

Thrombos. Diathes. haemorrh. (Stuttg.), 1975, 33, 632

Studies on the Thrombogenicity of Scottish Factor IX Concentrates in Dogs*

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Introduction

During the early studies of factor IX containing concentrates the concept of thrombogenicity as a potential hazard was clearly recognised (Ménaché et al. 1959), and although in most subsequent reports on the clinical use of such preparations this problem did not appear to arise (Bidwell et al. 1967; Gilchrist et al. 1969; Diko et al. 1972; Bruning et al. 1971; Josso 1970; Nilsson et al. 1971), in others there seemed little doubt that thrombogenicity was a real hazard (Tullis and Breen 1970; Kasper 1973; Steinberg and Dreiling 1973; Edson 1974; Marchesi and Burney 1974).

In 1967 a factor IX concentrate (P.P.S.B.) was first prepared in Scotland using the method described by Soulier et al. (1974), using EDTA plasma. This has been widely used in congenital and acquired factor IX deficiency with no reports received of thrombogenicity. In 1971 further protein fractionation developments led to the introduction of DEFIX, a concentrate containing factors II, IX and X prepared from citrated, factor VIII depleted plasma (Middleton et al. 1972). This product has been distributed routinely for the management of haemophilia B patients in Scotland since that time, again without reports of thrombogenicity. More recent developments have concerned the further purification of DEFIX, using polyethylene glycol PEG 4000 in order to produce a more potent concentrate and possibly carrying less risk of transmitting serum hepatitis (Johnson et al. 1973). This PEG concentrate (PEG-IX) has not yet been distributed for routine clinical use, but its development prompted us to consider whether efforts designed to increase purity might lead to a final product which was potentially thrombogenic. In this study an *in vivo* model was used and the dog selected as its size permitted serial blood sampling over prolonged periods of time. The factor IX concentrates currently prepared in the Scottish National Protein Fractionation Centre are summarised in Table 1.

Materials and Methods

Healthy female mongrel or Collie dogs weighing 15-25 kg, previously fed ad-lib, were used. Anaesthetic induction was obtained using intravenous pentobarbitone sodium. Their brachial (infusion) and femoral (blood sampling) veins were cannulated. The patency of these cannulae was maintained by an infusion of 5% dextrose. The urinary bladder was catheterised with a self-retaining Foley catheter and urine collected at 10 minute intervals. Pulse rates were monitored using an electrocardiograph. Not less than 60 minutes after the Foley catheter had been inserted (the absolute time depending on the steady urine flow) the infusion schedule was begun by control period (50 ml isotonic saline)

* Task Force Report

Studies on the Thrombogenicity of Scottish Factor IX Concentrates in Dogs 633

Table 1. Some characteristics of the Scottish factor IX containing concentrates included in the study.

	P.P.S.B.	DEFIX	PEG-IX
Production method	Calcium phosphate absorption of E.D.T.A. plasma; Citrate elution, then ethanol fractionation of eluate. (Pool size 70 litres)	Absorption of supernatant from Factor VIII production with DEAE-Cellulose. Phosphate citrate elution. (Pool size 100 litres)	Absorption of supernatant from Factor VIII production with DEAE-Cellulose. Phosphate citrate elution followed by precipitation with PEG-4000. (Pool size 200 litres)
Reconstitution volume (ml)	10	10	10
Vial contents (units)			
Factor IX	300	300	1000
Factor II	300	300	1000
Factor X	200	200	700
Factor VII	300	<10	<10
Heparin content (units/vial)	100	Nil	100

for 30 minutes followed by a second 30 minutes during which the test infusate was administered in a volume of 50 ml using a Harvard Constant Infusion Pump. The timings of the blood sampling is shown in the figures (vide infra).

