A Novel Approach to Treating FVIII Inhibitors: In Vitro Results for Combination Therapy with Human Prothrombin Complex Concentrate and Plasma-Derived FVIII/VWF Complex

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Abstract

Coagulation factor VIII (FVIII) inhibitors are the most severe complications of haemophilia A treatment. We studied the effect of a non-activated prothrombin concentrate (PCC) combined with FVIII in vitro. FVIII antibodies minimised the in vitro thrombin generation of standard human plasma and prolonged the lag phase at a residual FVIII activity of $\leq 0.01$ IU/ml. Time to clotting of FVIII-inhibited fresh whole blood was not measurable until 55 minutes (observation time). Combining a PCC and a plasma-derived FVIII/VWF concentrate normalised TG and ROTEM® parameters. Our findings support an approach to moderately shift the haemostatic balance to a more procoagulant potential.

Introduction

Haemophilia A (HA) is caused by a deficiency in the amount or activity of coagulation factor VIII (FVIII) in plasma and is largely managed through the administration of plasma-derived or recombinant human FVIII concentrates. Inhibitory antibodies to exogenously administered FVIII can develop, thereby neutralising its therapeutic effect and resulting in severe bleeding complications. Inhibitory antibodies represent the most significant clinical complication in the modern day treatment of HA [1]. In patients who develop inhibitory antibodies, prophylaxis with FVIII cannot be performed until complete and sustained elimination of the inhibitor has been achieved. As a great deal of clinical progress has been made in recent years in the management of HA characterised by persistent FVIII inhibitors, several treatment options are now available in the armamentarium [2-4]. Immediate and persistent initiation of immune tolerance induction (ITI) therapy, involving repeated FVIII dosing over months or years, is the treatment of choice and the only proven strategy for achieving sustained inhibitor eradication [5]. ITI has generally been more successful when FVIII concentrates containing von Willebrand factor (VWF) were used [5-7]. Besides its direct haemostatic role, VWF has a key function in the protection, transport and presentation of FVIII with respect to the immune system, and this is an important area of current research. For the patients who do not respond to ITI treatment alone, bypassing agents represent the next therapeutic step. These bypassing agents include non-activated prothrombin complex concentrates (PCCs), activated PCCs (aPCCs) and recombinant factor VIIa (rFVIIa) [3,4]. Several case reports and in vitro studies indicate that combining different types of bypassing agents, either sequentially or as combination therapy, can be an efficacious way to treat monotherapy-resistant inhibitors [8-15]. In particular Klintman and colleagues showed in vitro that combining any two of aPCC, rFVIIa and FVIII concentrates resulted in an additive effect on thrombin production [15]. While aPCCs and rFVIIa are well established for patients displaying FVIII inhibitors, some individuals are refractory to these treatments, and although thrombotic side effects are rare, a potential risk remains with the application of high concentrations of activated coagulation factors [16-18]. Moreover, the high cost of these products limits their availability in some patient populations and countries. In clinical practice, the successful use of non-activated PCCs has been demonstrated in many cases throughout the last decades to counteract inhibitor-associated bleeding complications [19,20]. Human non-activated PCC (Octaplex® [Octapharma PPGmbH, Vienna, Austria]), which contains a balanced ratio of the coagulation factors II, VII, IX and X and the anticoagulant proteins C and S, is currently licensed for use in perioperative prophylaxis and treatment of bleeding following treatment or overdose with vitamin K antagonists and for congenital deficiencies of factors II and X when specific factor products are not available [21-23]. Following encouraging results with this PCC in reducing bleeding complications of HA patients displaying high-titre inhibitors, we were interested to establish whether plasma-derived (pd)FVIII/VWF concentrates may confer an additional clinical advantage when used as a
supplementary add-on therapy to PCC in acute bleeding situations [24]. Using a thrombin generation (TG) assay [25-27], thrombelastometry (ROTEM®) [28,29] and quantification of FVIII activity (FVIII:C), we investigated the effect of spiking samples with both the PCC and pdFVIII/VWF concentrates (Octanate® or Wilate®), while also experimentally adding FVIII antibodies to standard human plasma (SHP) or patient-derived plasma samples.

