Quantitative and qualitative analysis of coagulation factors in cryoprecipitate prepared from fresh-frozen plasma inactivated with amotosalen and ultraviolet A light

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BACKGROUND: There were no previous studies about the quality of cryoprecipitate prepared from fresh-frozen plasma (FFP) inactivated with amotosalen and ultraviolet A (UVA) light. The aim of this study was to analyze the quantity and quality of coagulation factors in cryoprecipitate prepared from FFP treated with amotosalen and UVA light.

STUDY DESIGN AND METHODS: FFP was obtained from whole blood donations and inactivated with amotosalen and UVA light according to the manufacturer's instructions. Fibrinogen, factor VIII (FVIII), von Willebrand factor antigen (VWF : Ag) and activity (VWF : RCo), the von Willebrand factor cleavage protease activity (ADAMTS-13), and the multimeric structure of VWF were analyzed.

RESULTS: The content of fibrinogen, FVIII, and ADAMTS-13 was lower in cryoprecipitates prepared from amotosalen-treated plasma when compared with cryoprecipitates prepared from nontreated plasma (35, 40, and 18% loss, respectively). The quantity and quality of VWF as well as VWF multimer patterns were not affected by the inactivation method.

CONCLUSION: Cryoprecipitates prepared from amotosalen-treated FFP contained significantly reduced levels of fibrinogen, FVIII, and ADAMTS-13. However, the VWF quantity and quality was well preserved.

The cold-insoluble portion of plasma that precipitates when fresh-frozen plasma (FFP) is thawed between 1 and 6°C is known as cryoprecipitate in European countries[1] or cryoprecipitated antihemophilic factor in North American states.[2] The manufacturing process has changed little since first described by Judith Graham Pool.[3] This plasma component is prepared from one FFP obtained from whole blood (WB) donations. After the FFP is thawed overnight at 2 to 6°C, the component is recentrifuged using a hard spin at the same temperature and the supernatant is removed, leaving the cold-insoluble precipitate plus 5 to 15 mL of plasma in the original bag, according to guidelines provided in the American Standards,[4] while European guidelines[1] recommend a maximal final volume of 40 mL. Minimum requirements of fibrinogen and Factor (F)VIII are 150 mg and 80 IU/bag, respectively, according to AABB Standards, while European guidelines recommend at least

ABBREVIATIONS: ADAMTS-13 = von Willebrand factor cleavage protease activity; T&B = top and bottom; VWF : Ag = von Willebrand factor antigen; VWF : RCo = von Willebrand factor activity; WB = whole blood.

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140 mg of fibrinogen, at least 70 IU of FVIII, and more than 100 IU of von Willebrand factor (VWF).

Proactive technologies to improve transfusion safety have been developed to treat plasma for inactivation of pathogens that may remain undetected before transfusion.[5] Currently, two fundamentally different approaches to pathogen inactivation of plasma are in use: solvent/detergent (S/D) treatment or the addition of nucleic acid–binding heterocyclic sensitizers that inactivate DNA or RNA upon light exposure (photosensitizers).[6] One available photosensitizer is the use of amotosalen and ultraviolet A (UVA) light for the inactivation of pathogens in plasma components because of selectively targeting of nucleic acids and formation of covalent bounds with nucleic acid bases.[7]

There is extensive literature about the quality of plasma treated with amotosalen and UVA light[8-10] as well as clinical use of this treated plasma in patients with congenital coagulation factor deficiencies,[11] patients with acquired coagulopathy of liver disease,[12] and patients with thrombotic thrombocytopenic purpura.[13] However, to our knowledge, there are no previous studies about the quality of cryoprecipitate prepared from plasma bags inactivated with amotosalen and UVA light. The aim of this pilot study was to analyze the quantity and quality of coagulation factors in cryoprecipitate prepared from FFP bags treated with amotosalen and UVA light.

