, higher concentrations (up to 1.2%) of sclerosants were included to investigate a possible destructive effect of STS on target antigens. In flow

cytometry experiments, sclerosant concentrations were limited to 0.15% as platelets were significantly lysed at higher concentrations (see Results) and there were too few events for analysis.

2.1 Soluble Markers

Soluble (s) P-selectin (R&D Systems, Minneapolis, USA), von Willebrand factor (vWF) antigen (Hyphen-Biomed, Neuville-sur-Oise, France), sCD40 ligand (sCD40L, Alexis Biochemicals, Lausen, Switzerland) and serotonin (DRG, New Jersey, USA) levels were measured by ELISA. WB, PRP or PPP samples were incubated with serial dilutions of sclerosant for 15 minutes. All assays were performed in duplicate according to the manufacturers' instructions. For serotonin ELISA, as per the manufacturer's instructions, WB and PRP samples were incubated for 15 minutes, whereas PPP samples were incubated overnight. Absorbances were measured at 450nm using a plate reader (Rayto, Shenzhen, China). The inter-assay coefficients of variation for sP-Selectin, vWF, sCD40L and serotonin assays were 8.8%, 5-10%, 6.8% and 3.8%-6.6% respectively.

Control experiments were performed to determine the effect of sclerosants on target antigens in the absence of cells or cellular elements. Serial dilutions of sclerosants were prepared in 5% bovine serum albumin (BSA; Bio-Rad, CA, USA) and spiked in microtitre plates with target antigens derived from the standards in each kit (50ng sP-selectin, 10ng sCD40L, 125ng vWF or 390ng serotonin) before transfer to ELISA plates. The normal protocol for each ELISA was then followed.

Further control experiments were performed to exclude the possibility of a destructive effect of the detergents on the solid phase of the assays. All ELISA plates used in this study were coated by simple adsorption (direct communication with manufacturers). We identified two assays (sP-selectin and vWF) where high concentrations of STS caused a significant reduction in the target antigen in all media (WB, PRP, PPP and BSA) (see Results). In control experiments, 5% BSA samples were spiked with serial dilutions of STS and incubated in the ELISA wells for one hour. After five washes, 200μL of the supplied calibrator for each kit at the recommended concentration (7.25ng/mL sP-selectin, 10.1ng/mL vWF) was added to all wells and incubated for one hour. The normal protocol for each assay was then followed.

2.2 Membrane-Bound Markers

Flow cytometry was performed for CD62P (P-selectin) and CD63 (LIMP-1) expression and for PAC-1 (activated GPIIb/IIIa) binding on CD42b+ (GPIb) platelets and PMP. PRP samples (200μL) were incubated with serial dilutions of sclerosants (50μL) for 15 minutes at room temperature. 5μL of test sample was added to flow cytometry tubes containing 5μL of each antibody (anti-CD42b-PE-Cy5, anti-CD62p-APC, anti-CD63-PE, PAC-1-

FITC) or respective isotype control, with the volume made up to 50μL using PBS and as incubated for 20 minutes before the addition of 1mL of 0.2% paraformaldehyde in saline. Platelets were identified based on their expression of CD42b, with activation markers assessed based on their expression compared to 0.5% of their respective isotype control. A minimum of 10,000 CD42b+ events were acquired for analysis.

2.3 Platelet Calcium Kinetics

Platelet cytoplasmic calcium concentrations were measured using flow cytometry as previously described.[10] PRP (50μL) was added to 450μL PBS and 2.5μL of 1mM FLUO-3AM and incubated for 15 minutes. 25μL of this was added to flow cytometry tubes containing 5μL of CD41a-PerCP-Cy5.5, incubated for 15 minutes before the addition of 1mL PBS. Each sample was stirred and analyzed on the flow cytometer for 30 seconds before the addition of agonist or sclerosant and immediately returned for analysis. Samples were analyzed for a minimum of five minutes.

3. Platelet Aggregation

The effects of sclerosants on platelet aggregation were studied using several methods. Given the progressive lysis of platelets by high concentration sclerosants, these experiments were limited to concentrations up to 0.15%.

3.1 Platelet Function Analyzer

Platelet aggregation under controlled shear conditions was assessed by PFA-100 (Siemens, Marburg, Germany).[11] Whole blood samples (960μL) were incubated with serial dilutions of sclerosants (40μL). Samples were then analyzed and closure times (CT) in both collagen/ADP and collagen/epinephrine (EPI) cartridges measured.

3.2 Light Transmission Aggregometry

Light transmission aggregometry (LTA) was performed using an Aggregation Remote Analyzer Module (AggRam, Helena Laboratories, Texas, USA). All reagents were obtained from Helena Laboratories except for ADP (Sigma-Aldrich). Preparation of PRP and aggregation tests were performed as per published protocols.[12, 13] PRP samples (200μL) were incubated with serial dilutions of sclerosants (25μL). Samples were then incubated for 2 minutes at 37°C on the aggregometer. 25μL of each agonist was added at a final concentration of ADP (2.5 μM), collagen (5μg/mL), epinephrine (300μM) and arachidonic acid (0.5mg/mL). This was incubated for a minimum of 3 minutes, with results interpreted as the percentage of the maximum aggregation.

3.3 Impedance Aggregometry

This was assessed using Multiplate® (Dynabyte Medical).[14] WB samples (300μL) were

added to the test cells and diluted 1: 1 with saline preheated to 37°C. The mixtures were incubated with serial dilutions of sclerosants for 3 minutes before the addition of agonists which included TRAP (TRAPtest, 32μM), collagen (COLtest, 3.2μg/mL), arachidonic acid (AA, ASPItest, 0.5mM), ADP (ADPtest, 6.4μM) and ADP and prostaglandin (PG) E1 (ADP-HS; 6.4μM ADP + 0.3nM PGE1). All samples were run for 6 minutes. Results were interpreted as the area under the aggregation response curve.

4. GPIIb/IIIa Receptor

4.1 Subunit and Heterodimer Expression

Flow cytometry was performed to assess CD42b (GPIb), CD41a (GPIIb), CD61 (GPIIIa) and GPIIb/IIIa heterodimer expression. 5μL of test sample was added to flow cytometry tubes containing 5μL of each relevant antibody (anti-CD42b-PE, anti-CD41a-PerCP-Cy5.5, anti-CD61-FITC or anti-GPIIb/IIIa heterodimer) and made up to a total volume of 50μL using PBS. For GPIIb/IIIa heterodimer, samples were incubated for 10 minutes before the addition of 5μL of PE-labeled anti-mouse IgG. All reactions were performed for a total of 20 minutes before the addition of 1mL of 0.2% paraformaldehyde in saline and analyzed by flow cytometry. The mean fluorescence intensities corresponding to each antibody were then used as a measure of antigen density on the CD41a+ or GPIIb/IIIa heterodimer+ platelet and PMP populations.

