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Efficacy of A Collagen Hemostat Versus A Carrier-bound Fibrin Sealant

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Abstract:

Aim: A fast activation time of hemostatic agents with blood must be sufficient to effectively stop bleeding within surgical procedures. However, there are no functional *in vitro* tests of hemostatic agents which mimick such a clinical application.

Method: The efficacy of two common hemostatic agents was examined with heparinised human whole blood (0.7 IU/ml) and a contact time of only three minutes between blood and hemostatic agents. Traditional biochemical assays were compared with a new rheometric method for measuring clot formation.

Results: Blood without previous material contact (negative control) induced a basal thrombin-antithrombin (TAT, 240 ± 85 µg/l) or ß-thromboglobulin (TG, 1000 ± 216 U/ml) complex formation. Stainless steel (positive control) or a thrombin coated equine collagen fleece failed to increase TAT or ß-TG. However, a bovine collagen fleece significantly increased formation of TAT (1426 ± 378 µg/l) or ß-TG (3829 ± 857 U/ml). In rheometric measurements of the negative control the clotting time (CT) was 17 ± 4 min and the clot strength (CS) was 71 ± 45Pa. In the positive control CT (stainless steel) was 9 ± 3 min and CS was 298 ± 68Pa. The equine collagen fleece caused no detectable stimulation of CT and CS whereas the bovine collagen fleece (CT 13 ± 3 min, CS 186 ± 86Pa) was almost as effective as stainless steel.

Conclusion: Traditional biochemical parameters fail to indicate thrombogenicity under the tested conditions but oscillatory shear rheometry is a sensitive tool to analyse blood coagulation *in vitro*. Furthermore, mimicking the clinical relevant application times, the rheometric method detects functional differences of hemostatic agents. Since these differences correlate with *in vivo* data, the rheometric method is a valuable tool during the development of hemostatic agents.

Keywords: Collagen; Thrombin; Biochemical parameters; Bleeding

Introduction

Surgical interventions on parenchymatous organs may result in diffusive bleedings which are hard to control. Additionally heavy blood loss might occur while suturing vascular anastomoses. Apart from different surgical measures to stop such bleedings, a very common measure is the use of topical agents. These hemostatic agents are wound dressings which promote coagulation within several minutes of application. Bone wax, oxidized regenerated cellulose, gelatine, collagen, fibrin sealants and synthetic glues are available interventions. Recently, some compendial reviews about the use of different hemostats appeared, especially on fibrin and collagen sealants [1-3].

Collagen based hemostats favour adhesion and aggregation of platelets which in turn promote coagulation [4-7]. Tachosil® is a combination product of an equine collagen fleece containing the fibrin glue components human thrombin and human fibrinogen. The coagulation is induced by platelet adhesion combined with local fibrin generation. The efficacy and safety of Tachosil® has already been demonstrated in human, prospective randomized trials where Tachosil® was found to be superior to standard hemostatic suturing, argon beamer coagulation, and conventional hemostatic materials [8-14]. Sangustop®, a hemostatic device, is a bovine collagen based material without any coagulation activators. In a recent preclinical comparison Sangustop® showed superior hemostatic effects in pigs compared with Tachosil® [15]. With Sangustop®, diffuse liver bleeding time was significantly shorter and significantly fewer fleeces were needed to stop liver bleeding. This finding was surprising since no functional *in vitro* test supported this improved *in vivo* function. The objective of this study was to install oscillation rheometry as a simple but reproducible and economical method to determine the efficacy of hemostatic agents while using individual blood samples from different patients.

Materials and Methods

Materials

Human blood (500 ml blood bag containing 0.7 U /ml heparin) was obtained from the local blood bank. Blood was incubated in 50 ml polystyrene test tubes (Greiner, Germany). Protamine was from Sigma (Germany). Dilution of protamine was performed with phosphate buffered saline (Lonza, Belgium). The ELISA for Thrombin-Antithrombin-complex determination (TAT) was Enzygnost (Siemens healthcare diagnostics, Eschborn, Germany). The ELISA for ß-Thromboglobulin (ß-TG) was Asserachrom ß-TG (Roche Diagnostics, Mannheim, Germany). Stainless steel sticks used as positive controls were from Rocholl (Aglasterhausen, Germany). Since endotoxins are known to interfere with the coagulation cascade [16,17] they have to be removed from steel surfaces. Sticks cleaned with isopropanol were additionally depyrogenated at 250°C for 60 min in a heating coil (Heraeus, Germany) according to the present EP monography [2.6.14]. Two commercially available hemostatic agents were tested. Sangustop®(BIBraun, Germany) is a bovine collagen fleece and Tachosil®(Takeda, Germany) is an equine collagen fleece with Fibrinogen and Thrombin. The expiry date of both test samples was well within the experimental execution time.

Preincubation of blood with various materials

Blood incubation experiments with the hemostatic agents were performed in 10 ml blood aliquots prepared in 50 ml centrifugation tubes. Stainless steel or hemostatic agents (surface area 12 cm^2) were added and incubations were performed at 37°C under soft agitation for a period of three minutes. Preincubation of blood was performed in a Titramax 1000 (Heidolph, Germany). Immediately after incubation, protamine was added to neutralise heparin and a sample was placed within the rheometer for detection of coagulation. The amount of protamine was titrated for each individual blood bag to result in a coagulation time frame of approximately 15 min. Blood was used only on the day of delivery since storage for more than 24 h activates the coagulation cascade [18].

