Coagulopathy during Induced Severe Intracranial Hypertension in a Porcine Donor Model

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Background: Development of coagulopathy is a serious complication arising from isolated traumatic brain injury, and it predicts poor outcome. The underlying mechanism has not yet been established, although coagulopathy arising from brain tissue injury and the release of tissue factor may represent the pathophysiology. The authors investigated dynamic whole-blood clot formation (ROTEM®) in a recently developed porcine model of induced severe intracranial hypertension.

Methods: In this prospective, randomized experimental study, 17 pigs were designated for severe intracranial hypertension or sham operation. Intracranial hypertension was induced by inflation of an intracranial balloon. Whole-blood clot formation was assessed by clot initiation, and clot propagation and clot strength through thrombelastometry. The authors also assessed thrombin generation and prothrombin time, which were obtained at baseline, immediately after intervention, and 5 h after intervention.

Results: A dramatic shortening in time to clot initiation and an increase in clot propagation were observed after induction of intracranial hypertension as compared to the control group. These results were further substantiated by a pronounced increase in thrombin generation and a significantly shortened prothrombin time in the intervention group. No difference in clot strength was detected between the groups.

Conclusions: In a porcine model, induction of increased intracranial pressure causing severe intracranial hypertension was associated with a pronounced activation of the coagulation system. Taken together, the various results indicate that tissue factor probably represents the main trigger of hypercoagulopathy found in these pigs.

ISOLO TED traumatic brain injury with subsequent development of coagulopathy has been reported as a hazardous complication predictive of poor clinical outcome.1–5 A plausible explanation for development of coagulopathy after brain injury may be an excessive release of tissue factor from glia cells to the blood, causing disseminated intravascular coagulation, which is accompanied by consumption of coagulation factors, resulting in a bleeding diathesis.6,7 So far, the suggested mechanisms responsible for the development of coagulopathy after traumatic brain injury have been based on measured changes in markers of procoagulant as well as anticoagulant activities.1–3 Furthermore, controversy exists within the literature regarding the functionally most important determinants of the development of coagulopathy after brain injury.8,9 Finally, intensive research on the mechanisms of traumatic coagulopathy has shown that multiple factors, such as metabolic acidosis, hypothermia, anemia, consumption, fibrinogen deficiency from colloidal resuscitation, hyperfibrinolysis, and multiple blood transfusions, may critically affect overall hemostatic performance.10,11

During the past decade, global functional coagulation assays, such as recording of continuous thrombin generation and dynamic thromboelastometry whole-blood coagulation profiles, have been developed and validated.12,13 In comparison with standard plasma clotting tests and markers of procoagulation or anticoagulation, the global assays are highly sensitive and have been reported to more optimally reflect *in vivo* biology. In the case of whole-blood clotting tests, the overall integrated function of blood cells, platelets, and plasma coagulation factors influences the results. Such methods have proven useful for characterizing a variety of bleeding disorders and other conditions with compromised hemostatic capacity.14–16 as well as for detecting a tendency to hypercoagulation and thrombosis.17,18,19

In an attempt to obtain further knowledge on the overall functional changes in the hemostatic system after severe intracranial hypertension, we investigated whether dynamic whole-blood clot formation and thrombin generation might be useful for characterizing the hemostatic balance by using a recently developed porcine model of severe intracranial hypertension. We hypothesized that brain injury results in an immediate state of hypercoagulation, as evaluated by shortened time to whole-blood clot initiation, accelerated whole-blood clot propagation, and enhanced thrombin generation. In addition, *ex vivo* spiking studies were performed whereby blood was substituted with tissue factor in high amounts to explore the possibility that tissue factor might be involved in the predicted functional activation of coagulation.

