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 publications⁷,⁸ 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...
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 cloting 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 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.

Factor Xa activated clotting time (XACT) tests were carried out as previously described. 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.

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 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 and the kaolin clotting time 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 dilutions.
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 CaCl₂. Control tubes contained 2.5 mM K₂EDTA. This was incubated for 30 minutes before the addition of 1 mL HEPES buffer containing 2.5 mM CaCl₂. 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.
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

<table>
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<th>FACTOR</th>
<th>PNP with 0.3% POL</th>
<th>PNP with 0.3% STS</th>
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<tr>
<td></td>
<td>T = 5 min</td>
<td>T = 30 min</td>
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<tr>
<td>II</td>
<td>102%</td>
<td>97%</td>
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<tr>
<td>V</td>
<td>70</td>
<td>59</td>
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<td>XII</td>
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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. 2d). 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. An interesting paradox appears with sporadic reports of DVT following direct vision sclerotherapy of reticular veins using low concentration agents.

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. 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 fibrinogen (ogen). We can only postulate that such deposits can possibly occlude small vessels leading to arteriolar ischaemia 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

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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.
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 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 effect. There are other agents reported to have a similar effect on clotting tests. 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.

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 vivo studies.

Acknowledgements

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