Material and Methods

**FVIII: C quantification for inhibitor determination**
Inhibitory activity was determined using the FVIII chromogenic method and an inhibitor reagent kit (Technoclone GmbH, Vienna, Austria).

**Residual FVIII: C activities after incubation**
FVIII activity was determined in the low range, 0.088-0.006 IU FVIII:C/ml using the chromogenic assay (Coamatic FVIII from Chromogenix [IL Spa, Milano, Italy]) in a microplate format as follows: an adapted standard curve was prepared between 0.006 and 0.088 IU FVIII:C/ml from dilutions of standard human plasma (SHP) (Siemens Healthcare Diagnostics [SHD] GmbH, Marburg, Germany) calibrated against fresh normal plasma and the buffer blank. The samples were diluted, if necessary, into the range of the standard curve. Control SHP calibrated against fresh normal plasma served as control. The incubation steps and the final dilution for samples, standards and controls were performed according to the manufacturers’ instructions. The absorbance was read at 405 nm.

**Thrombin generation assay**
Thrombin generation was measured using the Technothrombin TGA kit and RC high reagent (Technoclone GmbH, Vienna, Austria). The readout parameters were lag time (phase) and peak thrombin concentration. To mimic plasma with a FVIII inhibitor, monoclonal antibodies (mAb, ESH-4 and ESH-8, American Diagnostica Inc., Stamford, USA) and/or polyclonal (pAb) sheep FVIII antibodies (Enzyme Research Laboratories Ltd., South Bend, USA) were added to SHP in order to achieve final inhibitory activities of 10 and 31 Bethesda Units (BU)/ml. To mimic plasma with a platelet inhibitor, a monoclonal antibody (anti-platelet, ESH-4 and ESH-8, American Diagnostica Inc., Stamford, USA) was added to SHP in order to achieve a final platelet count of 200,000/µl (± 10%), resulting in a corresponding plasma dilution. Inhibition of FVIII was performed in the presence and absence of platelets (1 hour, 37°C) so as to exclude any FVIII-related activity displayed by the reconstituted platelets themselves. The FVIII/VWF concentrate was reconstituted according to the manufacturer’s instructions to obtain a solution of 100 IU FVIII:C/ml.

The PCC was reconstituted with water for injection to a FIX concentration of 25 IU/ml. Because the PCC used contains an average of 0.45 IU heparin per 1 IU FIX (with low variation; 0.2-0.5 IU heparin/IU FIX according to specification), pre-experiments were performed including the heparin-free in-process bulk solution to exclude any significant impact on test parameters [30]. In general, the PCC concentrations indicated refer to the final FIX concentrations added. A concentration of 0.25 IU FIX/ml (corresponding to < 0.12 IU heparin/ml) in the assay neither prolonged the lag phase nor reduced the peak TG (data not shown). No impact was observed on the ROTEM® results. As expected, the PCC at 0.5 IU FIX/ml showed some impact and, therefore, the heparin-free in-process solution was used for studies ≤ 0.5 IU FIX/ml.

The PCC and pdFVIII/VWF concentrate were not mixed before spiking in the experiments illustrated here, but were added to samples separately. Lag phase (minutes) and peak thrombin concentration (nM) were assessed for the PCC and pdFVIII/VWF concentrate separately and in combination in SHP containing inhibitors. TG assays were performed for the pdFVIII/VWF at FVIII concentrations of 0.1, 0.3, 0.5, 0.75, 1.0 or 1.5 IU/ml. In addition, the PCC was investigated separately at a concentration of 0.25 IU/ml, and combined with 0.5 IU/ml pdFVIII/VWF (Octanate® or Wilate®). In the absence of haemostatic treatments, TG parameters were investigated for SHP with or without pAb/mAb inhibitors, and as controls, in SHP containing platelets with or without pAb/mAb inhibitors.
In addition, in vitro/ex vivo effects of aPCC using FEIBA® (2 U/ml) or rFVIIa (2 µg/ml) in inhibitor plasmas were studied, without or in combination with 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF in the presence of platelets.