4.2 GPIIb/IIIa Activation

Flow cytometry was performed for PAC-1 binding (as described earlier) using PAC-1-FITC antibody with the addition of platelet agonists TRAP (80μM), ADP (5μM) and CRP (200μg/mL) to flow cytometry tubes during staining.

RESULTS

1. Platelet and PMP Count

In PRP, sclerosants induced platelets lysis at ≥0.15% STS (Fig. 1A) and ≥0.09% POL (Fig.1B). PMP counts increased at ≥0.075% but then progressively decreased to reach zero at higher concentrations of detergents (1.2% STS, ≥0.6% POL). At >0.3% STS, PMP progressively failed to bind annexin V, but still demonstrated lactadherin binding (Fig. 1C).

2. Platelet Activation

2.1 Soluble Markers

At low concentrations (≤0.15%), STS increased the antigen levels of sP-selectin, vWF, sCD40L and serotonin in WB and PRP (Fig. 2). High concentration STS, reduced all antigen levels in all sample types with the exception of serotonin, where the level of antigen remained high.

Low concentration POL increased the antigen levels of sP-selectin in WB and PRP. By contrast to STS, sP-selectin antigen levels remained high at higher concentrations of POL. Low concentration POL induced smaller rises in vWF and sCD40L antigen levels which returned to baseline levels at higher concentrations of this detergent. Serotonin levels were not affected by POL in these experiments.

In control experiments performed in PPP and BSA, no increase in the antigen levels was detected. In solid phase control experiments, STS had no effect on the solid phase of the ELISA assays tested (results not shown).

2.2 Membrane-Bound Markers

The treatment of platelets with STS and POL increased the platelet expression of CD62p and CD63 (Fig. 3A and 3C). STS induced CD62p+/CD63+ PMP while POL only caused a mild increase in PMP exposure of CD63 (Fig. 3B and 3D).

2.3 Platelet Calcium Kinetics

At low concentrations, both sclerosants progressively increased the cytoplasmic concentrations of calcium reaching a peak at 0.0075% STS and 0.005% POL (Fig. 3E). This effect was rapid (<20s) in POL-treated samples, but there was a longer lag-time (approximately 200s) for STS-treated samples. At higher concentrations (>0.01% STS, >0.0075% POL), platelets were lysed within seconds, PMP were formed and there was no detectable release of calcium (results not shown).

3. Platelet Aggregation

3.1 Platelet Function Analyzer

The normal range for PFA-100 CT was established at 80-170s for EPI-CT and 65-120s for ADP-CT. The CT was progressively increased by both agents in both cartridges and at 0.15% was indefinitely prolonged (>300s) (Fig. 4).

3.2 Light Transmission Aggregometry

Both sclerosants inhibited agonist-stimulated platelet aggregation in a dose-dependent manner, with aggregation to ADP and epinephrine more affected than collagen and arachidonic acid (Fig. 5A and 5B). When stimulated by ADP or collagen, low concentration POL demonstrated a stronger inhibitory effect on platelet aggregation than equivalent concentrations of STS. At 0.1%, the maximal aggregation response to ADP was reduced to 35%-75% by STS but to 6%-35% by POL. The primary aggregation response (slope) to ADP and epinephrine was retained but the secondary aggregation response was inhibited by both agents. This was achieved by POL at a much lower concentration (0.0125%) compared with STS (0.025% for ADP and 0.05% for epinephrine). In the absence of any platelet agonists, 0.1% STS induced an immediate spontaneous aggregation of up to 30% which was reversed completely within 2 minutes (Fig. 5C).

3.3 Impedance Aggregometry

Agonist-stimulated platelet aggregation was inhibited by both sclerosants in a concentration-dependent manner (Fig. 6). Complete inhibition was achieved at 0.1% in all assays with the exception of TRAP where approximately 50% inhibition was achieved. In the absence of platelet agonists, low concentration STS induced a small rise in platelet aggregation.

4. GPIIb/IIIa Receptor

4.1 Subunit and Heterodimer Expression

The surface expression of GPIIb, GPIIIa, GPIIb/IIIa heterodimer and GPIb on platelets and PMP was not affected by sclerosants (results not shown).

4.2 GPIIb/IIIa Activation

Both sclerosants reduced the ability of platelets to bind PAC-1 following agonist stimulation (Fig. 7A and 7B). This decrease was most prominent in ADP-stimulated samples. In the absence of agonist stimulation, PAC-1 binding was increased by 0.1%STS, but not affected by POL (Fig. 7C).

DISCUSSION

Detergent sclerosants are typically administered as a foam or liquid at concentrations ranging from 0.1% to 3.0% depending on the diameter of the target vessel. Although the initial concentration of the agent may be high, the subsequent mixing with blood results in a decrease in the free concentration due to dilution and neutralisation by plasma proteins.[4, 15] These agents function by inducing endothelial lysis, exposing the underlying collagen with the ultimate aim of achieving endovascular fibrosis. However, whether this is achieved via a transitional state of thrombosis, direct induction of endovascular fibrosis or induction of a transient inflammatory state is unclear and debated.[16, 17] At low concentrations, both agents demonstrate procoagulant activity and initiate clot formation in the absence of vascular injury *in vitro*.[5, 6, 8, 18] In this study, low concentration sclerosants induced platelet activation but inhibited platelet aggregation by interfering with the agonist-stimulated activation of GPIIb/IIIa receptor.