Thirty dog studies were performed: 10 different infusates with 3 dogs in each group (Table 2). The fibrinogen, albumin, gammaglobulin and factor IX containing concentrates were prepared in the Protein Fractionation Centre. The blood coagulation tests undertaken were the platelet count (Dacie and Lewis 1963) plasma fibrinogen (Ellis and Stransky 1961), partial (Kaolin) thromboplastin time (Hardisty and Ingram 1965), serum fibrin/fibrinogen degradation products (Hoq and Das 1971) using homologous tanned red cells,

Table 2. Summary of the types of infusions given to the ten groups of dogs.

Group	Infusate
I	Citrate saline Control (50 ml: 0.01 M Sod. citrate: 0.45% Sod. chlorido)
II	Thrombin (150 u/Kg) (Bovine, Parke Davis)
III	P.P.S.B. (150 u. IX/kg)
IV	DEFIX (150 u. IX/kg)
V	PEG-IX (500 u. IX/kg)
VI	PEG-IX (50 u. IX/kg)
VII	Normal pooled human (ACD) plasma (50 ml)
VIII	Normal pooled human gammaglobulin (50 ml: 3.6 G)
IX	Normal human albumin (50 ml: 7.5 G)
X	Human fibrinogen (50 ml: 1 G)

fibrinogen and antisera and the ethanol gelation test (Kierulf and Godal 1971). These assays were performed immediately after blood withdrawal with the exception of fibrinogen and serum F.D.P. estimations which were undertaken some days later on appropriate aliquots stored at -36°C in plastic tubes.

The units of the factor IX concentrates shown in the results refer to the content of assayed factor IX. The bovine thrombin was obtained from Parke Davis Ltd.

Results

1. Citrate-saline: Normal plasma: Albumin: Fibrinogen: Gammaglobulin defix (150) an PEG-IX (50).

The results of the citrate-saline infusion are summarised in Fig. 1. No significant changes in the parameters measured were recorded during the 240 minute observation period with the exception of a small but consistent rise in serum F.D.P. in all animals. The results of infusions of normal plasma, albumin, fibrinogen, gammaglobulin, DEFIX (150 u/Kg) and PEG-IX (50 u/Kg) were not significantly different from the citrate-saline controls.

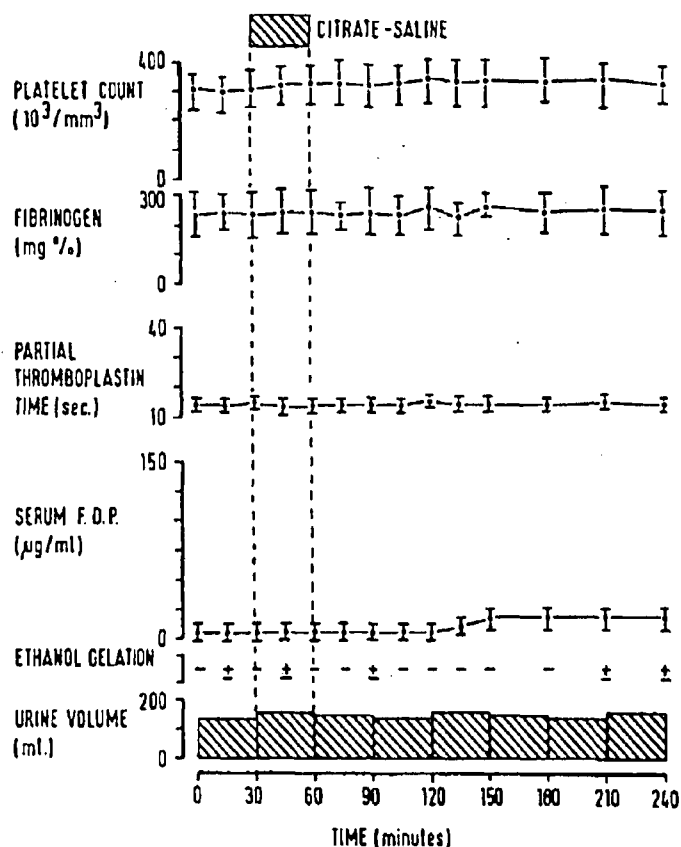


Fig. 1. Coagulation changes in three dogs infused with 50 ml citrate-saline. The close circles represent arithmetical means and the vertical bars the range. Urino volumes are given as arithmetic means only.

