Haematologica 1998; 83:1009-1014 original paper

von Willebrand factor contained in factor VIII concentrates of different purities supports platelet adhesion in blood samples from a heterogeneous group of patients with von Willebrand disease

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Abstract

Background and Objective. **Plasma derived FVIII-VWF concentrates in which the VWF structure is reasonably maintained are recommended as substitutive therapy in VWD. Our aim was to assess platelet deposition and binding to subendothelial structures of VWF present in FVIII concentrates.**

Design and Methods. **Cryoprecipitate (CRY), intermediate-purity (IPC), or high-purity (HPC) FVIII concentrates were added *in vitro* to citrated blood samples from 11 patients affected by different subtypes of VWD, with the aim of normalizing VWF levels. Measurements of VWF:Ag, ristocetin cofactor (RiCof) activities, FVIII coagulant activity (FVIII:C), and platelet interaction with subendothelium under flow conditions (Baumgartner's perfusion method, computer-assisted morphometry, shear rate 1000 s^{-1}, 10 min, 37°C) were determined. Binding of VWF to the luminal surface of the perfused vessels was assessed by immunofluorescence microscopy. Paired t-test statistics were performed.**

Results. **Addition of FVIII-VWF preparations raised VWF:Ag from baseline (BSL) values of 0.3 (SD 0.2) to averages of 1.4 (SD 0.5, p<0.001), 1.2 (SD 0.6, p<0.001), and 0.4 (SD 0.3) IU mL^{-1} after CRY, IPC, and HPC, respectively. A positive labeling for VWF was observed by immunofluorescence in vessels perfused with blood containing any of the concentrates. Platelet adhesion of 13.2 (SD 7.6), 22.4 (SD 10.8), 24.8 (SD 7.8, p<0.03), or 22.5 (SD 4.8)% was measured in BSL, CRY, IPC, or HPC tests, respectively.**

Interpretation and Conclusions. **Our observations support the hypothesis above the mechanisms involved in the beneficial effects of commercial concentrates in von Willebrand disease: the VWF in these concentrates has functional capacity to bind to subendothelium and to support platelet adhesion.**

Key words: von Willebrand disease, FVIII concentrate, platelet adhesion, hemostasis

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Von Willebrand disease (VWD) is a variable bleeding disorder whose severity is dependent upon quantitative and qualitative deficiencies of plasma von Willebrand factor (VWF), in which clinically severe hemorrhage is mainly manifested following invasive procedures or trauma. Both prophylaxis and treatment of the bleeding are easily managed in mild forms of the disease. This may not be the case for severe, or variant forms of VWD. Selection of the proper treatment for the VWD patient is based on adequate classification of the VWD type, and includes D-arginine vasopressin (DDAVP) and plasma-derived concentrates.[1]

Due to the clinical experience gained, factor VIII/von Willebrand factor concentrates which are able to normalize VWF and factor VIII (FVIII) levels have been recommended for several VWD types, mainly type 3 and 2, to prevent or stop bleeding.[2] Additionally, VWF-depleted reconstituted blood incubated with a factor VIII-VWF concentrate was able to increase platelet deposition on subendothelial surfaces in an experimental system,[3] confirming that the VWF present in that preparation promoted platelet adhesion.

In the present study, we have further evaluated the hemostatic effect of different factor VIII preparations containing variable amounts of VWF. The preparations tested included regular cryoprecipitates, and intermediate and high-purity factor VIII concentrates. Aliquots of these preparations were added to blood samples from type 1, 2 or 3 VWD patients with the aim of reaching physiological VWF concentrations (>0.60 IU mL^{-1}). Platelet deposition and VWF distribution on subendothelial surfaces perfused under arterial blood flow conditions were evaluated morphometrically.[4]

Materials and Methods

Study medication

Standard single donor cryoprecipitates (CRY), locally produced at the study centers, were always thawed before use. Vials of freeze-dried intermediate

