Efficacy of Hemostatic Agents in Humans With Rotational Thromboelastometry: An *in-vitro* Study

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ABSTRACT Objectives: Hemorrhage is the leading cause of preventable death in military conflicts. Different types of hemostatic dressings have been compared in animal studies for their ability to control bleeding. However, the effects of hemostatic agents in animals may be different from those in humans. The aim of this study was to assess the efficacy of hemostatic dressings in human blood. Methods: Clotting time, clot formation time, α-angle, maximum clot firmness, and lysis index of human blood incubated with QuikClot Gauze, Celox Gauze, QuikClot ACS+, and standard gauze, were compared using rotational thromboelastometry (ROTEM). Nonactivated, intrinsically activated, extrinsically activated, and fibrin-based ROTEM were used to elucidate different mechanisms of action of those dressings. Results: QuikClot Gauze was the most efficacious hemostatic dressing, followed by Celox Gauze and standard gauze. QuikClot ACS+ was clearly outperformed. Conclusions: Modern hemostatic dressings such as QuikClot Gauze and Celox Gauze should be preferred to previous generations of hemostatic dressings, such as QuikClot ACS+. In vitro studies like ROTEM can provide valuable information about the mechanisms of action of hemostatic dressings. A combination of different mechanisms of action may increase the efficacy of hemostatic dressings.

INTRODUCTION

Hemorrhage is the most common cause of death in military conflicts and many of those hemorrhagic deaths are potentially preventable with appropriate treatment.[1–4] In the prehospital setting, wound packing is often the only treatment for extremity hemorrhage at junctional zones that are unsuitable for tourniquet application.[1,5] In the past 15 years, a wide variety of hemostatic dressings have been developed for the management of external bleeding. These dressings are now used worldwide for military and civilian prehospital hemorrhage management.[5–7]

Most studies that compare hemostatic products employ models of lethal hemorrhage and use large animals, mostly swine.[6] However, it is possible that a hemostatic agent has species-specific effects, and studies comparing human to porcine coagulation revealed differences in the coagulation system and predominantly hypercoagulable blood in swine compared to humans.[8–10] The efficacy of different hemostatic dressings in humans has been investigated in case reports, but only very few comparative studies exist.[6,11,12]

Viscoelastic methods for hemostatic testing like rotational thromboelastometry (ROTEM) can assess coagulation parameters such as the velocity of clot formation, maximum clot firmness, and clot lysis, and can thus monitor the functioning of the entire coagulation process. Some animal studies on the efficacy of hemostatic agents included both a lethal model of hemorrhage and an in vitro viscoelastic coagulation test in which the studied hemostatic agents were analyzed.[13–17] The investigators were thus able to compare the efficacy of different hemostatic dressings and to obtain additional insights into the mechanisms of action of the various agents. Viscoelastic coagulation tests, however, allow hemostatic dressings also to be studied in humans.[18]

Against this background, the objective of our study was to use ROTEM with human blood samples to quantify the hemostatic efficacy of hemostatic dressings that are used in the military setting, i.e., QuikClot Gauze (CG; Z-Medica, Wallingford, Connecticut), Celox Gauze (CX; MedTrade Products Ltd, Crewe, United Kingdom), and QuikClot ACS+ (ACS+; Z-Medica) in comparison to standard gauze (SG).

METHODS

Study Groups

All procedures were approved by the ethical committee of the University of Ulm (ref. no. 231/11).

Eight male human subjects between 25 and 35 years of age gave their written informed consent. Subjects were excluded if they had a coagulation disorder, if they had not given their written informed consent, if they had taken any medications during the 10 days preceding blood collection, or if they had a coagulation abnormality. Two citrated blood

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samples (each 12 mL) were taken from the antecubital vein of each subject.

Hemostatic Dressings

CG is a hemostatic dressing impregnated with kaolin, an aluminum silicate and known activator of the intrinsic clotting cascade.[6,7,12] It is the Conformité Européenne (European Conformity) marked version of QuikClot Combat Gauze and, besides the name, these are identical products.

CX is a hemostatic dressing which contains chitosan granules. Chitosan is cationic and adheres to negatively charged surfaces of erythrocytes. In addition, platelet adhesion may contribute to hemostatic function. It acts as a biodegradable mucoadhesive agent that seals wound surfaces.[6,7,12]

ACS+ is a hemostatic dressing of a chemically modified mineral (zeolite), which consists of beads enclosed in loose mesh bags. These beads rapidly absorb water in an exothermic reaction and thus concentrate cellular and protein components in the wound.[6,12]

H&H PriMed Compressed Gauze (H&H Associates, Ordinary, Virginia) is made of cotton and does not contain any hemostatic agents. It was used as a control material.