MATERIALS AND METHODS

Plasma units

Plasma units used in the present study were obtained from WB donations according to our current method for preparation of blood components.[14] Briefly, blood was collected from volunteer donors meeting European guidelines criteria for blood donation. We collected 450 mL (±10%) of blood in a conventional, top-and-bottom (T&B), quadruple-bag system (Grifols, Barcelona, Spain). The contents in the T&B WB bags were cooled to and kept at 22 ± 2°C after donation using butane-1,4-diol plates until the following morning. The T&B WB bags were centrifuged at 22°C in a low-speed centrifuge (Cryofuge 6000i, Heraeus Instruments GmbH, Osterode, Germany). The centrifugation settings were 4497 × *g* for 18 minutes with an acceleration rate of 2 and deceleration rate of 5. After centrifugation, the components were separated using an automated device (Terumo Europe, Leuven, Belgium). The plasma was extracted from the WB bag via the top outlet and the red blood cells were extracted via the bottom outlet. The buffy coats containing platelets remained in the original WB collection bag.

Fifteen plasma pools were prepared each from three ABO-matched WB-derived plasma components. After pooling, each pool was split into two portions: one aliquot (235-315 mL) was used as nontreated FFP, and a second aliquot (385-635 mL) was treated with amotosalen. The plasma bags were rapidly frozen in a freezer at –40°C within 24 hours after WB collection and stored in an electric freezer at –30°C.

Plasma inactivation

Amotosalen treatment (Cerus Europe B.V., Amersfoort, the Netherlands) of the units was performed according to the manufacturer's instructions. The entire plasma content was allowed to flow through the amotosalen solution container into the illumination container. After heat seal and separation of the amotosalen container along with the original plasma bag, the plasma mixture was illuminated with the illumination device (Cerus Europe B.V.) at 3 J/cm^2 for approximately 7 minutes. After illumination, the plasma was passed through the compound adsorption device by gravity into the final storage containers. The compound adsorption device step took approximately 20 minutes.

Preparation of cryoprecipitates

We thawed plasma units overnight at 2 to 6°C and we recentrifuged the plasma bags at 5000 × *g* for 6 minutes at 2 to 6°C. We then removed the supernatant, leaving the cold-insoluble precipitate in a final plasma volume of 40 mL. We froze the resulting cryoprecipitate rapidly at –30°C.

Measurements of in vitro quantitative and qualitative protein analysis

After 1-month frozen storage –30°C, the plasma samples were thawed immediately before testing. Fibrinogen (FI) was measured with a modified Clauss assay in which the clotting time of a diluted plasma sample, after conversion by thrombin into insoluble fibrins, was compared to a standard curve prepared with reference plasma of known fibrinogen concentration.

Coagulation FVIII was assayed with activated thromboplastin time–based clotting assay. The clotting time of a mixture of diluted test plasma sample and plasma deficient in the factor being quantified was compared with a reference curve constructed from the clotting times of five dilutions, ranging from 1:2 to 1:50, of plasma with known activity mixed with deficient plasma. This test was performed on an automated coagulation analyzer (Izasa, Barcelona, Spain). Reagents included human recombinant tissue factor (Instrumentation Laboratory Co., Lexington, MA), calcium chloride (Instrumentation Laboratory Co.), synthetic phospholipids (Instrumentation Laboratory Co.), and congenital factor–deficient substrate. The end-

TABLE 1. Coagulation factors in cryoprecipitates prepared from nontreated and amotosalen-treated plasma

Coagulation factor levels*	Number	Nontreated	Amotosalen treated	p value
Fibrinogen (mg/unit)	15	558 ± 151 610 (214-878)	362 ± 120 342 (214-610)	<0.001
FVIII (IU/unit)	15	203 ± 60 178 (142-342)	121 ± 27 117 (78-182)	<0.001
VWF : Ag (IU/unit)	15	229 ± 75 200 (129-354)	226 ± 100 185 (134-488)	0.9
VWF : RCo (IU/unit)	15	153 ± 95 110 (65-316)	137 ± 99 67 (61-333)	0.6
ADAMTS-13 (IU/unit)	7	77 ± 9 78 (59-86)	62 ± 10 62 (46-75)	0.02

* Values are given as mean ± SD and median (range).

point of all tests was the formation of a clot detected photooptically and measured in seconds. The level of the factor being measured was inversely proportional to the time it takes for a clot to form. Factor assay control plasma (Instrumentation Laboratory Co.) was used as the reference standard for the coagulation factor assay.