Consistent with previous reports, both detergents induced platelet and PMP lysis at higher concentrations.[4, 5, 18, 19] A number of *in vivo* studies have shown a significant decrease in the platelet count, 1-3 days after sclerotherapy treatment with STS[20-22] and POL [20] while some have reported no significant change with POL sclerotherapy[16, 21]. Lysis of biological membranes requires a minimum effective concentration of the detergent (critical solubilisation concentration) [23] and the apparent discrepancy is most likely due to a decrease in the final free concentration of POL after significant mixing with blood.[4]

Both STS and POL induced platelet activation in the absence of other platelet agonists. This was demonstrated by a number of methods and evident by a rise in the free cytoplasmic calcium and both soluble and membrane-bound markers of platelet activation. We also demonstrated that both detergents increased the platelet and PMP surface expression of phosphatidylserine, an anionic phospholipid usually expressed on the cytoplasmic surface of the membrane. Exoplasmic exposure of phosphatidylserine can result in the activation of coagulation enzyme complexes such as tenase and prothrombinase, thrombin generation and activation of further platelets. Hence the mechanism of activation probably involves the non-specific but direct interaction of detergents with surface membrane lipids and the subsequent exoplasmic exposure of phosphatidylserine. At high STS concentrations, sP-selectin, vWF and sCD40L levels were reduced in all sample types. This was most likely due to denaturation of the protein molecules by this anionic detergent.[23]

Experiments to assess cytoplasmic calcium were performed in diluted PRP and hence much lower sclerosant concentrations were required to achieve calcium release due to reduced protein binding.[4] Treatment with POL induced an immediate increase, whereas there was a prolonged lag-time in the STS treated samples. This was not unexpected as the polyoxyethylene chains of POL contain hydrophilic-hydrophobic regions which

allow the surfactant to flip-flop rapidly across the membrane. By contrast, detergents with strong hydrophilic heads such as STS flip-flop at a slower rate, resulting in delayed solubilisation.[23]

In all platelet aggregation studies, both sclerosants induced a dose-dependent inhibition of agonist-stimulated platelet aggregation while the PFA-100 CT was progressively prolonged. ADP and epinephrine are considered weak platelet agonists[24] while thrombin is the most potent platelet activator.[25] Consistently, both agents showed a stronger inhibitory effect on ADP and epinephrine stimulation in LTA experiments and a weaker inhibitory effect on TRAP when tested by impedance aggregometry. The presence of platelet aggregation in TRAP stimulated samples suggests that the capacity of platelets to aggregate was not completely diminished.

Normally, agonist-stimulated activation of platelets results in a sequence of events that leads to a conformational change in the GPIIb/IIIa heterodimer and its subsequent ability to bind fibrinogen.[26] We have previously shown that fibrinogen levels are not affected by low concentration sclerosants.[6] In this study, the inhibition of platelet aggregation was not due to a reduced expression of the individual GPIIb/IIIa subunits or dissociation of the heterodimer complex but due to a decreased activation of this receptor. Therefore, the sclerosants appear to interfere with the signal transduction pathways that converge on GPIIb/IIIa receptor. One possible mechanism may involve the gradual incorporation of the detergent molecule in the cell membrane, membrane destabilisation and interference with the function of membrane cytoskeletal proteins such as talin[27], necessary for GPIIb/IIIa activation.

Although agonist-stimulated platelet aggregation was inhibited, low concentration STS induced a spontaneous reversible platelet aggregation. This was detected by LTA and impedance aggregometry and corresponded to a rise in PAC-1 binding in the absence of any platelet agonists. Low concentration STS has been previously reported to induce platelet aggregation on LTA.[18] The mechanism possibly involves platelet activation leading to aggregation followed by a gradual destabilisation of membrane lipids resulting in disaggregation. This finding was specific for STS and is consistent with the prolonged lag time and the longer solubilisation time required by this detergent as against POL.[23]

Consistent with our previous reports, low concentrations of both agents stimulated PMP release. The majority of microparticles were capable of binding annexin V but at higher STS concentrations (0.3%-0.6%), annexin V binding was reduced. Further experiments using lactadherin, a calcium-independent phospholipid probe, confirmed these events to be phosphatidylserine+ PMP. Annexin V binding was most likely reduced due to interference of STS, an anionic detergent, with extracellular cationic calcium required for detection of phosphatidylserine with annexin V.[28]

Clinically, sclerotherapy has been associated with a range of thrombo-occlusive, inflammatory and ischemic neurological adverse events. Although platelets play a limited role in venous thromboembolism [29], platelet activation plays an important role in arterial ischemic events. We have previously shown that low concentrations of both agents initiate strong clots in a platelet-dependent fashion and in the absence of vascular injury.[6] The direct effect of sclerosants on platelets and the subsequent activation and degranulation would contribute to the process of thrombus formation. Platelet activation may lead to recruitment of inflammatory cells such as leukocytes and formation of platelet-leukocyte aggregates.[30] Such interactions may play an essential role in the local thrombo-inflammatory complications of sclerotherapy such as thrombophlebitis.

In this study, the phenotype expressed by sclerosant-induced PMP suggests a potential capacity for initiation of thrombo-inflammatory processes at distant sites. Both agents induced phosphatidylserine+/CD63+ PMP while STS-induced microparticles also expressed P-selectin. P-selectin+ PMP may bind to tissue factor (TF) expressing monocytes, stimulating further TF release and initiation of the coagulation cascade.[31] A systemic release of microparticles has not been investigated in clinical studies but such an event may play a pathogenic role in the neurological and possibly other thrombotic adverse events of sclerotherapy.

STS (but not POL) stimulated the release of serotonin from platelets. Serotonin has been implicated in the pathogenesis of migraine, with a number of studies associating decreased platelet [32-34] and increased plasma serotonin [33] with the onset of migraine. Migraine is known to be a possible adverse consequence of sclerotherapy.[35] Based on our current findings, serotonin release from platelets is an unlikely cause of migraine in patients treated with POL. Serotonin is a potent vasoconstrictor and may contribute to the clinically observed vasoconstrictive effects of STS.

This study had a number of limitations. The mechanism of sclerosant-induced platelet activation and pathways leading to inhibition of GPIIb/IIIa activation require further investigations. The effect of sclerosants on thrombin generation, vWF activity and the *in vivo* effects of sclerosants on platelets and PMP are being investigated by the authors in other studies.

SUMMARY

Both sclerosants lysed platelets and PMP at high concentrations. At low concentrations (<0.15%), both agents activated platelets while progressively inhibiting platelet aggregation by interfering with GPIIb/IIIa activation. At ≥0.075%, both stimulated the release of phosphatidylserine+ PMP that did not express the active form of GPIIb/IIIa.