Measurements were performed with 1.5 mg of each of the three hemostatic gauzes, which were cut into small pieces of roughly 1 mm diameter using nail scissors, and with 1.5 mg of ACS+ beads, which were crushed with a pestle and mortar. In previous tests done in preparation for the research, no errors occurred when we placed 1.5 mg of a sample into a cuvette and performed a ROTEM analysis.

Viscoelastic Coagulation Testing With ROTEM

In our study, coagulation was analyzed with a ROTEM delta analyzer (ROTEM, Team International GmbH, Munich, Germany).

In ROTEM, appropriate quantities of citrated blood (300 µl) and the required reagents (20 µl) are placed in a cuvette in the correct order using the automatic pipette of the system. The cuvette is then loaded onto the analyzer using one of the measurement channels provided by the system. A pin is immersed in the sample and rotates back and forth (± 4.75°). As the blood starts clotting, the clots restrict the rotation of the pin. A graphical display is created and shows the amplitude of pin rotation versus time. For example, a decrease in pin rotation reflects an increase in clot formation and leads to a larger amplitude (Fig. 1).[19] Different reagents can be used to investigate special aspects of coagulation separately, e.g., the intrinsic and extrinsic pathways.[19,20]

The following reagents were used in the study presented here:

(1) Non-activated thromboelastometry (NATEM). Classical thromboelastometry was performed using star-tem (0.2 mol/l $CaCl_2$ in HEPES buffer pH 7.4 and 0.1% sodium azide) as a recalcification reagent. Coagulation is activated by the contact of blood with the surfaces of the cuvette and the pin.

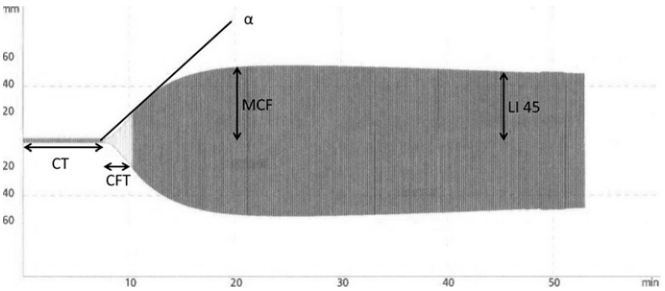

FIGURE 1. Scheme of ROTEM parameters The figure shows clotting time (CT) in seconds, clot formation time (CFT) in seconds, maximal clot firmness (MCF) in millimeters, lysis index at 45 minutes after the onset of coagulation (LI 45) in percent, and alpha angle (α) in degrees.

(2) Intrinsically activated thromboelastometry (INTEM). The INTEM assay was performed using in-tem (partial thromboplastin made of rabbit brain (chloroform extract), ellagic acid, buffer, and preservatives) as an activator of the intrinsic coagulation pathway.
(3) Extrinsically activated thromboelastometry (EXTEM). The EXTEM assay was performed using r ex-tem (recombinant tissue factor and phospholipids, heparin inhibitor, preservatives, and buffer) as an activator of the extrinsic coagulation pathway.
(4) Fibrin-based thromboelastometry (FIBTEM). The FIBTEM assay was performed using fib-tem (cytochalasin D/DMSO solution 0.2 mol/L, $CaCl_2$ in HEPES buffer pH 7.4, preservative). FIBTEM eliminates the platelet contribution to clot formation. As a result, a fibrin clot is formed.

The following parameters were measured (Fig. 1):

(1) Clotting time (CT) in seconds (s): CT is the time from the beginning of measurement to initial blood clot formation (clot firmness of 2 mm). CT represents the onset of clotting.
(2) Clot formation time (CFT) in seconds (s): CFT is the time from the onset of clotting (clot firmness of 2 mm) until a clot firmness of 20 mm has been reached. It represents the speed at which a clot forms.
(3) Alpha angle in degrees (α°): The alpha angle is the angle of the slope and represents the acceleration of clot formation.
(4) Maximum clot firmness (MCF) in millimeters (mm): MCF is the maximum amplitude and reflects the maximal strength of a clot.
(5) Lysis index (LI) in percent (%): LI is the percentage of remaining clot stability in relation to MCF. It describes the extent of lysis at a specific time point.