Materials and Methods

Study Subjects

The animal studies were approved by the Danish Inspectorate of Animal Experimentation (Copenhagen, Denmark), and the experiments were conducted accord-
ing to the guidelines in the Guide for the Care and Use of Laboratory Animals.\textsuperscript{20} 

In total, 17 female landrace pigs (mean weight, 29 kg; range, 28--32 kg) were used in the study. After randomization, 10 pigs were allocated to the interventional group and subjected to induced severe intracranial hypertension according to the experimental setup outlined below; another 7 pigs were randomized to a control group (sham operation). The animals were deprived of food overnight but allowed free access to water. Premedication consisted of an intramuscular injection of midazolam (0.25 mg/kg). Anesthesia was induced by intravenously injected ketamine (10 \( \mu g/kg \)) and midazolam (0.5 mg/kg) and was maintained by continuous infusion of fentanyl (60 \( \mu g \cdot kg^{-1} \cdot h^{-1} \)) and midazolam (6 mg \cdot kg^{-1} \cdot h^{-1} \)). The pigs were tracheotomized and mechanically ventilated. Ventilation and fresh gas flow were adjusted to maintain an end-tidal carbon dioxide between 5.5 and 6.0 kPa and pH between 7.35 and 7.45. Adopting sterile techniques, an intravenous catheter for fluid and drug administration was placed in the right external jugular vein. Another line was placed in the right internal carotid artery for continuous blood pressure measurements and sampling of blood. A pulmonary artery catheter (Swan-Ganz; Edwards Lifesciences, Irvine, CA,) was inserted through the right external jugular vein. All animals were initially given 500 ml of Ringer’s acetate within the first hour before infusion of 10 ml \cdot kg^{-1} \cdot h^{-1} \). To avoid hypotension, 500 ml of hydroxyethyl starch 130/0.4 (Voluven; Fresenius Kabi AB, Uppsala, Sweden) was given 30 min after severe intracranial hypertension. In the sham-operated animals, Ringer’s acetate infusion was discontinued while hydroxyethyl starch was infused. The mean arterial pressure was maintained above 60 mmHg by bolus injection of 250 ml of Ringer’s acetate, repeatedly if required. No vasopressors or inotropic drugs were used. Severe intracranial hypertension induces diabetes insipidus,\textsuperscript{21} and urine volumes greater than 200 ml/h. Replacement with Ringer’s acetate was administered within the following hour.

Procedure of Anesthesia, Increased Intracranial Pressure, and Intracranial Hypertension

The experimental setup is depicted in figure 1. Two holes were drilled in the cranium: one hole positioned 15 mm anteriorly, and the other hole 15 mm posterior to the coronal suture, using separate sides of the sagittal suture. On the basis of randomization using the closed envelope principle, pigs allocated to severe intracranial hypertension were provided with a 20-French Foley catheter (Euromedical Industries, Kedah, Malaysia) inserted into the anterior hole, and the balloon of the catheter was placed in the epidural space as previously described.\textsuperscript{22,23} A steel needle from a 22-gauge Venflon\textsuperscript{®} (Becton Dickinson, Franklin Lakes, NJ) was introduced subdurally into the posterior hole to measure intracranial pressure. Sham-operated pigs were not subjected to catheter placement; however, holes were drilled using the same technique as for pigs in the intervention group. The Foley catheter balloon was inflated with isotonic saline at a rate of 0.25 ml/min. After a median of 64 min (range, 52--108 min), the cerebral perfusion pressure became negative (mean arterial pressure less than intracranial pressure), indicating that the blood flow to the brain of the animal had ceased off. Immediately after this event, a bolus of isotonic saline was added to the balloon to give a total volume of 30 ml, which was kept constant throughout the experiment. The induction of severe intracranial hypertension produced a hemodynamic response as previously described.\textsuperscript{24,25} To avoid brain stem reflex-induced myoclonies, a bolus of cisatracurium (0.6 mg/kg) was given just before the final bolus in the balloon; sham-operated pigs were subjected to the same treatment. Anesthesia was maintained continuously throughout the whole experiment in both groups.