(IPC: Haemate-P, Centeon GmbH, Germany), or high-purity factor VIII concentrates (HPC: Beriate-P, Centeon GmbH, Germany), containing 500 IU FVIII were dissolved in sterile water immediately before use. For calculations, the amounts declared by the manufacturer, both in the IPC (25 IU FVIII:C mL^{-1}, 55 IU VWF mL^{-1}) and HPC (100 IU FVIII:C mL^{-1}, <20 IU VWF mL^{-1}) were used. CRY were assumed to contain an average of 5 IU VWF mL^{-1}, as determined by periodic quality testing in the blood bank. Under our experimental conditions, low-resolution multimeric analysis of VWF contained in the different concentrates reveals 12-15 bands in the standard cryoprecipitates. The IPC lacks one third to one fourth of the bands corresponding to the high molecular weight multimers. The HPC lacked the highest molecular weight and a portion of the intermediate molecular weight forms.

Blood donations and preparation of perfusates

Men or women aged ≥18 years, previously diagnosed with von Willebrand disease according to standard criteria,[1] and not actively bleeding at the time of the experimental procedures, were selected. On enrolment, all patients gave informed consent to donate blood for the study, which was conducted in accordance with the current version of the Declaration of Helsinki, once notified the institutional review board. Blood was anticoagulated with citrate-phosphate dextrose (CPD, final concentrate in blood 19 mM). Platelet counts and hematocrit were within the normal limits. None of the patients had taken drugs affecting platelet function in the previous 15 days.

One IU VWF mL^{-1} for CRY and IPC was added to citrated blood samples with a total volume of 30 mL from the different patients, with the aim of normalizing VWF levels. The latest preparation (HPC) was added to greatly exceed physiological FVIII:C levels. Perfusates were incubated for 30 min in a water bath at 37°C prior to the perfusion experiments. Samples of the perfusates were used for the determining hematocrit, platelet count, FVIII:C, RiCof and VWF:Ag. All the experimental procedures were conducted according to a blind design.

Blood perfusion and morphometry

The evaluation of platelet deposition on subendothelium was performed on everted de-endothelialized vascular segments placed in a perfusion chamber and exposed to flowing blood.[3] Flow was obtained by pumping the blood through a hemodialysis blood pump (Renal Systems, Minneapolis, MN, USA) at the appropriate flow rate (175 mL min^{-1}) to produce a wall shear rate equivalent to 1000 s^{-1}. After 5 min perfusion, the segments were rinsed, fixed with paraformaldehyde-glutaraldehyde and further processed for light microscopy to evaluate platelet interaction with subendothelium morphometrically, according to well established criteria.[4]

Assessment of FVIII/VWF related activities and structure

Coagulant factor VIII activity (FVIII:C) was measured by the one-stage clotting time.[5] Ristocetin cofactor activity (RiCof) was measured by using formaldehyde-fixed platelets.[6] Values were expressed in IU mL^{-1} with reference to plasma calibrated against the 2nd international standard for FVIII related activities in plasma (87/718, National Institute for Biological Standards and Controls, Potters Bar, UK). Von Willebrand factor antigen (VWF:Ag) was measured by ELISA.[7] The VWF multimeric structure was analyzed by sodium dodecyl sulphate agarose gel electrophoresis followed by electrotransfer to nitrocellulose membranes.[8]

Immunofluorescence microscopy

The presence of VWF:Ag bound to perfused vessels was assessed with an immunofluorescent technique.[9] Eight micrometer thick cryosections of perfused vascular segments were initially incubated with a 1/100 dilution in PBS of a rabbit antibody against human VWF (Dako A082, Dakopats, Denmark) previously conjugated with fluorescein (60 min, 20°C). After incubation, slides containing the sections were rinsed with PBS and finally mounted on coverslips using an antifading solution containing p-phenylenediamine in PBS-glycerin. Preparations were then studied through epifluorescence optics and micrographs taken on high sensitivity panchromatic film.