Kinetics of FVIII inhibition after addition of PCC and pdFVIII/VWF

After 1 hour incubation of antibody-spiked SHP, lag phases and peak thrombin concentrations were assessed at baseline and after addition of 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF. FVIII:C was determined in parallel. These parameters were assessed for the following incubation times of this combination of concentrates: 0, 15, 30, 45, and 60 minutes. SHP with and without inhibitors were run as controls.

Rotation thrombelastometry (ROTEM®) of whole blood

ROTEM® (TEM Innovations GmbH, Munich, Germany) was performed at 37°C using the NATEM® system according to the manufacturer's description. pAb was added to freshly drawn whole blood samples after a resting time of 30 minutes at room temperature, and incubated for 1 hour at 37°C in order to achieve a final inhibitory activity of 31 BU/ml. Samples for ROTEM®/NATEM® analysis were treated with the following haemostatic interventions: 0.5 IU/ml PCC and 1.0 IU/ml pdFVIII/VWF or 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF. Samples were analysed at the following time points after the addition of the two concentrates: 0, 5, 15, 30, 45 and 60 minutes. Whole blood samples with and without antibodies were run as controls.

Ex vivo studies

Plasma samples obtained from HA patients were obtained and stored as described previously [31]. The inhibitory potencies of the four individuals ranged from 30 to 159 BU/ml. TG parameters with or without addition of 0.5 IU/ml PCC and 0.5 IU/ml or 1.0 IU/ml pdFVIII/VWF, FEIBA® or rFVIIa were investigated.

Results

Impact of pdFVIII/VWF and PCC/pdFVIII/VWF on TG parameters in plasma with inhibitors

As characteristic of these studies, the addition of pAb or pAb/mAb to SHP resulted in the minimisation of TG and a prolongation of the lag phase. In the presence of platelets, these effects were still considerable, although thrombin generation was not completely eradicated (Illustration 1). After incubation of SHP and platelets with antibodies, the addition of pdFVIII/VWF within the concentration range of 0.1-1.5 IU/ml resulted in a general trend towards reduced lag time with increasing pdFVIII/VWF concentrations, but this did not reach the control level (SHP, Illustration 1, Panel A and platelets). Peak thrombin concentrations increased with higher pdFVIII/VWF level, but reached a plateau at 0.5 IU/ml FVIII corresponding to approximately half of that observed for the control (Illustration 1, Panel B). The addition of 0.25 IU/ml PCC to SHP with platelets containing pAb/mAb normalised the lag time, whereas peak thrombin concentration was significantly improved but not normalised. The addition of 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF from Octanate® resulted in normalisation of both lag time and peak thrombin concentration. Also, the addition of the other pdFVIII/VWF product (Wilate®) to the PCC revealed comparable effects at the same FVIII:C concentrations (data not shown).

Correspondingly, ROTEM® analysis performed with freshly drawn blood showed prevention of clot formation upon addition of FVIII antibodies. Normalisation of ROTEM® parameters was achieved after addition of 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF (Illustration 2; Panels A, B and C).

Kinetics of FVIII inhibition after addition of PCC/pdFVIII/VWF: Impact on TG and clot formation (ROTEM®)

SHP incubation in the presence of platelets and after addition of pAb showed a minimum residual FVIII:C of 0.01 IU/ml after 60 minutes at 37°C, which reproducibly did not further decrease upon longer incubation times (up to 180 minutes, data not shown). Accordingly, this procedure was defined as the standard pre-incubation time.

After standard pre-incubation of SHP with pAb in the presence of added platelets, 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF was added and aliquots were drawn for immediate measurements of FVIII:C activity and TG at time point 0 and up to 60 minutes after addition. According to the FVIII:C added (0.5 IU/ml) and the residual activity after inhibitor incubation, a calculated FVIII:C of 0.51 IU/ml resulted after addition of PCC/pdFVIII/VWF. Immediate measurement repeatedly resulted in residual FVIII:C of 0.26 to 0.29 IU/ml, which decreased to 0.08-0.10 IU/ml and 0.05 IU/ml after 15 and 30 minutes, respectively, reaching a stable baseline activity of 0.03 IU/ml at 60 minutes (Illustration 3; Panel A).