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FIGURES

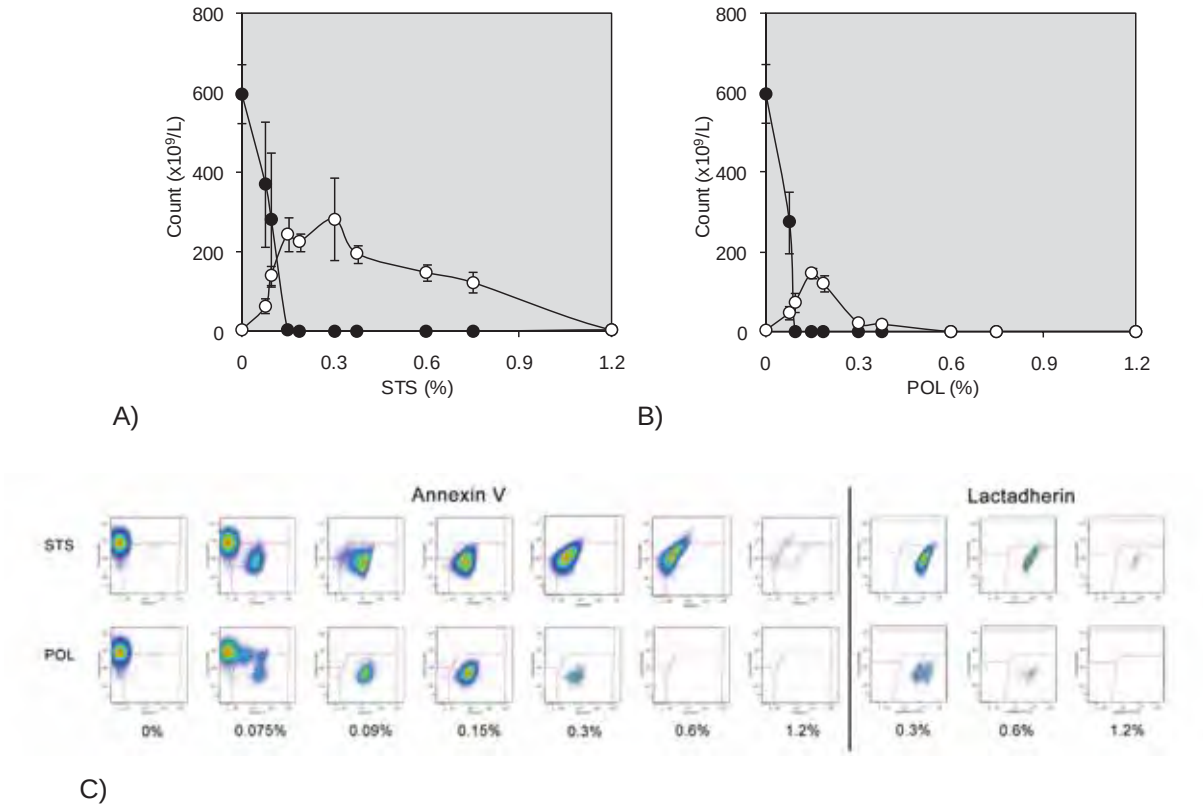

Figure 1

The effect of (A) STS and (B) POL on platelet (•) and platelet microparticle (PMP) count (o) in platelet rich plasma (PRP). Results are the mean±SEM (n=4). (C) Representative scatter plots demonstrating annexin V or lactadherin binding (X-axis) versus forward scatter (Y-axis) of sclerosant-treated PRP on platelet and PMP counts (n=4).

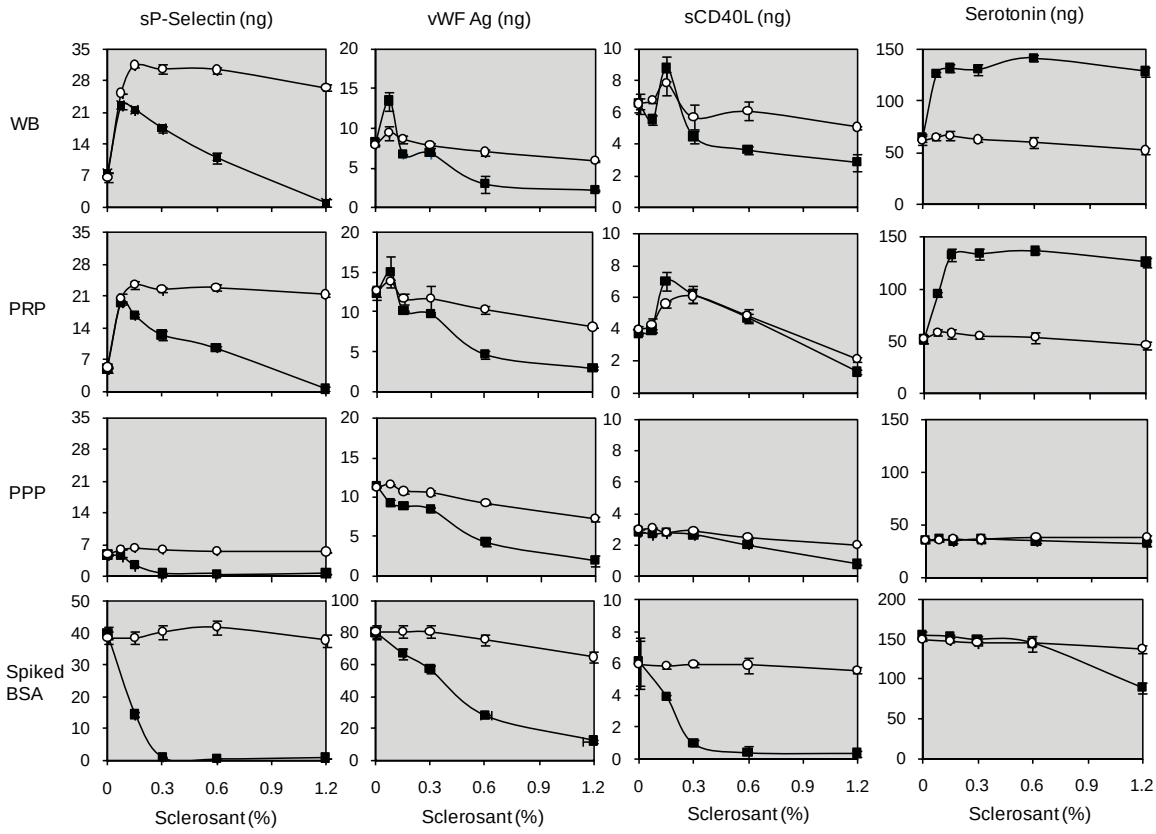

Figure 2

The effects of STS (□) and POL (○) in whole blood (WB), platelet-rich (PRP) and platelet poor plasma (PPP) on soluble (s)P-selectin, von Willebrand Factor (vWF) antigen, sCD40L and serotonin. Control experiments were performed in 5% bovine serum albumin (BSA) spiked with each standard. Results are the mean±SEM (n=4).