Statistical analysis

Computer-derived data, FVIII:C, VWF:Ag, RiCof and bleeding time values for each set of experiments were entered twice in a database (MS-Access, Ver. 2.0, 1989-1994, Microsoft Corp., Richmond, OR, USA) specifically designed for the present study. Data were translated to the SAS statistical package (ver 6.08., SAS Institute, NC, USA, 1988) for evaluation. The results of the experiments were expressed as mean (standard deviation). Group differences were analyzed by a paired t-test. Significance was considered at a p value <0.05.

Results

Subjects

Seven females and four males aged 32 to 62 years, weighing 50 to 90 kg were enrolled in the study. Von Willebrand disease was diagnosed as type 1 in 3 patients, as type 2A in 7 patients and as type 3 in 1 patient. Patients were not actively bleeding at the time of blood sampling and had not received any coagulation product, DDAVP or acetylsalicylic acid for at least the previous 15 days. The characteristics of the study population are described in Table 1.

Table 1. Study population. Pooled data are expressed as percentage (ordinary variables) or mean (SD) (quantitative variables).

Patient no.	*Sex*	*Age*	*Weight (kg)*	*Height (cm)*	*FVIII:C (IU mL^{-1})*	*Bleeding time (min)*	*VWF:Ag (IU mL^{-1})*	*RiCof (IU mL^{-1})*	*VWD subtype*
01	M	62	61	158	0.45	>15	0.46	0.01	2A
02	M	60	70	175	0.08	>15	0.01	0.02	1 (severe)
03	F	32	67	173	0.47	>15	0.34	0.09	2A
04	F	55	80	169	0.47	25	0.16	0.16	1
05	M	34	90	175	0.01	>15	0.01	0.01	3
06	M	36	60	170	0.05	18	0.07	0.25	1
07	F	32	83	183	0.36	>15	0.64	0.22	2A
08	F	58	56	168	0.32	>15	0.39	0.08	2A
09	F	32	50	164	0.30	>15	0.64	0.10	2A
10	F	34	61	159	0.34	>15	0.24	0.09	2A
11	F	52	83	158	0.23	>15	0.31	0.11	2A
	M (36%) F (64%)	44 (12)	69 (12)	168 (8)	0.30 (0.17)	>15	0.29 (0.1)	0.14 (0.11)	1 (27%) 2A (64%) 3 (8%)

M: male; F: female.

Baseline characteristics of perfusates

Variations in VWF:Ag, RiCof and FVIII:C levels in the final perfusates are shown in Table 2. Statistical differences were found between Group I (baseline) and groups II and III (CRY and IPC) for VWF:Ag, RiCof, and FVIII:C ($p<0.001$). Ristocetin cofactor values and FVIII:C in Group IV (HPC) reached levels of statistical difference when compared with Group I ($p<0.03$, $p<0.001$, respectively).

At the doses used, VWF:Ag reached normal values (>0.60 IU mL^{-1}) in 10/11 samples from Group II, 9/11 samples from Group III and 2/11 samples from Group IV. All samples from Group II, 4/11 samples from Group III and 1/11 samples from Group IV normalized RiCof (>0.60 IU mL^{-1}). Coagulant factor VIII reached normal values in 8/11 perfusates for Group III, and in all perfusates for Groups II and IV. Results are summarized in Figure 1.

Table 2. Baseline characteristics of perfusates. Data are expressed as mean (standard deviation), n = 11.