TG assays revealed that lag time in SHP containing pAb was severely prolonged (55 minutes until the initiation of clotting) (Illustration 3; Panel A). Incubation of SHP containing pAb inhibitors with 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF for 0 and 15 minutes resulted in normalisation of the lag phase (effect was
Impact on TG parameters of PCC/pdFVIII/VWF compared with aPCC and rFVIIa

TG assays were performed in order to assess the haemostatic effects of 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF, aPCC and rFVIIa after addition to SHP/platelets containing pAb/mAb. rFVIIa shortened the lag time somewhat below the SHP/platelet control, but did not normalise peak thrombin concentration (Illustration 4; Panels A, B). In contrast, the combination of PCC and pdFVIII/VWF normalised both parameters. aPCC revealed a significant shortening of the lag phase at the concentration used, while peak thrombin concentration was in the normal range. The CT was prolonged 3-fold, correspondingly (Illustration 2; Panel A). The MCFs remained in the normal range. After 60 minutes, the CT was prolonged 3-fold, correspondingly (Illustration 2; Panel E).

Platelets contribute to the effect of PCC/pdFVIII/VWF on TG in SHP in the presence of pAb/mAb

In order to evaluate the contribution of platelets in the described setting, TG assays were performed using SHP containing pAb/mAb. In contrast to the experiments performed with pAb alone (data not shown), platelets were required for the PCC/pdFVIII/VWF combination to bring peak thrombin concentrations into the normal range. In contrast, conditions without platelets were associated with only a low response (Illustration 6). There was no significant difference between thrombin generation of SHP and SHP/platelets.

Discussions and Conclusion

In HA patients with inhibitors, recent data suggest that the PCC used in this study may be a useful alternative to aPCCs or rFVIIa as a bypassing agent [24]. The data presented in this in vitro/ex vivo study demonstrate that PCC, given in combination with pdFVIII/VWF concentrates (Octanate® or Wilate®), results in normalisation of plasma/platelet TG. This held true for experimentally (FVIII) inhibited normal plasma as well as for the investigated HA inhibitor plasmas.

Both assay systems, TG and ROTEM®, have been extensively investigated for monitoring of aPCC and rFVIIa effects in the treatment of HA patients with specific factor inhibitors and rare coagulation disorders [16,32-34]. In the present study, ROTEM® analyses...
supplemented the data gained by the TG assay, showing that clotting activity was eliminated in whole blood containing pAb, and application of a combination of PCC and pdFVIII/VWF restored clot formation parameters in vitro.

In general, rFVIIa alone confirmed the poor in vitro response reported by other working groups [35-37], even in the presence of platelets, though rFVIIa has been shown to be clinically effective [38]. It thus poorly facilitates a prediction of in vivo effects. In contrast, aPCC application resulted in the expected pronounced shortening of lag time and increased peak thrombin concentration [39]. The in vitro/ex vivo effects observed upon combination of PCC/pdFVIII/VWF and rFVIIa or aPCC appeared not to be synergistic, but this will have to be carefully observed clinically with regard to thromboembolic adverse events if used to treat emergency bleeding episodes.

Based on the fact that non-activated PCCs show a clinical usefulness for treating bleeding events in HA inhibitor patients, cases responding poorly to monotherapy of either PCC, aPCC or rFVIIa may become of particular interest for a potential PCC/pdFVIII/VWF combination treatment. In vitro results indicated that even a moderate increase in non-activated clotting factors can shift the haemostatic balance to a more procoagulant state, which can be brought about via intervention with a PCC. It is plausible that application of exogenous FVIII may drive coagulation if exceeding the total inhibitor capacity or if escaping the immediate neutralisation by the antibodies. The significant haemostasis enhancing effect of even very low FVIII:C is known, and thus the kinetics of FVIII inhibition are decisive for the extent and duration of cofactor effect and treatment efficacy. Therefore, we used the model to mimic moderate to high FVIII inhibitory capacities in plasma by spiking samples with pAb or a mixture of pAb/mAb. Epitope specificities and neutralising capabilities of the latter were reported by another working group [40].