Blood Sampling and Processing

Blood samples were drawn from the centrally located arterial catheter into Venoject\textsuperscript{®} tubes (Terumo Europe, Leuven, Belgium; trisodium citrate 0.129 mol/l; 3.2 W/V %), at a volume ratio of 1:10. Samples were obtained: (1) at baseline, (2) immediately after intervention, and (3) 5 h after intervention. To circumvent contact activation, tubes collected for thromboelastometry analyses received corn trypsin inhibitor (Hematologic Technologies Inc., Essex Junction, VT) giving a final concentration at 100 \( \mu g/ml \) of blood. Citrated whole blood was rested for 30 min at ambient temperature before thromboelastometry analysis. Remaining tubes were centrifuged (2,800 g, 3,000 g, 3,800 g, and 5,000 g).
25 min, 4°C), and plasma was separated and frozen at −80°C in 500-μl aliquots.

**Thromboelastometry Whole-blood Coagulation Analysis**

Dynamic whole-blood coagulation profiles were recorded in parallel using thromboelastometry (ROTEM®, Pentapharm, Munich, Germany). In brief, prewarmed (37°C) ROTEM® plastic cups were loaded with 300 μl of whole blood and 20 μl of buffer (Hepes 20 mM, NaCl 150 mM, pH 7.4). In previous studies, activation of the whole-blood clotting process was initiated with small amounts of tissue factor. To increase the sensitivity of the test to endogenous tissue factor in the present context, the coagulation process was recalculated by the addition of 20 μl of CaCl2 (200 mM) not activated. Hence, the final volume in the ROTEM® cup was 340 μl in all cases. All analyses were processed in duplicate for a minimum of 45 min. Standard thromboelastometry parameters such as the clotting time (sec) and maximum clot firmness (mm) were recorded. The digitalized raw signal was further processed using a software program (DyCo-DerivAn; Avordusol, Risskov, Denmark) to obtain the dynamic velocity parameters: maximum velocity (MaxVel, [mm × 100/s]) and time to maximum velocity (t, MaxVel [sec]). The initiation phase of whole-blood clot formation was defined by the clotting time, whereas the propagation phase was defined by MaxVel and t, MaxVel. The characteristic clot formation time was not included in the current study because this parameter possesses extensive variability and low reproducibility.

**Thrombin Generation**

Thrombin generation was measured according to the protocol devised by Hemker by using a calibrated automated thrombogram (Thrombinscope BV, Maastricht, The Netherlands). Blood samples were centrifuged at 2800 g for 25 min to obtain platelet-poor plasma. A 96-well microtiter plastic plate (Immuno1 H2B clear 96-well; Thermo Electron Corporation, Vantaa, Finland) was prepared with 80 μl of platelet-poor plasma followed by 20 μl of activator (consisting of a mixture of tissue factor, [Innovin®, Dade Behring, Marburg, Germany] at a final dilution of 1:7000 and phospholipid-TGT [Rossix, Möln达尔, Sweden] at a final concentration of 4 pm. After a short incubation, 20 μl of thrombin substrate (Fluo-Substrate, Thrombinscope) was added automatically. All reagents were prewarmed to physiologic temperature (37°C). Using the “slow” fluorogenic thrombin substrate and continuous comparison to a simultaneously run calibrator, the continuous development of thrombin was recorded on the Fluroscan Ascent fluorometer (Thermo Electron). The measurements were performed in triplicate, calibrating each well to a parallel well with a thrombin calibrator (Thrombin calibrator TS 20.0, Thrombinscope) with predefined thrombin-like activity. Using the software provided by Thrombinscope, the following parameters were calculated: lag time (min), endogenous thrombin potential, peak level of thrombin generation (nm), and time to peak thrombin (min).

**Measurement of Prothrombin Time**

Diluted prothrombin time was analyzed on a BCT Coagulation Analyzer (Dade Behring) using minute amounts of tissue factor (Innovin Reagent, Dade Behring) at a final dilution of 1:200 as a test reagent.