Study group	*Study drug*	*VWF:Ag (IU mL^{-1})*	*RiCof (IU mL^{-1})*	*FVIII:C (IU mL^{-1})*
I	BSL	0.29 (0.21)	0.14 (0.11)	0.30 (0.17)
II	CRY	1.37 (0.50)*	0.92 (0.22)*	1.12 (0.20) *
III	IPC	1.16 (0.63)*	0.42 (0.08)*	0.78 (0.19)*
IV	HPC	0.41 (0.25)	0.25 (0.07)#	1.09 (0.23)*

*Baseline (BSL), cryoprecipitate (CRY), intermediate-purity (IPC), high-purity (HPC) FVIII concentrate. *$p<0.001$ when compared with group I; #$p<0.03$ when compared with group I.*

Perfusion experiments

1. Pooled data. Due to technical reasons, baseline values could not be obtained from 5 patients. Therefore, the statistical evaluation compared baseline, CRY, IPC and HPC groups by paired t tests for only 6 complete experiments, which resulted in unfair influence on the treatment's effect.

Baseline perfusates (Group I), which corresponded to native VWD blood samples, showed 13.2 (7.6)% total surface coverage with platelets. Group II experiments (CRY) induced a surface coverage of 22.4 (10.8)%. Group III (IPC) increased total surface coverage to 24.8 (7.8)%. Group IV (HPC) induced a covered surface of 22.5 (4.8)%. Statistical significance was obtained for IPC surface coverage ($p<0.03$) when compared to baseline values (n=6). Figure 1 summarizes the profiles of the individual responses to the preparations added.

2. Subpopulations. Blood samples from the two patients presenting severe quantitative deficiencies (patients #02 and #05) raised VWF:Ag and RiCof to normal levels when CRY or IPC (Groups II and III, respectively) were added. Group IV (HPC) raised both parameters to a minimum of 0.40 IU mL^{-1}. Consistently, platelets interacting with subendothelium varied from baseline surface coverage of 8.0% to 38% for Group II, 30% for Group III, and 24% for Group IV.

Experiences with blood samples belonging to VWD type 2 patients (patients #01, 03, 07, 08, 09, 10, 11) showed heterogeneous responses. Increased surface coverages were detected in 2 out of 7 samples from Group II, 3 out of 7 samples from Group III and 2 out of 7 samples from Group IV. These results were in part explained by the basal VWF:Ag and RiCof activity found, which ranged from 0.01 to 0.64 IU VWF:Ag mL^{-1}, and from 0.01 to 0.25 IU RiCof mL^{-1}.