Studies on the Thrombogenicity of Scottish Factor IX Concentrates in Dogs 635

2. Thrombin

During the infusion of thrombin ample laboratory evidence of disseminated intra-vascular coagulation (DIC) was observed (Fig. 2). There was a profound fall in the platelet count, fibrinogen and a rise in the partial thromboplastin times and serum F.D.P. Severe oliguria was also associated with the infusion of thrombin.

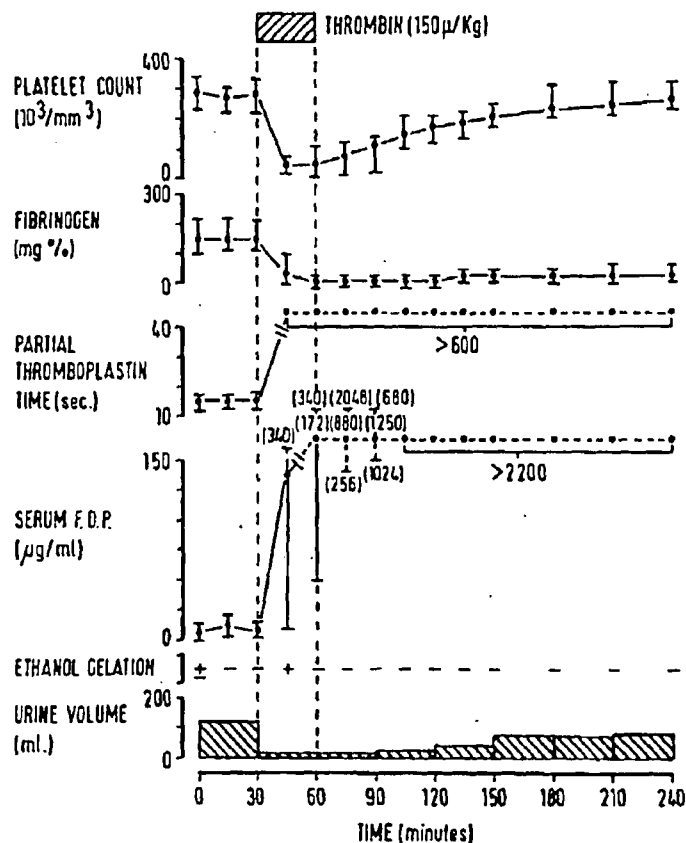


Fig. 2. Coagulation changes associated with the infusion of bovine thrombin (150 u/Kg) into three dogs. Graphs expressed as Fig. 1.

3. P.P.S.B.

The results of the infusions of P.P.S.B. (150 u/Kg) into three dogs are summarised in Fig. 3. Sixty minutes after the infusion had been terminated there was a fall in the platelet count in two dogs, which returned spontaneously towards the pre-infusion values. Satisfactory interpretation of fibrinogen assays was not possible in these studies; after thawing the frozen plasma it was noted that a significant number of post-infusion samples had clotted, making fibrinogen estimations by the Ellis and Stransky (1961) method impossible. The partial thromboplastin time lengthened slightly during the infusion and this was assumed to be related to the heparin present in our preparations of P.P.S.B. The results of the serum F.D.P. and ethanol gelation tests in conjunction with evidence of spontaneous clotting of fibrinogen during freeze/