Measurements

When the blood samples were collected, care was taken to completely fill the citrated tubes and to ensure the correct mixing ratio of citrate to blood. Measurements were

completed within a maximum period of 4 hours after sampling and were performed strictly in accordance with the manufacturer's instructions using original disposable test materials. Routine quality controls of the measuring instrument were carried out on a weekly basis according to the manufacturer's instructions. All measurements were performed by the same investigator (M.M.). The coagulation was analyzed before and after the addition of a hemostatic agent. For the second measurements, small pieces of each hemostatic dressing (1.5 mg) were placed into a cuvette and a mixture of blood and a reagent was added immediately after its preparation.

Statistical Analysis

Data were tested for normality using the Kolmogorov–Smirnov test. An analysis of variance with Bonferroni's multiple comparison test was then performed.

Hemostatic dressings were compared using descriptive statistics. The hemostatic dressing with the largest number of significant differences was considered to have the greatest hemostatic potential. In addition, data were analyzed to elucidate different mechanisms of actions of the products.

In addition, data were statistically compared with human reference values and with each other on the basis of the NATEM assay (i.e., in the absence of possible effects of specific activators). The level of significance was set at $p \leq 0.05$. Calculations were performed using GraphPad Prism 5.0 (GraphPad, San Diego, California).

TABLE I. Coagulation of Human Blood With and Without Hemostatic Agents

	CT (s)	CFT (s)	α (°)	MCF (mm)	LI 45 (%)
NATEM					
Native	851 ± 108	383 ± 102	37 ± 7	41 ± 4	94 ± 5
CG	**181 ± 81**[a]	**167 ± 65**	**60 ± 9**	**56 ± 7**	93 ± 3
CX	**555 ± 122**	517 ± 178	35 ± 5	**50 ± 5**	**99 ± 1**
ACS+	718 ± 151	291 ± 29	45 ± 5	45 ± 3	94 ± 2
SG	**374 ± 98**	448 ± 105	35 ± 4	**49 ± 4**	**98 ± 2**
EXTEM					
Native	48 ± 6	101 ± 21	70 ± 4	55 ± 3	89 ± 2
CG	30 ± 18	92 ± 20	75 ± 4	60 ± 5	**94 ± 2**
CX	**26 ± 8**	95 ± 24	76 ± 6	59 ± 6	**95 ± 2**
ACS+	49 ± 14	121 ± 42	68 ± 7	55 ± 8	**92 ± 3**
SG	**25 ± 11**	104 ± 34	71 ± 5	60 ± 6	**93 ± 2**
INTEM					
Native	176 ± 22	82 ± 13	74 ± 3	52 ± 4	89 ± 3
CG	**128 ± 19**	82 ± 18	74 ± 4	**58 ± 5**	**93 ± 3**
CX	150 ± 33	82 ± 16	75 ± 4	**59 ± 5**	**93 ± 2**
ACS+	164 ± 23	79 ± 20	75 ± 4	56 ± 4	91 ± 3
SG	146 ± 12	75 ± 12	76 ± 2	**58 ± 4**	**93 ± 2**
FIBTEM					
Native	43 ± 7	—	—	11 ± 3	90 ± 7
CG	**24 ± 6**	—	78 ± 5	**19 ± 5**	**99 ± 3**
CX	35 ± 8	—	76 ± 9	**20 ± 6**	**100 ± 0**
ACS+	48 ± 12	—	55 ± 20	12 ± 4	**99 ± 2**
SG	34 ± 9	—	70 ± 9	17 ± 4	**98 ± 2**

Results are expressed as means ± standard deviations ($n = 8$ samples in each group). LI 45, LI at 45 minutes after the onset of coagulation. [a]Bold values indicate significant differences between coagulation with and without a hemostatic agent ($p \leq 0.05$).

RESULTS

CG, followed by CX and SG, caused the most significant changes when compared to normal human coagulation (Table I). In blood samples without specific activators (NATEM), the investigated agents influenced coagulation in different respects and to different degrees. CG had the greatest effect on the kinetics of clot formation (CT, CFT, and α°) and increased clot firmness (MCF). CX activated coagulation (CT) but had no significant effect on the speed of clot formation (CFT and α°). It increased clot firmness (MCF) and stabilized the clot (LI 45 [LI at 45 minutes after the onset of coagulation]). ACS+ had the lowest hemostatic potential and had almost no significant influence on coagulation. SG (control material) activated coagulation (CT) and increased the firmness (MCF) and stability (LI 45) of the clot, too. All hemostatic agents had such a strong effect on the speed of clot formation in the FIBTEM assay that α° values were obtained. In the INTEM assay and the FIBTEM assay, in which the platelet contribution to clot formation is eliminated, CG caused the most significant changes. By contrast, in the EXTEM assay, CX and SG showed the greatest effects.