In Vitro Titration Experiments with Tissue Factor

Additional in vitro spiking experiments were performed in one pig from the intervention group. Thrombelastometric analyses were performed on both a baseline sample and samples with increasing amounts of Innovin® (Dade Behring) at final concentrations of 1:100,000, 1:50,000, 1:10,000, and 1:1000.

**Statistics**

Statistical analyses was performed using statistical software Sigma Plot (version 11.0; Systat Software, Inc, San Jose, CA). The minimum sample size was estimated as n = 10 in the intervention group, based on pilot studies showing an expected increase of 25% in maximum velocity of clot formation (alpha = 0.05, power = 0.8). Pilot animals were not included in the current study. Data followed a Gaussian distribution as evaluated by interpretation of Q-Q plots and histograms. Two-way analysis of variance (ANOVA) with Holm-Sidak post test was applied to test changes from baseline over time in groups. Differences between the groups were compared using unpaired t test or Mann–Whitney test. Two-tailed P values less than 5% (P < 0.05) were considered statistically significant.

**Results**

One pig in the intervention group experienced intracranial bleeding during the surgical preparation and was excluded from data analysis. Another pig in the intervention group died suddenly 3 h after induction of severe intracranial hypertension; in this case, values from baseline and after intervention only were included.

There was no difference in the fluid balance defined as infusion minus diuresis in the intervention group (1,677 ml ± 299 ml) as compared with the control group (1,847 ± 114 ml; P = 0.62).

**Thromboelastometric Whole-blood Coagulation Analyses**

At baseline, the dynamic whole-blood clotting profiles were similar in the two groups. Compared to the control group, pigs with severe intracranial hypertension experi-
experienced a distinct and significant shortening of the initiation phase as well as an acceleration of the propagation phase of whole-blood clot formation. This acceleration was illustrated by a shortened clotting time, increased maximum velocity of clot formation, and shortened time until maximum velocity. Five hours after intervention, the thromboelastometry parameters in the intervention group were partly normalized, although time to maximum velocity remained significantly shortened. Maximum clot firmness showed no statistically significant changes between the two groups. Compared to the baseline values, the thromboelastometry parameters recorded in the control group were unchanged except for clot formation time after intervention and 5 h after intervention (fig. 2).

Measurement of Diluted Prothrombin Time

The two study groups showed indistinguishable results at baseline. The intervention group revealed a pronounced and significant reduction in the diluted prothrombin time. The difference was balanced 5 h after intervention, comparable with the control group, and not statistically different from baseline. No changes in diluted prothrombin time were observed in the control group (fig. 3).

Thrombin Generation

The two study groups showed no difference in thrombin generation at baseline. Pigs with severe intracranial hypertension experienced a marked enhancement of thrombin generation, as illustrated by a significant reduction in the lag time, as well as increased peak levels and shortened time to peak levels of thrombin. All observed differences were balanced 5 h after intervention, comparable to the control group and similar to baseline values. No significant changes in the measured variables of thrombin generation were observed in the control group.

In Vitro Titration Experiments with Tissue Factor

In vitro spiking experiments with increasing concentrations of tissue factor caused changes in the dynamic whole-blood clot formation very similar to those observed immediately after intervention. Hence, in a dose-dependent manner, the presence of tissue factor shortened the clotting time and increased the maximum velocity of whole-blood clots, as well as induced a minor increase in the maximum clot firmness. At final tissue factor dilutions of 1:10,000 and 1:1000, the dynamic whole-blood clotting profile was equivalent to the profile obtained immediately after induction of severe intracranial hypertension in the same pig (fig. 4).