Von Willebrand factor antigen (VWF : Ag; Grifols) and activity (VWF : RCo; Axis-Shield Diagnostics Limited, London, UK) were both determined by using standard techniques. The VWF cleavage protease (ADAMTS-13) activity was carried out by the assay developed by Kokame and coworkers.[15] Assay calibration was performed by using a pool of normal human plasma diluted 1:25 (100% of activity) in the assay buffer. Further calibration samples were obtained by serial dilutions of normal human plasma of 3:4 (75%), 1:2 (50%), 1:4 (25%), and 1:20 (5%) in heat-inactivated FFP; incubated for 30 minutes at 56°C; and followed by 15 minutes of centrifugation at 15,000 × *g*. All the samples were subsequently diluted 1:25 in the assay buffer. The substrate FRETS-VWF73 was diluted with dimethyl sulfoxide and distilled water to obtain a concentration of 100 µmol/L and finally diluted 1:25 in assay buffer. Samples were incubated for 10 minutes at 37°C in a fluorometer (Thermo Electron Corp., Waltham, MA) and the substrate FRETS-VWF73 was added to initiate the reaction. Fluorescence was measured using an excitation filter of 340 nm and an emission filter of 450 nm every 5 minutes for a total of 1 hour, and the reaction rate was calculated by linear regression analysis.

Analysis of VWF multimers was carried out by sodium dodecyl sulfate–agarose discontinuous gel electrophoresis followed by protein transfer to nitrocellulose membranes by Western blotting. Blots were probed using horseradish peroxidase (HRP)-conjugated rabbit anti-VWF; visualization of VWF multimers was achieved using a commercially available enhanced chemiluminescence kit for detecting HRP-labeled antibodies on Western blots.[16]

Statistical analysis

The variables were normally distributed and were reported as mean and standard deviation (SD). Because of our small sample size, statistical comparisons were made with the nonparametric Wilcoxon test to determine whether there were differences between amotosalen-treated group and nontreated group. A p value of less than 0.05 would indicate significant differences between the groups. Statistical analysis was carried out with computer software (SPSS, Inc., Chicago, IL; and GraphPad Software, Inc., San Diego, CA).

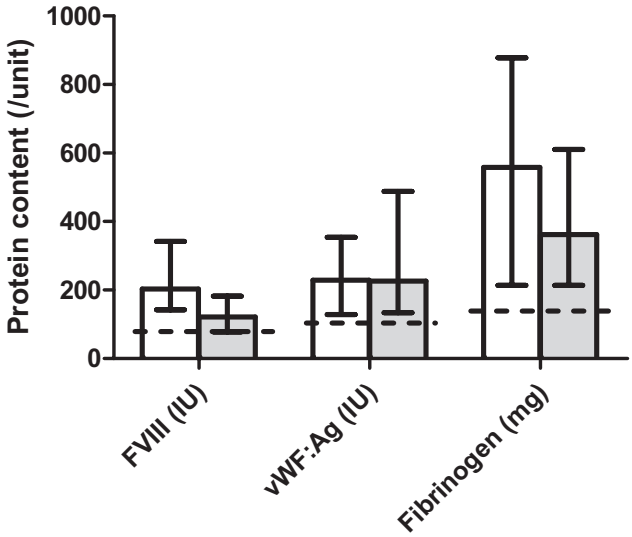

Fig. 1. Protein content of FVIII, VWF : Ag, and fibrinogen in cryoprecipitates prepared from nontreated (□) and amotosalen-treated plasma (▨). Columns and error bars indicate mean protein content and range, respectively. (- - -) Minimum requirements by the European guidelines (FVIII, ≥70 IU/unit; VWF : Ag, >100 IU/unit; fibrinogen, ≥140 mg/unit).

RESULTS

Table 1 shows the results of coagulation factor levels and ADAMTS-13 activity in cryoprecipitates prepared from nontreated and amotosalen-treated plasma. We observed that cryoprecipitates prepared from plasma treated with amotosalen contained a lower quantity of all coagulation factors analyzed when compared with cryoprecipitates prepared from nontreated plasma. The reduction was

significant for fibrinogen, FVIII, and ADAMTS-13. However, cryoprecipitates prepared from amotosalen-treated plasma met the European requirement (Fig. 1).