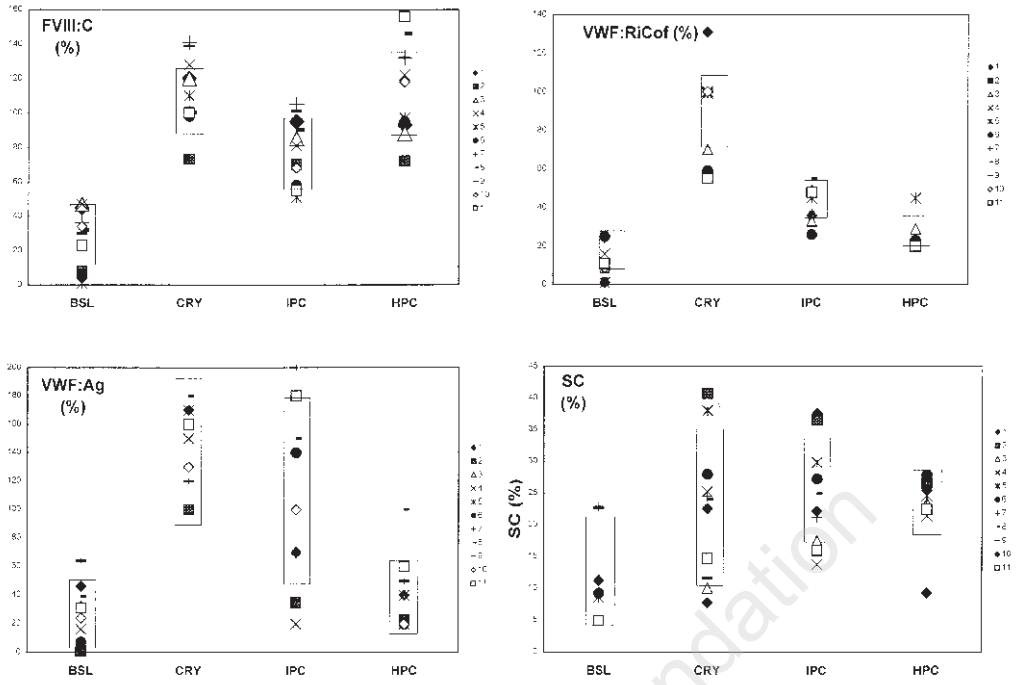

Figure 1. Coagulant factor VIII (FVIII:C), von Willebrand factor antigen (VWF:Ag), ristocetin cofactor activity (RiCof) and surface coverage (SC) results from blood samples from von Willebrand disease patients. Legends mean: Baseline (BSL), cryoprecipitate (CRY), intermediate-purity (IPC), high-purity (HPC) FVIII concentrate. Boxes represent the standard deviations of the data.

Immunofluorescent pattern of von Willebrand factor

Immunofluorescence studies confirmed differences between the study groups with respect to VWF deposition. A diffuse fluorescence without a predetermined pattern was always observed over the luminal surface of the vessel subendothelium perfused with VWD native blood. Clusters of fluorescence, mostly located in relation to platelet aggregates, were observed over subendothelial structures exposed to perfusates receiving CRY or IPC. A similar qualitative pattern was observed for HPC (Figure 2).

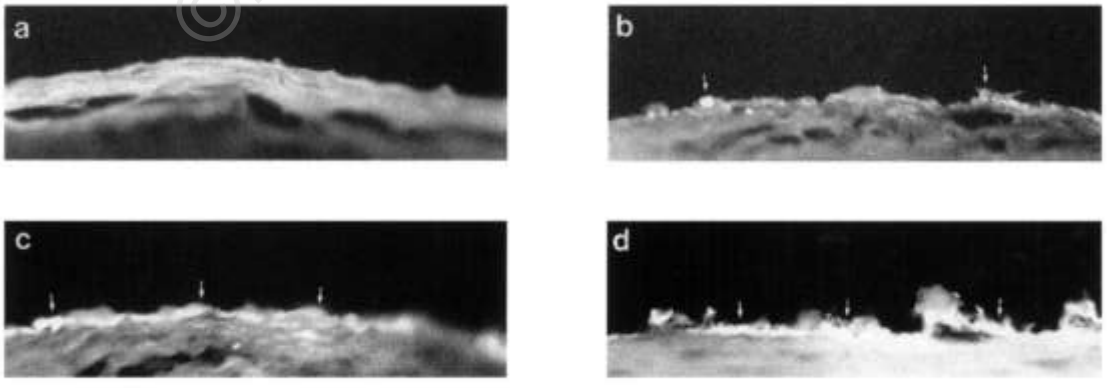

Figure 2. von Willebrand factor distribution on cross-sections of rabbit aorta subendothelial structures perfused with blood samples from von Willebrand disease patients: (a) baseline, (b) high-purity FVIII concentrate, (c) cryoprecipitate, (d) intermediate-purity FVIII concentrate. The antigen was detected by means of a double-indirect labelling technique performed on cryosections. Fluorescence was detected in relation to the luminal surface of the vessel. Original picture magnification ×400.

Discussion

Results of the present study show that the VWF contained in factor VIII preparations binds to vascular subendothelium and enhances platelet adhesion when added *in vitro* to blood samples from patients with von Willebrand disease.