A comparison of the agents in the NATEM assay showed that CG was significantly superior to all other products in terms of kinetic properties, but was inferior to CX and SG in terms of clot stability. CX was equal to SG and outperformed ACS+ in the speed of clot formation and clot stability. CX and SG achieved the highest clot stability. Compared with the other products, ACS+ was at best equally efficacious in only a few hemostatic parameters. Although SG does not contain hemostatic agents, it was superior to ACS+ with respect to the kinetics of clot formation (Table II).

DISCUSSION

The objective of this study was to assess the efficacy of hemostatic agents in human blood using ROTEM. CG was found to have the greatest hemostatic potential, closely followed by CX and SG (Table I).

Except for the LI, CG achieved a significant improvement in all coagulation parameters in human blood (Table I, NATEM) and significantly outperformed the other hemostatic agents in CT, α°, and CFT (Table II). This was reflected by an early activation of coagulation and a high speed of clot formation. Compared with the other hemostatic agents, CG achieved the most significant changes in the INTEM assay (Table I). Our data thus demonstrated that kaolin-containing gauze activated the intrinsic coagulation pathway.[5,6] Our results did not provide conclusive evidence as to whether, and if so to what extent, the raw

TABLE II. Comparison of Hemostatic Agents Using NATEM

NATEM	CG vs CX	CG vs ACS+	CG vs SG	CX vs ACS+	CX vs SG	ACS+ vs SG
CT (s)	*	*	*	ns	ns	*
CFT (s)	*	ns	*	*	ns	ns
α (°)	*	*	*	*	ns	*
MCF (mm)	ns	*	ns	ns	ns	ns
LI 45 (%)	*	ns	*	*	ns	ns

*Significant difference with $p \leq 0.05$. LI 45, LI at 45 minutes after the onset of coagulation; ns, not significant.

material of CG, and not kaolin, promoted coagulation. As in our study, other viscoelastic testings performed with thromboelastography (TEG) showed that CG caused an increase in the kinetics of clot formation and clot strength in swine.[14] TEG also demonstrated that CG significantly increased all parameters, with the exception of lysis, in human blood.[18] This finding is supported by the results presented here.

CX led to less significant changes than CG but showed a high LI 45 value and thus formed stable blood clots (Table I, NATEM). CX was significantly superior to CG and ACS+ in clot stability (LI 45) and was equal to SG in all other parameters (Table II). The high stability of blood clots suggested by our data can be explained by the general mechanism of action of chitosan, which involves the cross-linking of different blood components.[5–7] Watters et al[18] investigated the effects of CX in human blood by TEG and found no significant activation of coagulation. In our tests, however, CX led to significant changes in human coagulation (Table I). A possible explanation for this difference is the use of different methods of incubation in the in vitro experiments. In most studies with in vitro testing, several milliliters of blood were incubated with the product to be tested and the quantity of blood required for the viscoelastic tests was pipetted from this mixture.[13,14,18,21] Our method of incubation was associated with a longer contact with blood, which is likely to be required for the formation of a mesh of blood components that stabilizes a clot. In recent studies on the effects of hemostatic gauzes in animals, CX was reported to have a hemostatic potential similar to that of CG and was associated with a considerable decrease in the loss of blood after wound packing.[7,18,22,23] The high clot stability that we measured in vitro appears to offset the significantly poorer results for the kinetics of clot formation and may explain the excellent results obtained with CX in in vivo tests.