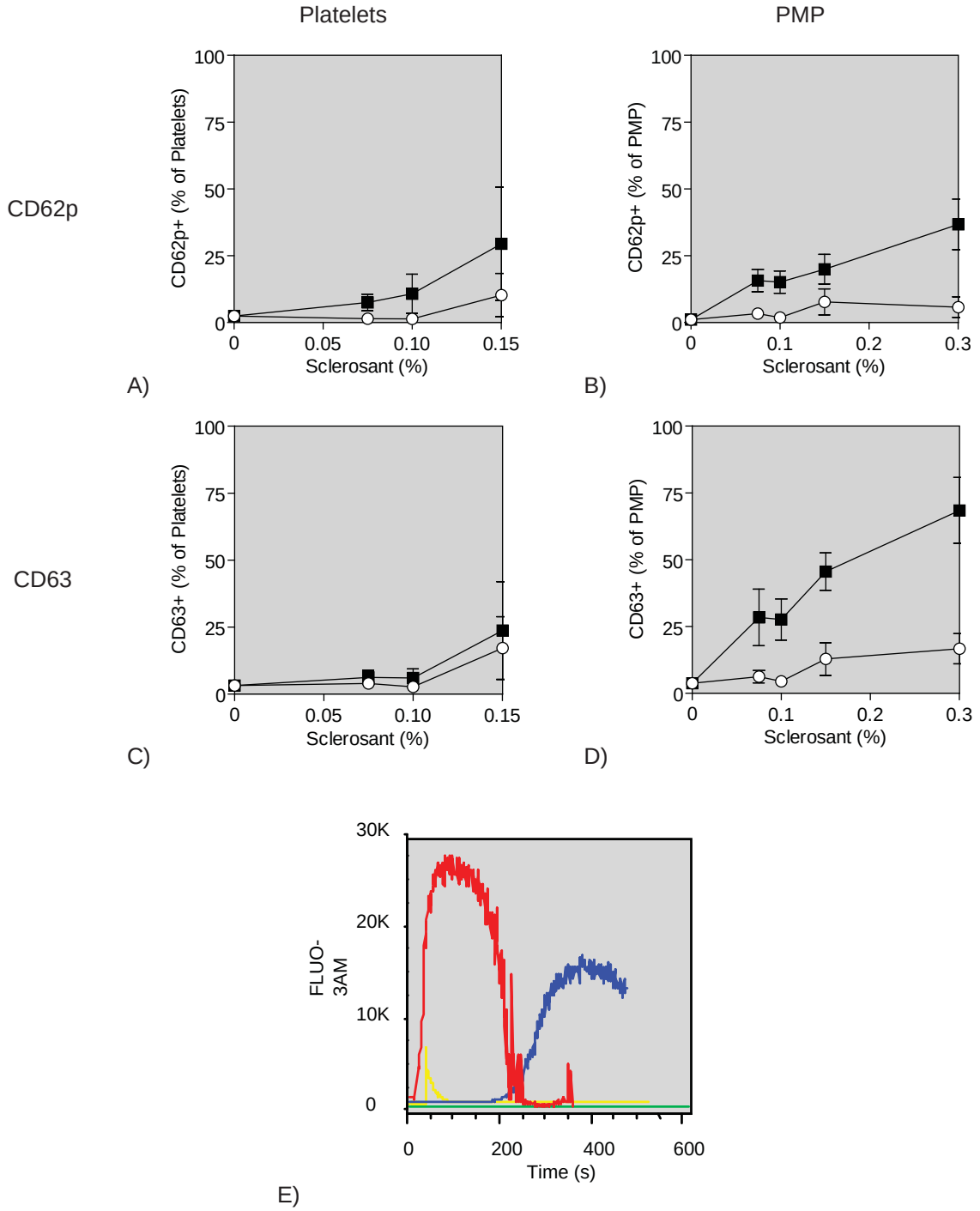

Figure 3

The effects of STS (■) and POL (○) in platelet-rich plasma (PRP) on the exposure of (A) platelet P-Selectin (CD62p), (B) platelet microparticle (PMP) CD62p, (C) platelet CD63 and (D) PMP CD63. Results are the mean±SEM (n=4). (E) Representative traces demonstrating cytoplasmic calcium release from un-stimulated (green) or 0.0075% STS (blue), 0.005% POL (red) or 2.5μM ADP (yellow) treated FLUO-3AM loaded platelets (n=4).

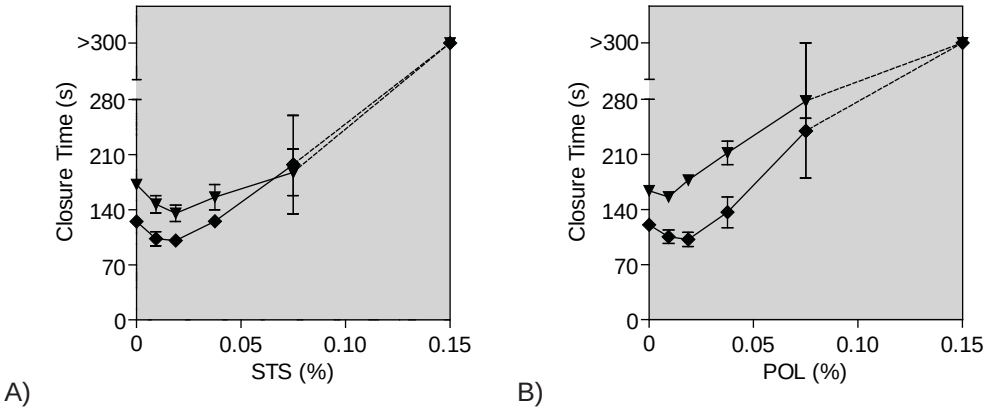

Figure 4

The effects of (A) STS and (B) POL on platelet function under high shear stress as assessed by platelet function analyzer (PFA-100) using Collagen-ADP (◆) or Collagen-Epinephrine (▼). Results are the mean±SEM (n=4).