Biochemical analysis

For biochemical analyses following blood incubation, samples were centrifuged at room temperature with 2500 g for 20 min, the supernatant (plasma) was taken in aliquots and frozen to -80°C. Plasma sample aliquots from the negative control, steel (positive control), Sangustop® or TachoSil® were thawed and used in ELISA analysis according to the manufacturer's instructions. Sample concentrations were pretested with some representatives before all samples were diluted to be tested in the assays.

Rheologic measurements

A calibrated Kinexus pro rheometer (Malvern Instruments, Herrenberg, Germany) was used for analysis of blood coagulation as recently described [18]. In brief, experiments were with 1650 µl blood aliquots. Blood was placed on the bottom plate of the rheometer. The upper cone (stainless steel, angle 4°, 4 cm diameter) was immediately adjusted leaving a gap of 400 µm. Oscillation started with 1 Hz and a target shear strain of 2%. Storage modulus (G´) was determined every 5 sec over a period of 30 min at a temperature of 37°C. Data are presented as time course of G´ alterations.

Statistics

Mean values are calculated for clotting time (CT, in min) and clot strength (CS, in Pa). CT was calculated as time point when G´ exceeded the mean value of the baseline by more than 3 standard deviations (SD). CS was calculated as the maximal G´ value observed within 30 minutes of observation.

6 blood bags from different individuals were used for experiments. Mean values of these 4 groups were compared after performing ANOVA with Dunnett multiple comparisons test against the negative control. Significance was determined at a level of P<0.05.

Results

Biochemical analysis

The incubation of blood with different materials provoked the formation of TAT or ß-TG. TAT indicates activation of the coagulation cascade, ß-TG indicates activation of platelets. The formation of TAT or ß-TG after 3 minutes of blood incubation with the indicated agents is summarised in Table 1. Whereas there was no significant activation of coagulation by steel after 3 minutes of incubation, TAT or ß-TG significantly increased after 5 minutes of incubation. Thus the TAT concentration after 5 min of incubation reached 1164 ± 341 µg/l and ß-TG reached 2616 ± 491 U/ml.

S. No	Material	TAT (µg/l)	ß-TG (U/ml)	N
1	neg. control	240 ± 85	1000 ± 216	6
2	Stainless Steel	670 ± 327	1415 ± 295	6
3	Sangustop®	1426 ± 378[a]	3829 ± 857[b]	6
4	Tachosil®	632 ± 196	1647 ± 420	6

Table 1: Formation of Thrombin-Antithrombin Complex (TAT) or ß-Thromboglobulin (ß-TG) during incubation of human whole blood with various agents. Blood aliqouts were incubated for 3 minutes with the indicated materials in the presence of heparin 0.7 U/ml. TAT and ß-TG were determined from the same blood aliqout taken from one blood bag. A total of 6 bags was used. Values are means ± SD. Mean values are compared with Dunnett multiple comparisons test against negative control. P<0.05 for TAT concentration induced by Sangustop *vs.* group 1, 2 and 4. P<0.05 for ß-TG activity induced by Sangustop *vs.* group 1, 2 and 4.

Time course of the storage modulus (G´) during rheometric measurements

Three minutes of blood incubation with steel or hemostatic agents were sufficient to provoke large alterations of coagulation reactions in the subsequent rheometric measurement. Blood in polystyrene test tubes (negative control) provoked a very slow coagulation reaction, whereas contact of blood with stainless steel sticks provoked a rapid blood coagulation. Sangustop®, the bovine collagen fleece, was almost as effective as stainless steel to stimulate coagulation (Table 2). This activation is material specific since Tachosil®, consisting of equine collagen fleece coated with fibrinogen and thrombin, was without any effect on blood coagulation (Figure 1a and 1b).

S No	Material	Clotting time CT (min)	Clot strength CS (Pa)	N
1	neg. control	16.7 ± 4.2	63 ± 43	6
2	Stainless Steel	9.3 ± 2.8 a	257 ± 86 b	6
3	Sangustop	13.0 ± 3.1	189 ± 81 b	6
4	Tachosil	17.1 ± 2.9	63 ± 38	6

Table 2: Effect of various materials on human whole blood coagulation in rheometrical measurements. 10 ml aliquots of human blood containing 0.7 U/ml heparin were incubated for 3 min at 37°C with the indicated materials. After appropriate protamine addition coagulation

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was monitored in an oscillating rheometer. Clotting time (CT) is the time point at which the actual G´ value exceeds the mean G´ baseline value by 3 x SD; clot strength (CS) is the maximal G´ value observed within a time period of 30 min. Mean values are compared with Dunnett multiple comparisons test against negative control. One experiment comprised a measurement of all 4 materials with one blood bag. A total of 6 individual blood bags were used. Values are means ± SD. Mean values are compared with Dunnett multiple comparisons test against negative control. $P<0.05$ for CT induced by group 2 and 3 *vs.* group 1 and 4. $P<0.05$ for CS induced by group 2 and 3 *vs.* group 1 and 4.