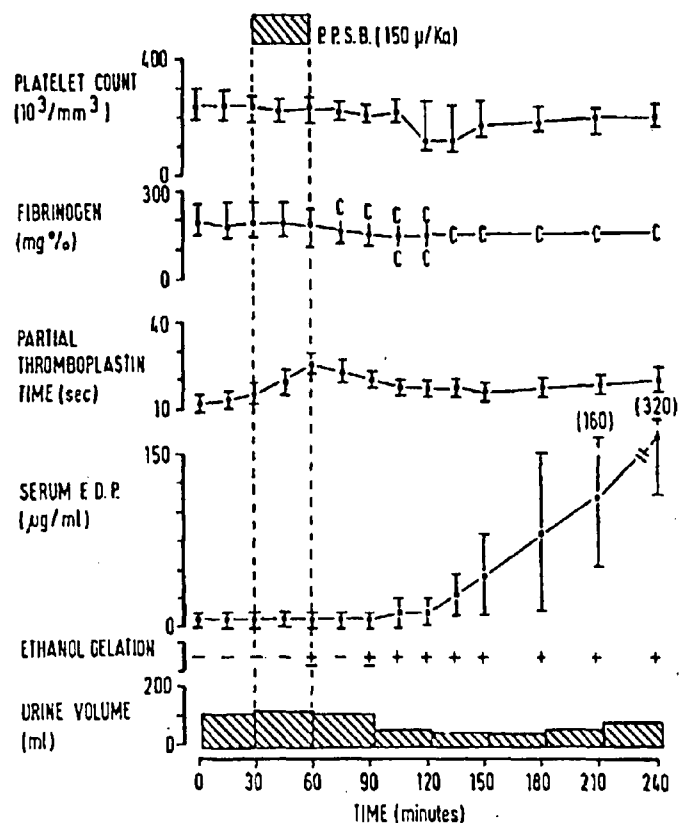


Fig. 3. Coagulation changes following the infusion of P.P.S.B. (150 μ /Kg) into three dogs. The changes are expressed as in Fig. 1, with the exception that C appearing in the fibrinogen levels indicates spontaneous clotting of sample (see text).

thawing was taken as evidence of low grade DIC in all three dogs. It was noted that this phenomenon did not occur during the infusion but was delayed in onset. In all dogs laboratory evidence of DIC was associated with a modest reduction in urine output.

4. PEG-IX (500)

The results of the infusion studies of PEG-IX at a dose of 500 units per Kg are summarised in Fig. 4. All three dogs showed a marked fall in platelet count, which occurred between 30-60 minutes after the infusion had been completed. Complete defibrination was also observed in all dogs but its onset again occurred some time after the termination of the infusion. The partial thromboplastin time lengthened slightly during the infusion, presumably due to the added heparin in this preparation; it then shortened, but was followed by a dramatic lengthening due to the developing defibrination. The serum F.D.P. and ethanol gelation data was also consistent with DIC in all dogs. There was a period of severe oliguria which coincided with the episode of DIC.