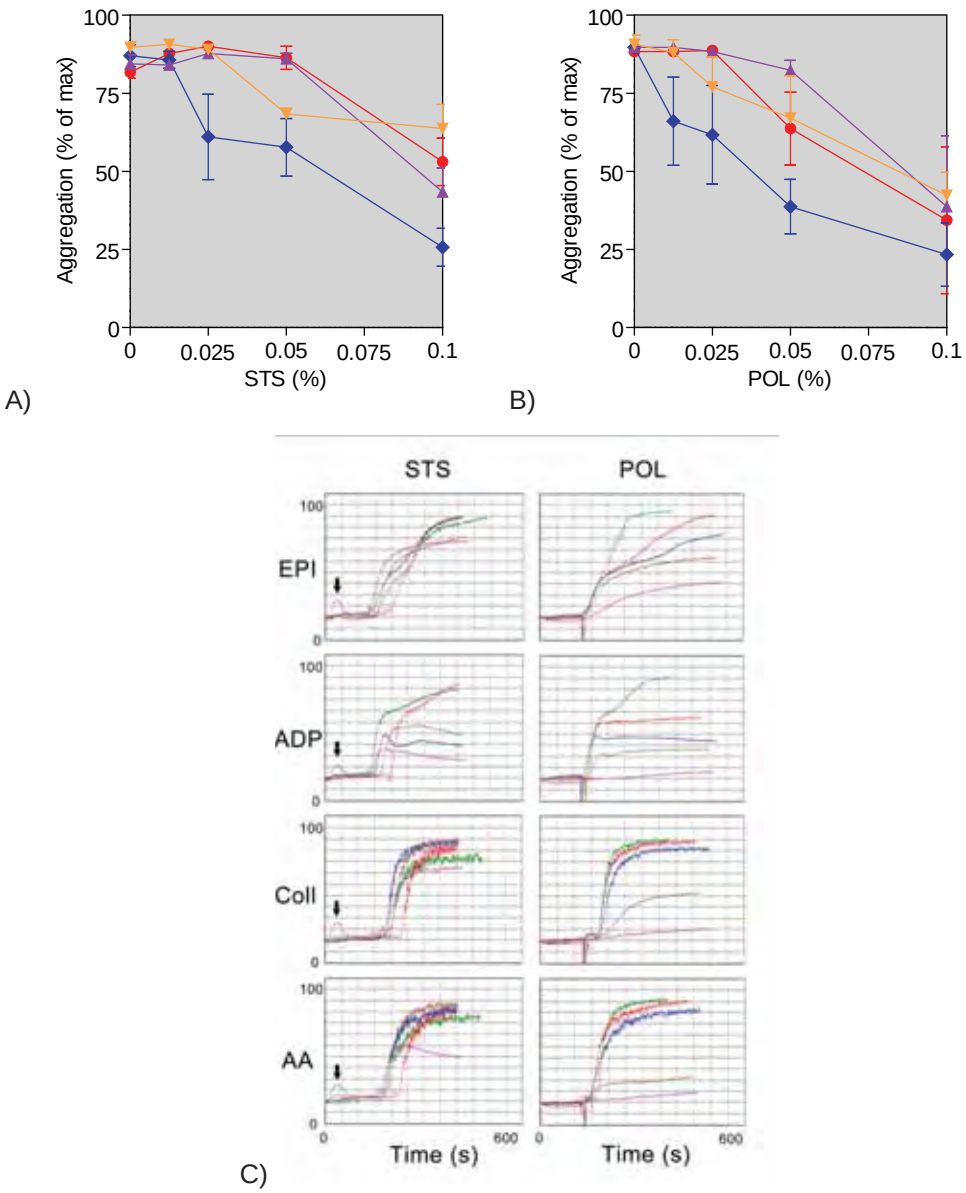

Figure 5

The effects of (A) STS and (B) POL on platelet aggregation in presence of platelet agonists: ADP (blue, ◆), collagen (red, ●), epinephrine (yellow, ▼), arachidonic acid (purple, ▲). Results are the mean±SEM (n=3). (C) Representative light transmission aggregometry (LTA) traces for both sclerosants. The X-axis corresponds to time and the Y-axis corresponds to percent aggregation. Sclerosant concentrations: 0.0125% (red), 0.025% (blue), 0.05% (brown) and 0.1% (pink). The arrow indicates a reversible aggregation in 0.1% STS treated samples. EPI, epinephrine; ADP, adenosine diphosphate; Coll, collagen; AA, arachidonic acid.

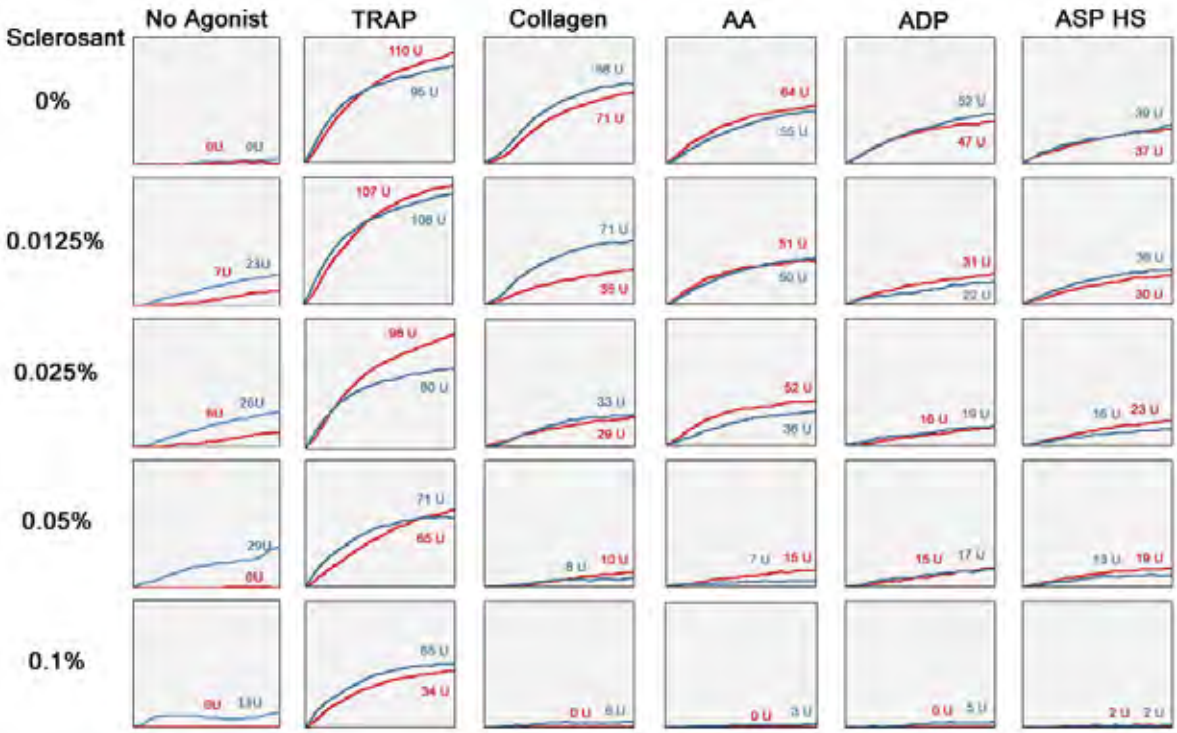

Figure 6

Representative traces of the effect of sclerosant treatment on platelet aggregation by Multiplate® impedance aggregometry. Whole blood (WB) samples were incubated with STS (blue) or POL (red) in presence of no agonist or thrombin receptor-activating peptide (TRAP), collagen, arachidonic acid (AA), adenosine diphosphate (ADP), and ADP/ prostaglandin E1 (ADP-HS). X-axis represents time (total 6 minutes) and Y-axis represents units of aggregation.