Regarding VWF, the content of VWF : Ag and VWF : RCo was lower in cryoprecipitates prepared from amotosalen-treated plasma when compared with non-treated plasma, although the difference was not significant (Table 1). Moreover, the VWF multimer patterns were not affected by the inactivation method (Fig. 2).

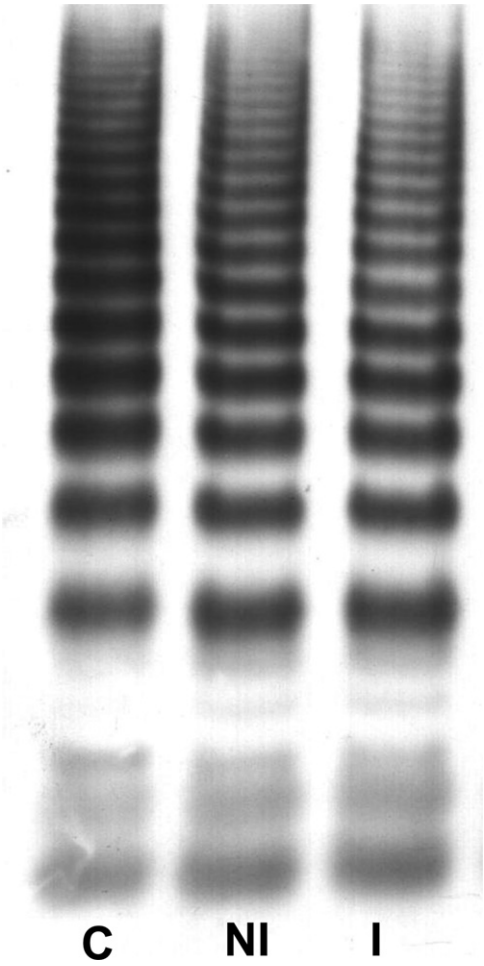

Fig. 2. Multimer analysis of VWF in cryoprecipitates prepared from nontreated plasma (NI) and amotosalen-treated plasma (I). Internal control (C).

DISCUSSION

To our knowledge, we report the first study about the quantity and quality of coagulation factors in cryoprecipitates prepared from plasma inactivated with amotosalen and UVA light. Our data showed that this pathogen inactivation process produced a significant decrease of fibrinogen, FVIII, and ADAMTS-13 values. However, the quantity and quality of VWF was not reduced because of the amotosalen inactivation method.

In our hands, cryoprecipitates prepared from amotosalen-treated plasma showed a 35% mean reduction of the fibrinogen level. Other authors have previously published the quality of cryoprecipitate prepared from inactivated plasma with the S/D method or the methylene blue plus light (Table 2). Regarding the S/D method, Keeling and colleagues[17] reported a 28% decrease of fibrinogen content in cryoprecipitates prepared from approximately 750-units-pooled plasma inactivated with that method. To improve these results, other authors used a minipool process for S/D treatment of five to six cryoprecipitates. With this approach, the authors reported an excellent 97% to 101% recovery of fibrinogen content and they recommended this technology to blood centers because it can be performed in a closed-bag system and using simple techniques and equipment.[18,19]

Regarding the methylene blue plus visible light photoinactivation method, the authors reported an acceptable 18% decrease of fibrinogen levels in cryoprecipitates prepared from plasma bags inactivated according to the original procedure of the German Red Cross of Springe.[20,21] With those results, the authors concluded that cryoprecipitates could be useful for replacing fibrinogen deficiencies in countries lacking the economic resources to obtain virally inactivated concentrates. Other authors reported a 41% to 43% decrease of fibrinogen levels in cryoprecipitates prepared from plasma units that were treated with methylene blue plus light according to either the method marketed by Baxter[22] or the method marketed by MacoPharma.[23] Because the obtained cryoprecipitate units do not satisfy the UK specifications, the same authors conditioned plasma units before preparing cryoprecipitates to improve those results. Conditioning plasma units means that FFP units were stored at +2 to +6°C for 8 hours before

TABLE 2. Comparison of mean coagulation factor loss in cryoprecipitates prepared from pathogen-inactivated plasma when compared with cryoprecipitates prepared from noninactivated plasma