Studies on the Thrombogenicity of Scottish Factor IX Concentrates in Dogs 637

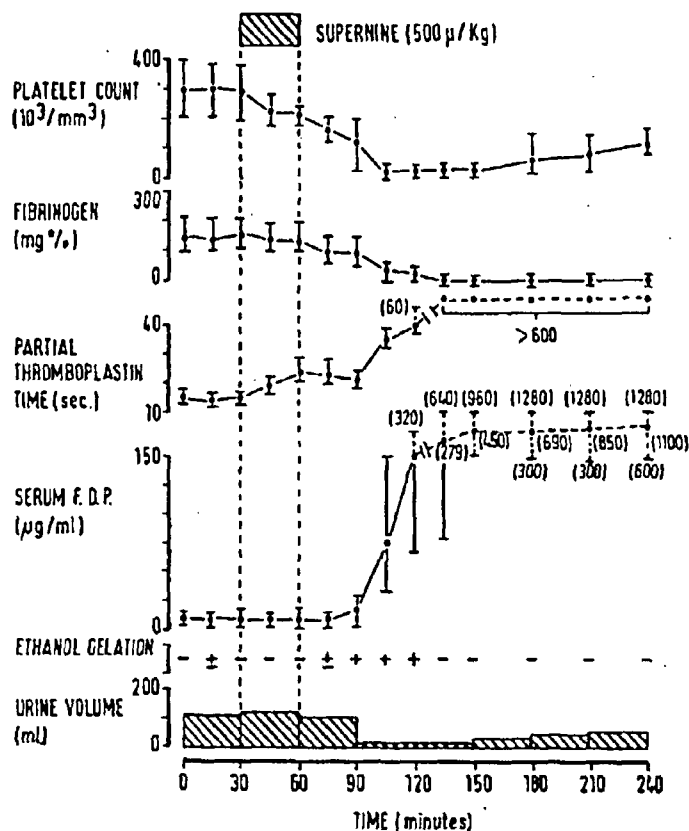


Fig. 4. Coagulation changes following the infusion of PEG-IX (500 u/Kg) into three dogs. The changes are expressed as in Fig. 1.

Discussion

All the factor IX containing concentrates used in this study had passed the then existing 'in house' *in vitro* thrombogenicity tests, based on the effects of the concentrate on the clotting time of purified fibrinogen and the recalcification time of normal citrated plasma. A concentrate containing more than 0.1 U thrombin per ml was considered unsuitable for clinical use. The batches studied on two of these concentrates (P.P.S.B. and DEFIX) had been given to many patients with no untoward effects reported.

The results of our investigation clearly demonstrated that in the very high doses used the particular batches of human P.P.S.B. and PEG-IX given to dogs were associated with low grade and extensive DIC, respectively. The evidence in favour of this conclusion was largely laboratory in origin but histology performed, on a separate run, of the kidney removed at the height of the DIC episode following PEG-IX (500 u/Kg) revealed widespread intra-renal microcirculatory thrombosis (Davidson 1975). This finding we presumed was the cause of the reversible renal failure observed. That this phenomenon was closely related to the administration of factor IX containing concentrates was evident by the absence of significant changes following the infusion of human plasma, fibrinogen, albumin and gammaglobulin.

Of particular interest was the observation that a considerable time elapsed (at least 30 minutes) after the infusion of both P.P.S.B. and PEG-IX before DIC was detected. This finding is in contrast to the report of Triantophyllopoulos (1972) who observed DIC arising during the infusion of prothrombin complex. However, these studies were conducted on rabbits using concentrates prepared by a different method from those used in Scotland. At the present time we have concluded that the delayed DIC response in our studies must be related to the gradual generation of thrombin *in vivo*, but beyond this there is no evidence on which to base a further hypothesis.

The clinical significance of these canine observations must also remain in doubt. The doses used have little clinical relevance, as that given to patients is of the order of 30 u/Kg; quantities which we are certain would have no thrombogenic effects in terms of DIC in dogs. However, they might be relevant in the context of managing patients with factor IX inhibitors or neonates and patients with liver disease. For this reason, simple *in vitro* techniques are currently being developed designed to assess the thrombogenic potential of different factor IX concentrate batches and laboratory observations are in hand on patients receiving these concentrates as part of their medical management.

Résumé

La thrombogénicité de plusieurs concentrés de facteur IX obtenus par différentes méthodes, adsorption sur phosphate calcique, chromatographie sur DEAE-cellulose suivie ou non de précipitation par le polyéthylène glycol a été essayée sur le chien. Bien qu'ayant passé les tests de thrombogénicité *in vitro*, deux types de préparation PPSB et PEG-IX ont provoqué à dose élevée une CID modérée pour la première et grave pour la deuxième préparation.

Zusammenfassung

Die Thrombogenizität mehrerer Faktor-IX-Konzentrate, mit verschiedenen Methoden – Adsorption auf Kalziumphosphat, DEAE-Zellulose-Chromatographie mit und ohne PEG-Fällung – gewonnen, wurde beim Hund geprüft. Obschon alle Präparate *in vitro* negative Resultate gezeigt hatten, provozierten hochdosierte Präparate, PPSB und PEG-IX, eine leichte resp. starke DIC beim Hund.

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Studies on the Thrombogenicity of Scottish Factor IX Concentrates in Dogs 639

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Received and accepted 30th January, 1975