There is biochemical, clinical and safety evidence in the literature supporting a positive role of the VWF contained in factor VIII concentrates in von Willebrand disease.[2] Available data on the efficacy of VWF-containing FVIII concentrates are based on bleeding time, VWF:Ag, RiCof recovery data and multimeric analysis after infusion. There is a large body of evidence that correlates these tests with VWF function, especially the bleeding time. In contrast, there is also consensus that standard *in vitro* determinations of VWF activities do not predict clinical effectiveness of concentrates.[10,11] For this reason, it was considered of interest to investigate the VWF functionality of different preparations further. As the main physiological function of VWF is to support platelet adhesion, the best way to study this physiological function should be through the evaluation of its ability to promote platelet adhesion. Such an objective can be readily evaluated by perfusion models.[12]

In the present study, the preparations under investigation were added to blood obtained from VWD patients with the aim of normalizing VWF activities. The study was carried out on blood samples from a group of VWD patients who were candidates to receive replacement therapy with FVIII/VWF concentrates. Moderate type 1 VWD patients were excluded from the study since this population of patients is dependent on DDAVP treatment.[1]

Binding of VWF to subendothelium is an essential step for VWF to support platelet adhesion mediated through glycoprotein Ib.[13] A common finding of the present study was that all the preparations tested promoted VWF binding to subendothelial structures as assessed by an immunofluorescent technique. Morphometric data revealed an improvement of platelet coverage. Even the high-purity preparation, with a RiCof/FVIII:C rate < 0.2, was capable of supporting platelet adhesion to subendothelium up to the levels of CRY and IPC. The HPC amount added increased VWF levels from 0.29 (0.21) IU mL^{-1} to 0.41 (0.25) IU mL^{-1}. Von Willebrand factor levels above 0.40 IU mL^{-1} are able to support platelet adhesion to subendothelium under the experimental conditions tested.[3] Moreover, treatment guidelines indicate that levels of VWF of 50% might be adequate to support hemostasis in the case of minor bleeding events.[2] These results confirm the possible role of residual VWF activities contained in some commercial high-purity FVIII concentrates when administered at high dosages (40-60 IU RiCof kg^{-1} ≈ 100-200 IU FVIII kg^{-1}). It is important to note that such high FVIII concentrations would be equivalent to dosages used for immunotolerance protocols to overcome hemophilia A inhibitors.[14] Additional studies carried out in a type 3 VWD patient confirmed that the HPC preparation containing residual VWF:Ag still improves platelet deposition, but this effect was lost in experiments performed with a recombinant factor VIII preparation which did not contain any VWF (data not shown).

Although it is perceived from general practice that substitutive treatment of VWD should provide a correction of the biological functions of FVIII/VWF, clinical experience with type 3 patients suggests that bleeding symptoms are not always corrected despite transfusional normalization of biological activities.[15] In our study, the cryoprecipitate produced the optimal correction of biological activities (FVIII:C, VWF:Ag, RiCof), though it did not provide the most consistent correction of platelet adhesion. The intermediate-purity concentrate produced a less dramatic correction of FVIII/VWF activities, but promoted a more reliable correction of platelet adherence as assessed by the narrower variation of platelet coverage values. A similar trend was observed with the high-purity preparation.

A statistical study with our pooled data was unable to demonstrate any correlation between VWF biological activities and abilities of the different preparations to support platelet adhesion. Thus, results of the present study indirectly suggest that a partial correction of the VWF activities might be sufficient to correct platelet adhesion. We think that the lack of correlation between VWF activities in plasma and correction of platelet adhesion may depend on the levels and function of the intraplatelet VWF pool. Recent literature reports seem to point in this direction.[16,17] Assessment of intraplatelet VWF could be a useful marker to classify VWD patients further according to hemorrhagic risks, and probably to indicate a patient-oriented substitutive treatment.

Taken together, our observations support the hypothesis of the mechanisms involved in the beneficial effects of commercial concentrates in von Willebrand disease: the VWF in these concentrates has functional capacity to bind to subendothelium, to platelet glycoprotein Ib[3] and, subsequently, to support platelet adhesion.