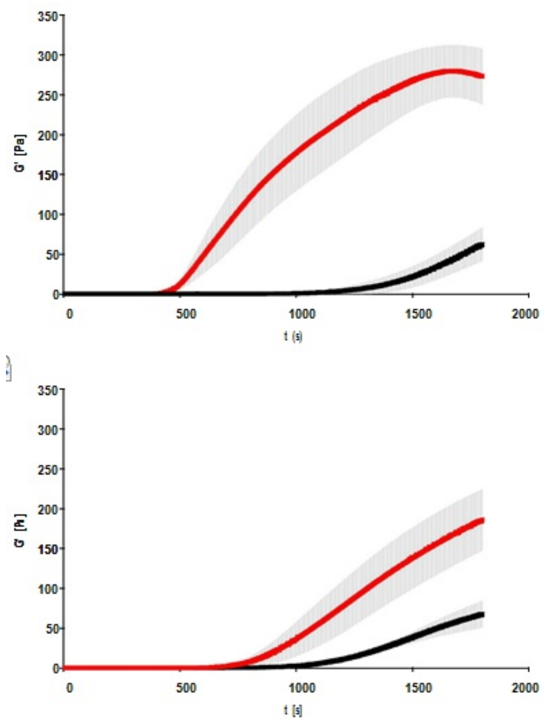

Figure 1 (a,b): Blood coagulation induced by Sangustop® or Tachosil®. Heparinised blood (0.7 U/ml) was incubated for 3 minutes in polypropylene tubes at 37°C. Immediately after incubation, protamine was added and blood was transferred to the rheometer to monitor coagulation. Experiments were performed at a shear strain of 2% at 1 Hz oscillation. The storage (G´) modulus was determined every 5 sec over a period of 30 min at a temperature of 37°C. a) black line: no preincubation (negative control), red line: preincubation with stainless steel (positive control). b) black line: preincubation with Tachosil®, red line: preincubation with Sangustop®. Values are means of 6 determinations ± SEM. Six blood bags from different patients were used for the determination.

With Tachosil neither CT nor CS were significantly altered compared with the negative controls whereas Sangustop® induced significant alterations. Data of CT and CS of all examined groups are summarised in Table 2.

Discussion

In general, conventional biochemical tests are excellent measures to detect the coagulation activation but might be not sensitive enough if the contact time of biomaterials and blood are reduced to very short periods mimicking a clinical situation. This is obvious from the TAT and ß-TG data obtained from stainless steel incubations. Nonpolished stainless steel is known to be procoagulant [19-21] but TAT and ß-TG values significantly increased first after 5 min of incubation. While the biochemical analysis would fail to reliably predict enhanced thrombogenicity, oscillation rheometry was sensitive enough to measure large effects. Only Sangustop® was equally potent as steel to induce thrombus formation whereas Tachosil® failed to effectively stimulate coagulation. The excellent coagulation activation of the collagen fleece might be due to the immediate and extensive adsorption and adhesion of the blood compounds into the open porous structure including the activation of the platelets. The surprisingly low coagulation activity of Tachosil® might be due to a slower adsorption of the blood or a decreased thrombin activity due to surface denaturation.

In this study we characterise the clotting formation of human whole blood after contact with different haemostatic agents using rheological parameters. The method was developed due to the lack of a reproducible *in vitro* technique to evaluate the efficiency of hemostatic agents in their clinical application. The experimental setup was chosen to mimic the routine clinical use of hemostatic agents as close as possible. Blood was heparinised with only 0.7 IU/ml which is near the therapeutic range of plasma heparin concentrations [22,23]. Lower heparin concentrations are not practically applicable since blood would be clotting within the blood bag already during transport and storage. The procedure of appropriate protamine additions is critical for the entire experiment. The protamine concentrations were chosen to result in a constant coagulation time (CT) of the negative control. With a precision of CT of 25% (relative standard deviation) the technique is similar to CT determination in thromboelastographs (23%) but superior to other free oscillating rheometers (40% [24]). The CT determination after protamine addition in the present study is, therefore, adequately controlled and reproducible. The contact time between blood and the two hemostatic agents was restricted to 3 min being the common timeframe of a clinical application for a hemostatic agent [14,15]. This contact time is, however, much shorter than the usual incubation time of 15 min previously established for quantifying thrombogenicity of blood contacting materials via thromboelastographs [25].

Conclusion

In conclusion, we established a reproducible rheological method to characterise coagulation induced by various hemostatic materials after a very short contact time with human blood. The method can distinguish strong acting hemostatic agents from weak acting hemostatic agents. It may provide a valuable tool during medical device or medicinal product development and may even help to distinguish individual coagulation cascade differences in a reproducible manner.

Disclosure statement

Heiko Steuer and Nicolas Lembert are researcher in the NMI (Naturwissenschaftliches & Medizinisches Institut of the University

Tübingen). E. K. Odermatt is an employee of Aesculap AG, which funded this research and has no conflicts of interest otherwise.

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