After inhibition of FVIII by a 1-hour incubation with pAb only, about 1% of residual FVIII:C was measured. Addition of 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF, immediate mixing and dilution for measurement, provoked a 40-50% increase in FVIII:C. Activities decreased with time, but even after 30 and 45 minutes, residual activities of 5% and 3%, respectively, were quantified. Peak thrombin concentrations remained in the normal to lower-limit–of–normal range, respectively, until 15 minutes after application. Minimum values were approached by 60 minutes; however, in the presence of platelets these parameters remained clearly measurable above baseline. Although ROTEM® results were obtained with whole blood, we utilised this methodology following inhibition of FVIII by the addition of antibodies (as in the TG kinetic studies) to demonstrate that small amounts of thrombin that are generated are sufficient to drive clot formation, as indicated in the plasma/platelet system. Taking into account that the plasma was diluted by 50% by platelet addition, extrapolated peak thrombin concentrations in whole plasma of equal or less than 60 nM should be sufficient to initiate clot formation, as observed 60 minutes after addition of PCC and pdFVIII/VWF. This is in agreement with recent publications suggesting that initial thrombin concentrations as low as 20-30 nM are sufficient for initiation of clotting, while the subsequent thrombin boost may be required for stability of the clot [41,42]. Nevertheless, at present there is no proven general threshold of peak thrombin generation sufficient to stop bleeding in HA inhibitor patients. Individual clinical conditions have to be considered.

The corresponding lag times increased with decreasing FVIII:C and peak thrombin concentrations over time, as expected. Notably, they did not reach the level of the inhibited plasma/platelet control, reflecting the basic effect of PCC in decreasing FVIII:C, which is in line with the ROTEM® results.

The experiments performed in this study underline the pivotal role of platelets in PCC/pdFVIII/VWF-mediated clotting activity and thrombin generation. Following treatment with the combination of PCC and pdFVIII/VWF, substantial clotting activity was observed in SHP containing lyophilised platelets with pAb/mAb inhibitors. However, this was not the case for conditions with no platelets, which warrants further investigation. Experiments in this study were performed using lyophilised platelets, which could be viewed as a consequential limitation of the results. While the lyophilised platelets have many of the molecular functions of fresh platelets, they do not perfectly reflect the multi-faceted functionality of viable fresh platelets, and they were not particular to the specific patients’ samples [43-45]. Nevertheless, the lyophilised platelets across all experiments here came from the same standardised batch. This standardisation of platelets thereby removed inter-patient or inter-batch platelet variability otherwise underlying differences in the results from the experimental samples.

Thrombin generation and clot formation were normalised for as long as 15 minutes after application of PCC/pdFVIII/VWF, remaining measurable until 60 minutes (limit of observation period). Other inhibitor profiles will also certainly have an influence on FVIII inhibition kinetics. However, coagulation factors
present in the PCC should remain present over a longer period of time relative to the decreasing FVIII:C following application of PCC and pdFVIII/VWF. This longer-lasting presence should hypothetically safeguard against recurrent bleeds [14,46,47]. The HA inhibitor patient samples spiked with platelets and PCC/pdFVIII/VWF revealed an excellent ex vivo response in all cases investigated. The addition of 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF concentrate (Octanate® or Wilate®), administered as separate preparations (i.e. not in immediate combination), would appear to be a reasonable starting point for clinical trials.

In conclusion, the in vitro/ex vivo results reported here are encouraging and support the investigation of the combination of PCC and pdFVIII/VWF in a proof-of-principle clinical trial, in order to further establish the viability of this approach as an effective and safe strategy for the treatment of bleeding events in HA inhibitor patients.