Discussion

The current study is the first prospective randomized case-control study in a porcine model to show the time-course of changes in hemostatic profiles as observed...
using dynamic whole-blood clot formation and continuous thrombin generation during induced severe intracranial hypertension. The coagulopathy immediately after increased severe intracranial hypertension was characterized by a hypercoagulation pattern as documented by a shortened clot initiation phase and accelerated propagation phase of whole-blood clot formation as compared to sham-operated controls. Furthermore, the intervention group displayed very similar changes in the profile of continuous thrombin generation. To date, clinical reports have primarily described the development of progressive consumptive coagulopathy as determined by derangements of standard coagulation markers, such as D-dimer, fibrinogen, platelets, activated partial thromboplastin time, and prothrombin time.27,28 However, the current study demonstrated the presence of a pronounced hypercoagulation that occurred immediately after development of severe intracranial hypertension. In contrast to previous reports, our data illustrate that the abnormal hypercoagulation during severe intracranial hypertension was reversed within 5 h, at which time the hypercoagulation tendency seems to be decreased and "normalized."

The most plausible explanation would be that the tissue factor stimulus has eased off due to completely abolished or significantly reduced brain blood circulation in the model. Another explanation could be emerging consumption of coagulation factors. However, this seems less likely because the prothrombin time was not prolonged compared with the control group. To explore the mechanisms underlying the state of hypercoagulation during severe intracranial hypertension, in vitro spiking experiments were performed in which increasing concentrations of tissue factor were added to pig blood. The changes brought about by tissue factor closely mirrored the changes observed during severe intracranial hypertension; therefore, these results point to the likelihood that excessive release of tissue factor to circulating blood from severe intracranial hypertension is involved in the hypercoagulable state seen. Measuring porcine tissue factor in the blood of pigs subjected to intracranial hypertension might further substantiate our conclusions. At the present time, we have not identified a suitable antibody specific for porcine tissue factor, and antibodies against human tissue factor do not react with porcine tissue factor. However, on the basis of our in vitro experiments, shortened clot initiation and accelerated clot propagation appear to be caused by tissue factor release. Notably, the thromboelastometry whole-blood coagulation analyses were performed on inactivated pig blood. Moreover, the blood-sampling tubes were incubated with corn trypsin inhibitor to abolish nonphysiological contact activation. Thus, activation of the clotting process in the present context is most likely driven by endogenous tissue factor. Finally, the hypothesis that tissue factor is the main contributor to the state of hypercoagulation was supported by a reduction in the diluted prothrombin time in the intervention group. The fluid balance was equal in the two groups, although the output and input was increased in the intervention group caused by diabetes insipidus. Hemodilution is known to induce coagulopathy; however, on the basis of continuous measurements of hematocrit (data not presented), the pigs in the intervention group did not develop hemodilution. If the hypothesis of excessive release of tissue factor is applicable, the source of the tissue factor is of great interest. The most obvious explanation would be release of tissue factor from glia cells in the brain tissue. Associated with surgery and induction of severe intracranial hypertension, an increase in endogenous inflammatory mediators like interleukin-6 and tumor necrosis factor alpha has been postulated. Among other pathologic and physiologic mediators, these factors are triggers for the expression of tissue factor on blood cells.30,31 However, as published
very recently by our group, no changes were detected in cytokine levels in a similar model of brain death.\textsuperscript{25}

It may be speculated that blocking or partial blocking tissue factor pathway may reduce the probability of developing hypercoagulation-related events, such as thromboembolism, in patients suffering from increased intracranial pressure. However, further investigations are needed to substantiate our findings.

In conclusion, induced severe intracranial hypertension was associated with a pronounced activation of the coagulation system. The state of hypercoagulation immediately after induction was illustrated by changes in dynamic whole-blood clot formation as well as by measurements of continuous thrombin generation.\textit{In vitro} spiking experiments suggest that tissue factor represents the main trigger of the hypercoagulable condition.

The authors thank Senior Biomedical Scientists Kirsten Christiansen (Department of Anesthesiology, Aarhus University Hospital, Aarhus, Denmark), Lisbeth Norengard (Department of Clinical Biochemistry, Centre for Haemophilia and Thrombosis, Aarhus University Hospital, Skejby, Denmark), and Senior Biomedical Scientist Lene Vestergaard (Department of Anesthesiology, Aarhus University Hospital, Aarhus, Denmark).

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