Contributions and Acknowledgments

GE and JAS were involved in study design, study analysis, discussion of the results, and production of the manuscript. MC was in charge of the experimental procedures and participated in their analysis and interpretation. MM and MQ selected the study population, participated in the experimental procedures, their analysis, and interpretation. CA was involved in study design, statistical analysis, and discussion of results. RC participated in study analysis, discussion of results, and manuscript production. All the authors reviewed and approved the manuscript. The authors wish to thank Mrs. Conchita Palacios, Mrs. Aránzazu de la Fuente, and Mrs. Montse Viñas for their technical support, and Mrs. Aurora Pedraza for her secretarial assistance.

Disclosures

Conflict of interest: the study was independently conducted at Hospital Clínic i Provincial, Barcelona and at Hospital La Paz, Madrid, Spain. JAS and CA, who work for the sponsor of the study, were involved in study design, study analysis, discussion of results and manuscript elaboration. The sponsor of the study produces and distributes plasma derived pharmaceuticals for commercial purposes.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received February 27, 1998; accepted July 3, 1998.

References

1. Sadler JE. A revised classification for von Willebrand disease. Thromb Haemostas 1994; 71:520-7.
2. Mannucci PM. Treatment of von Willebrand disease. Haemophilia 1998; 4:661-4.
3. Aznar-Salatti J, Escolar G, Arnau C, et al. An intermediate-purity factor VIII concentrate supports platelet adhesion under flow conditions. Haemophilia 1997; 3:14-20.
4. Baumgartner HR. The role of blood flow in platelet adhesion, fibrin deposition and formation of mural thrombi. Microvasc Res 1973; 5:175-9.
5. Hardistry R, MacPherson C. A one stage factor VIII assay and its use on venous and capillary plasma. Thromb Diath Haemorrhagica 1962; 7:215-28.
6. Zuzel M, Nilsson IM, Alberg MA. A method for measuring plasma ristocetin cofactor activity. Normal distribution and stability during storage. Thromb Res 1978; 12:745-54.
7. Ingerslev J. A sensitive ELISA for von Willebrand factor (vWF:Ag). Scand J Clin Lab Invest 1985; 45:17-26.
8. Ruggeri ZM, Zimmerman TS. The complex multimeric composition of factor VIII/von Willebrand factor. Blood 1981; 57:1140-3.
9. Escolar G, Garrido M, Aznar-Salatti J, Ordinas A, Bastida E. Comparison between human umbilical artery and rabbit abdominal aorta as substrata for platelet adhesion and platelet thrombus formation under flow conditions. Blood Vessels 1991; 28:520-31.
10. Berntorp E, Nilsson IM. Biochemical and in vivo properties of commercial virus-inactivated concentrates. Eur J Haematol 1988; 40:205-14.
11. Chang P, Aronson DL. Plasma derived von Willebrand factor preparations: collagen binding and ristocetin cofactor activities. Thromb Haemostas 1997; 78:930-3.
12. Sixma JJ. Interaction of blood platelets with the vessel wall. In: Bloom AL, Forbes CD, Thomas DP, Tuddenham EGD, eds. Haemostasis and Thrombosis, 3rd ed. Churchill Livingstone; Edinburgh; 1994. p. 259-85.
13. Sakariassen KS, Bolhuis PA, Sixma JJ. Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-von Willebrand factor bound to the subendothelium. Nature 1979; 279:636-8.
14. Gilles JGG, Jacquemin MG, Saint-Remy JMR. Factor VIII inhibitors. Thromb Haemostasis 1997; 78:641-6.
15. Menache D, Aronson DL. New treatments of von Willebrand disease: plasma derived von Willebrand factor concentrates. Thromb Haemostas 1997: 78:566-70.
16. Castillo R, Monteagudo J, Escolar G, Ordinas A, Magallón M, Martín Villar J. Hemostatic effects of normal platelet transfusion in severe von Willebrand disease patients. Blood 1991; 77:1901-5.
17. Castillo R, Escolar G, Monteagudo J, Aznar-Salatti J, Reverter JC, Ordinas A. Hemostasis in patients with severe von Willebrand disease improves after normal platelet transfusion and normalizes with further correction of the plasma defect. Transfusion 1997; 37:785-90.