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Conflict of Interest Statement

Erik Berntorp has received speaker fees from Octapharma. Juergen Roemisch, Katharina Pock, Isabella Laher-Kheirallah, Sandra Janisch, Olaf Walter and Sigurd Knaub are employees of Octapharma.

References

Illustrations

Illustration 1

Impact of PCC/pdFVIII/VWF on in vitro TG parameters in SHP containing FVIII antibodies

TG parameters are shown for 0.25 IU/ml of PCC and for a combination of 0.25 IU/ml of PCC with 0.5 IU/ml pdFVIII/VWF in SHP spiked with platelets and pAb/mAb (31 BU/ml). TG parameters are also shown for SHP with or without pAb/mAb, and SHP containing platelets with or without inhibitors (served as controls). SHP with platelets (Plt) and pAb/mAb are abbreviated as A in the legend.

Panel A
Panel B

![Graph showing peak thrombin concentration vs. various conditions]
Illustration 2

ROTEM analysis of PCC/pdFVIII/VWF in whole blood containing neutralising FVIII antibodies

Representative thrombelastograms are shown for freshly drawn whole blood (Panel A) and blood incubated with pAb (10 BU/ml) at 37°C for one hour (Panel B). Subsequently, 0.25 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF was added and ROTEM®/NATEM® was started immediately. Time points are shown for time 0 (Panel C), after 15 minutes (Panel D) and after 60 minutes of incubation (Panel E).

Panel A
Panel B
Panel C
Panel D
Panel E
Illustration 3

FVIII inhibition kinetics and impact on TG parameters upon application of PCC/pdFVIII/VWF.

Panel A: FVIII:C (line) and lag time (bars) were assessed in SHP incubated with 10 BU/ml of pAb. The impact of 0.25 IU/ml PCC/0.5 IU/ml pdFVIII/VWF were investigated at different time points. A theoretical value of 0.51 IU/ml of FVIII:C was calculated (first left point of line).

Panel B: peak thrombin concentrations (bars) and FVIII:C activities (line) according to Panel A. A: SHP with pAb.

Panel A
Panel B
Illustration 4

Impact of PCC/pdFVIII/VWF, rFVIIa and aPCC on TG parameters in SHP containing FVIII antibodies.

TG parameters (Panel A: lag phase; Panel B: peak thrombin concentration) are shown using SHP with platelets (Plt) and pAb/mAb (31 BU/ml). The impact of rFVIIa (2 µg/ml), aPCC (2 IU/ml), and rFVIIa/aPCC in combination with 0.25 IU/ml PCC/0.5 IU/ml pdFVIII/VWF are also shown. TG parameters are shown for SHP with platelets not containing pAb/mAb served as a control. A: SHP with platelets and pAb/mAb.

Panel A
Panel B

![Graph showing peak thrombin concentration in different conditions](image-url)
Illustration 5

Impact on TG parameters after ex vivo addition of PCC/pdFVIII/VWF, rFVIIa or aPCC to a plasma sample of an HA patient displaying inhibitors.

The plasma aliquots containing inhibitors (34 BU/ml) were spiked with platelets and TG curves were monitored for the following conditions: no treatment (squares), 0.5 IU/ml PCC and 0.5 IU/ml pdFVIII/VWF (crosses), 0.5 IU/ml PCC and 1.0 IU/ml pdFVIII/VWF (triangles), 2 µg/ml rFVIIa (circles), 2 IU/ml aPCC (dotted line) and SHP (diamonds, dashed line).
Illustration 6

The impact of platelets on the effect of PCC/pdFVIII/VWF on TG in SHP containing FVIII antibodies.

Peak thrombin concentrations were measured using SHP, SHP spiked with pAb/mAb, and SHP spiked with pAb/mAb and PCC/pdFVIII/VWF, each in the presence (grey bars) or absence of (added) platelets (black bars). Prior to addition of 0.25 IU/ml PCC/0.5 IU/ml pdFVIII/VWF, SHP with and without platelets had been incubated for 60 minutes at 37°C. SHP with pAb/mAb is abbreviated as A in the legend.
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