SG led to fewer significant changes than CG (Table I). There were no significant differences between SG and CX, but SG significantly outperformed ACS+ (Table II). In an animal model, SG acted via pressure and absorption of blood and allowed wounds to be packed more rapidly and more completely, showing no significant difference in hemorrhage control and survival between hemostatic dressings and standard gauze.[18] In our in vitro study, however, these mechanisms cannot explain the hemostatic effects of SG. It should be noted that SG is a very fine fabric and, when compared to CG or CX, offered the largest surface area that came into contact with blood. Apparently, SG provides a cotton matrix that promotes platelet aggregation and blood coagulation.[5] Our results are in line with other TEG studies reporting that gauze without a hemostatic agent (placebo gauze: 50% rayon and 50% polyester) led to improvements in coagulation.[14] By contrast, other investigators reported that SG did not activate in vitro coagulation of human blood.[18] The aforementioned differences in the methods of incubation can explain these divergent results. In animal studies too, regular gauze (without a hemostatic agent) was superior or at least equal to other hemostatic dressings in some respects.[18,24,25] However, one limitation of our study (and most of the in vivo studies mentioned before) is that the dressings were not used with manufacturer recommendation, because we did not apply direct pressure to the dressing packed into a wound. It is likely that this may contribute to the good results of SG compared to CX and CG.[7] Nevertheless, our findings confirm that the technique of packing wounds with regular cotton gauze, which has been practiced for decades, is a highly effective method of managing bleeding. In addition, SG is by far the cheapest product compared to all other hemostatic dressings.[7]

ACS+ was by far the least efficacious agent in our study and was significantly outperformed by all other dressings (Tables I and II). A possible explanation for the poor performance of this hemostatic dressing is that we used no more than 1.5 mg of ACS+ in our tests, as a result of which only a small quantity of water was absorbed by zeolite and only a minor local increase in coagulation activators was achieved. This explains why Ostomel et al,[26] who performed their tests with 20 mg of chemically modified zeolite, demonstrated good procoagulant properties of zeolite-based hemostatic agents by TEG although they used the same incubation method as we did. Other studies in the literature, however, are in line with our finding of a poor hemostatic effect of ACS+ or even report that TEG revealed a significant deterioration in almost all measurement parameters.[13] In in vivo studies, the efficacy of ACS+ was inconsistent. Although ACS+ showed higher efficacy than average in some studies,[27,28] its use was discontinued in another study when ACS+ failed to achieve hemostasis.[13] In conclusion, ACS+ was found to be generally inferior to modern hemostatic dressings such as CG. This finding was confirmed by our in vitro results for human blood.[5]

Our in vitro comparison suggested that CG was slightly superior to the other hemostatic dressings for use in humans. The in vitro method in our study, however, did not include a variety of factors such as packing time, loss of blood, rebleeding, dilutional and consumptive coagulopathy, effects of movement, and hypothermia.[7,29] And, as mentioned before, we did not apply direct pressure on a dressing placed in a bleeding wound cavity and therefore used the dressings not in accordance with the manufacturer's recommendations.[7] These factors, however, are essential for a comprehensive evaluation of a product and can only be assessed in tests on live animals. For this reason, experimental live animal use continues to play an important role in evaluations of the efficacy of hemostatic agents. Such animal studies, however, are very complex and expensive, and are likely to be even more so at least in Europe, where a new European directive on animal protection has come into force.[30] Viscoelastic coagulation tests are a cost-effective and practicable alternative that allows new agents to be analyzed in human blood in accordance with the principles of the Three Rs of alternative experimental methods (Refinement, Reduction, and Replacement) that were described by Russel and Burch as early as 1959 and that explicitly advocate in vitro studies.[26,31]

CONCLUSIONS

Viscoelastic coagulation tests are a valuable tool in the transfer of data from animal research to humans. Our in vitro results are in line with in vivo studies of hemostatic agents in animals and the current Tactical Combat Casualty Care Guidelines, which recommend CG and CX together with HemCon as the current hemostatic dressings of choice.[5–7]

Some authors believe that there is a lack of clear superiority of any hemostatic agent. This would suggest that, despite the different compositions and sizes of dressings, modern hemostatic dressing technology has potentially reached a plateau in terms of efficacy.[22] However, to date no single product has all ideal characteristics to treat hemorrhage.[7] The different mechanisms of action demonstrated in our study show that an appropriate combination may lead to an even higher level of efficacy. Some authors have mentioned such accumulative effects before, and they state that CG is both a factor concentrator (absorbs water with the gauze) and procoagulant (activates the clotting cascade with caolin component).[7] Furthermore, a strong activator of intrinsic coagulation (such as kaolin) may be combined with a material providing a large surface area which activates coagulation to a higher degree (such as cotton gauze). These additive effects may result in an overall improvement in hemostasis.

Finally, considering the limited data on efficacy of modern hemostatic dressings containing chitosan and kaolin in humans, our data suggest that the hemostatic effects of those dressings observed in numerous animal studies seem to be transferable to humans.[5,6,11,12,32–37]

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