	S/D method (%)		Methylene blue method (%)			Amotosalen method, this study (%)
Coagulation factor	Large pool[17]	Minipool[18,19]	Springe[20,21]	Baxter[22]	MacoPharma[23]	
Fibrinogen	28	1	18	41	43	35
FVIII	43	1	23	40	36	40
ADAMTS-13	NA	NA	NA	NA	NA	18
VWF : Ag	63	NA	3	14	13	1
VWF : RCo	64	7	13	20	NA	11

NA = not available.

cryoprecipitation. With this approach, the authors succeeded at significantly increasing the fibrinogen content of the cryoprecipitates with only a small loss of FVIII, and the components satisfied the UK guideline specification of at least 140 mg of fibrinogen and at least 70 IU of FVIII in 75% of cryoprecipitates tested.[24]

In our studies, FVIII levels were reduced by a mean of 40%. This figure is similar to reported data with other inactivation methods. Regarding the S/D method, the mean loss of FVIII was 43%.[17] Regarding the methylene blue method, the mean loss of FVIII was 23% with the original Springe method,[21] 40% with the Baxter method,[22] and 36% with the MacoPharma method.[23]

The ADAMTS-13 activity levels were reduced by an acceptable mean of 18%. To our knowledge, there are no previous reported data about the activity of this protease in cryoprecipitates prepared from other pathogen inactivation methods.

Of note, the quantity and quality of VWF as well as VWF multimer patterns were retained in cryoprecipitates prepared from amotosalen-treated plasma units. All these variables were much better preserved than when using the original S/D method. This method produced a high loss of VWF : Ag and VWF : RCo and the VWF multimeric distribution was abnormal, with loss of approximately the six highest molecular weight forms of a normal total of 24.[17] In addition, when comparing our present results with those obtained with the methylene blue method, we observed that the amotosalen inactivation was less aggressive with regard to the VWF integrity.[20-23]

There are two issues to take into account according to the present data. One of them is a regulatory issue because our results demonstrate a significantly quantitative reduction in fibrinogen, FVIII, and ADAMTS-13 levels when cryoprecipitates are prepared from amotosalen-treated plasma. In the absence of comparative studies with cryoprecipitates, the recent results by Rock in FFP deserve attention.[25] Although being a different product, inactivation of FFP caused similar reductions in coagulation factors. We believe that our present data could be useful for regulators if an inactivated cryoprecipitate is licensed in the future. However, other matters should be taken into account, such as resources, expenses, and cost-effectiveness analysis.

An additional issue is the clinical relevance of the analytical data observed in our studies. Standards of the AABB and European guidelines emphasize the contents of proteins per unit, with no specification on the quality of the blood product. It has been speculated that, apart from the quantitative changes, there could be qualitative alterations in the coagulation proteins after inactivation. Demonstrating possible differences among inactivated and noninactivated cryoprecipitates would require comparative clinical trials with great difficulties to perform taking into consideration the various inactivation procedures available. It is important in this respect that being fibrinogen the main component present in the cryoprecipitates, two recent opinion articles have raised the question on whether commercially available fibrinogen concentrate could be a more useful alternative to the cryoprecipitate.[26,27] Moreover, in this sense, a recent retrospective study compared the use of fibrinogen concentrate in acquired hypofibrinogenemia with noninactivated cryoprecipitate.[28] The median increment of fibrinogen after two bags of cryoprecipitate was 0.26 g/L (range, 0.01-1.64 g/L) compared with a median of 0.44 g/L (range, 0.02-1.98 g/L) after 2 g of fibrinogen concentrate. Although the difference was not significant, the authors concluded that because of its superior safety profile from infectious diseases, their study provided further evidence to support the use of fibrinogen concentrate. Taking together the previous information, we encourage performing prospective clinical studies focusing on dosing, efficacy, and safety, as well as evaluating methods of measuring fibrinogen and assessing fibrin polymerization.

In conclusion, we report the first study about the quantity and quality of coagulation factors in cryoprecipitate prepared from plasma inactivated with amotosalen and UVA light and, interestingly, our data showed that the VWF integrity was much better preserved than when using either the original S/D method or the methylene blue method.

CONFLICT OF INTEREST

The authors have no conflicts to disclose.

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