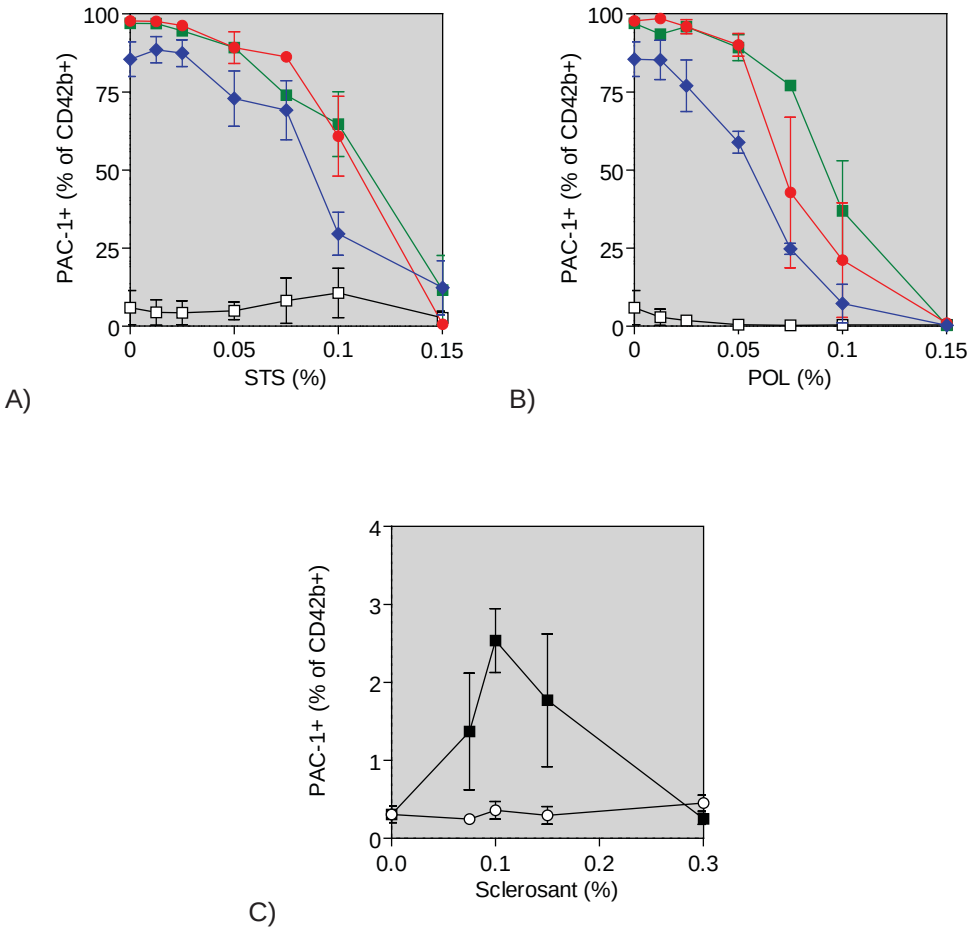

Figure 7

The effects of sclerosants on GPIIb/IIIa activation. PAC-1 binding was assessed in platelet rich plasma (PRP) samples incubated with (A) STS or (B) POL and either un-stimulated (black,□) or in presence of each agonist: adenosine diphosphate (ADP; blue,◆), collagen-related peptide (CRP; red,●) or thrombin receptor-activating peptide (TRAP; green,■). (C) In the absence of platelet agonists, 0.1%, STS (■) induced a rise in PAC-1 binding to platelets in PRP samples while POL (○) had no effect. Results are the mean±SEM (n=4).

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CHAPTER 8

Conclusion

Given the clinical incidence of thromboembolic and neurological ischemic complications of sclerotherapy and yet the conflicting literature on the haemostatic effects published to date, these investigations were initiated to examine the interaction of detergent sclerosants, STS and POL, with coagulation, antithrombotic and fibrinolytic mechanisms.

The general hypothesis of this thesis was that detergent sclerosants are *not* thrombogenic. The key finding of this thesis is that the effect of these agents on the haemostatic mechanisms is concentration-dependent. Both sclerosants indeed exhibit procoagulant activity and initiate strong clots at low concentrations. However, at higher concentrations both agents prevent clot formation and STS in particular exhibits strong anticoagulant activity.

The procoagulant activity of both detergents was limited to low concentrations and manifested by shortening of phospholipid-dependent clotting times (Chapter 2). The low concentrations were enough to activate platelets but not to induce significant platelet lysis (Chapters 2, 3 and 7). Platelet activation resulted in the release of procoagulant phosphatidylserine+ PMP and soluble factors from platelet granules. Low concentration POL demonstrated further prothrombotic activity by increasing the resistance of FVa to the proteolytic effects of APC (Chapter 4). This procoagulant activity was further confirmed in the global assays of coagulation where both agents enhanced strong clot formation at low concentrations (Chapter 6).

At slightly higher concentrations (mid-range concentrations of 0.15-0.3% in whole blood) both agents progressively lysed platelets and induced weak clots prone to lysis. In addition, due to its denaturing properties, STS reduced fibrinogen and FXIII levels. At higher concentrations (>0.3% in whole blood), platelet and PMP were lysed and the procoagulant activity was lost. STS destroyed clotting factors V, VII and X and demonstrated more anticoagulant activity than POL on conventional clotting tests (Chapter 2). In functional assays, STS exhibited potent anticoagulant properties as evident by significant anti-Xa, anti-IIa and anti-Va activity (Chapter 4). Furthermore, high concentration STS completely destroyed fibrinogen and prevented clot formation in the global assays of coagulation (Chapter 6). In general, POL showed less anticoagulant activity but nonetheless, this agent induced platelet and PMP lysis and prevented clot formation at high concentrations (Chapters 2, 3, 6 and 7).

In these studies, both sclerosants demonstrated anti-fibrinolytic activity (Chapter 5). At low concentrations, both agents enhanced the release of inhibitors of fibrinolysis such as PAI-1, antiplasmin and TAFI to demonstrate a net anti-fibrinolytic profile. At high concentrations, STS showed significant anti-fibrinolytic activity due to destruction and inhibition of fibrinolytic enzymes, plasminogen and t-PA. Neither agent had a direct lytic effect on cross-linked fibrin but high concentration STS destroyed non-cross-linked fibrin (Chapter 6).

An important hypothesis tested in this thesis was that albumin and other plasma proteins may inhibit the lytic activity of both sclerosants (Chapter 3). Given their surfactant nature, the main target of detergent sclerosants is lipids and in particular phospholipids of cell membranes. At high sclerosant concentrations, platelets, PMP, erythrocytes and endothelial cells were destroyed. Platelets appeared to be more resistant to lysis by sclerosants when compared with erythrocytes. It was demonstrated for the first time that plasma components and in particular albumin appeared to neutralise the lytic effects of sclerosants. The extent of the neutralisation process was dependent on the volume of blood with which the sclerosant was mixed. POL was significantly more inhibited in whole blood than STS.

These findings have partially explained why foam sclerosants are more effective than liquids. The displacement of blood by foam sclerosants reduces the neutralisation of detergents by serum albumin and other plasma components. Neutralisation of sclerosants by blood components possibly plays an important role in the low incidence of post-sclerotherapy venous thrombomebolic events and the low incidence of distant effects.

This study highlighted a number of differences between the two most commonly used sclerosants, STS and POL. STS, an anionic detergent, demonstrated protein denaturing properties as evident by destruction of clotting factors and fibrinolytic enzymes and most target antigens in ELISA assays. In functional assays, high concentration STS exhibited potent anticoagulant activity. Despite the observed anticoagulant properties of high concentration STS, this sclerosant is not necessarily a better or safer sclerosant than POL. STS certainly demonstrated stronger lytic activity in whole blood but POL could achieve the same or even higher potency if exposed to less intra-vascular blood.

The clinical relevance of these findings requires further investigations in the context of the free sclerosant concentration and the site of action. The final effect of the sclerosant on the target vessel depends on the degree of mixing and neutralisation by blood, the extent of endothelial lysis, the subsequent vasospasm and vessel wall approximation. Dilution and neutralisation by blood components would reduce the final free concentration of the detergents resulting in reduced lytic potency. But more importantly, the detergent can activate platelets and initiate clot formation at low concentrations. Organisation of such clots would result in vessel fibrosis and treatment success. However, exposure of such clots to running blood from adjoining vessels may result in thrombophlebitis, recanalisation and treatment failure (discussed in Chapter 6). Thrombophlebitis can lead to deep vein thrombosis and other complications of sclerotherapy such as post-inflammatory and haemosiderin pigmentation and telangiectatic matting (discussed in Chapters 6 and 7). Release of procoagulant PMP may also contribute to the local and distant thrombotic and other complications of sclerotherapy (discussed in Chapter 7). By contrast, a high final concentration, in particular in the absence of intra-vascular blood, would achieve

endothelial lysis, exposure of the underlying collagen and vessel fibrosis without thrombosis. However at high final concentrations, sclerosants can induce haemolysis of intra-vascular blood and such non-thrombosed haemolysed blood may persist within the lumen. Such non-coagulated 'trapped blood' can be clinically expressed and released weeks after the procedure and may possibly contribute to recanalisation of the treated vessels and persistent haemosiderin pigmentation.

Based on the present findings, it can be inferred that to maintain control over the final lytic and haemostatic effects of sclerosants, both from a safety and efficacy points of view, it is prudent to minimise the mixing and diffusion of sclerosants in blood. Empty vein techniques including the use of foam sclerosants and catheter-guided techniques that incorporate tumescent anaesthesia may achieve this clinically and need to be investigated in future studies.

These studies were not without limitations. Both STS and POL are produced as liquid but used by most practitioners as foam. Due to technical reasons, only liquid sclerosants were investigated in these experiments. The *in vivo* effects of both liquid and foam sclerosants on the haemostatic systems are currently under investigation by the candidate and co-authors. In these ethics-approved studies, samples from target veins, adjoining deep veins and systemic blood are collected from patients undergoing sclerotherapy and analysed for general systemic effects as well as procoagulant, anticoagulant and fibrinolytic parameters.

The experimental findings in these studies have raised further research questions. The mechanism of inhibition of platelet aggregation needs to be investigated in future studies. Also given the findings described in Chapter 7, future clinical studies should incorporate the possible effects of platelet inhibitors on sclerotherapy treatment outcomes. The activating effect of low concentration sclerosants may not be limited to platelets and these agents may activate other cells such as endothelial cells. Activation of endothelial cells would contribute to the angiogenic complications of sclerotherapy such as telangiectatic matting. This may be mediated via the release of angiogenic cytokines and/or endothelial microparticles (EMP). Detachment of endothelial cells may result in an increase in the number of circulating endothelial cells (CEC) that may contribute to distant complications of sclerotherapy. Further studies are being designed by the candidate and co-authors to investigate the effects of sclerosants on endothelial activation, CEC and EMP. Also of interest would be investigation of the effects of sclerosants on inflammatory cells and the subsequent release of inflammatory cytokines.

In summary, this study is a detailed investigation of the interaction of the two most commonly used detergent sclerosants, STS and POL, on coagulation, antithrombotic and fibrinolytic mechanisms. The effect of sclerosants was strongly influenced by the extent of neutralisation and the free final concentrations of these agents. Both detergents

demonstrated procoagulant and anti-fibrinolytic properties at low concentrations as evident by activation of platelets, release of procoagulant PMP, initiation of strong clots and potentiation of inhibitors of fibrinolysis. At high concentrations, both agents lysed platelets and PMP and inhibited clot formation but STS in addition exhibited potent anticoagulant and anti-fibrinolytic activity. Some of the key findings of this study, such as the neutralisation of sclerosants by plasma proteins and the procoagulant effects at low concentrations, have lead to a better understanding of the clinical outcomes of sclerotherapy. These experimental findings have resulted in modifications in the clinical practice and development of novel treatment techniques such as tumescent ultrasound guided sclerotherapy. Future studies following on from the understanding developed here may lead to increased safety and effectiveness of this popular procedure and would ultimately improve the treatment outcomes.

CHAPTER 9

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