参考資料1

平成20年度 薬事・食品衛生審議会
血液事業部会運営委員会・安全技術調査会合同委員会
議事概要（前半の部）

日時: 平成20年4月8日(火) 13:00～16:45
場所: 九段会館「鳳凰の間」
出席者:
　（運営委員会）高松委員長、大平、岡田、高橋、花井、山口（照）各委員
　（安全技術調査会）吉澤委員長、今井、内山、杉浦、高本、新津、半田、水落、山口（一）、脇田各委員（欠席：菊池委員）
　（事務局）黒川大臣官房審議官、新村血液対策課長、植村血液対策企画官、秋野補佐 他
　（採血事業者）日本赤十字社血液事業本部 田所経営会議委員、日野副本部長

議 題: 1. 不活化技術導入に関するヒアリング（公開）
　　信州大学医学部附属病院先端細胞治療センター
　　副センター長 下平 滋隆氏
　2. 不活化技術を有する事業者からのヒアリング（非公開）
　　（1）マコファルマ社及び株式会社アムコ
　　（2）BCT Japan 株式会社（旧ガンブロ株式会社）
　　（3）シーラス社及びバイオワン株式会社
　3. その他

（審議概要）

議題1について

信州大学医学部附属病院先端細胞治療センター副センター長 下平 滋隆氏から、不活化技術導入に関して説明後、委員から以下のような意見や質問が出された。

○ 輸血が導入された時点から、輸血による感染症への対策は長年の課題であったが、現状ではリスクはゼロとまではいかないまでも、安全性は非常に向上している。

○ 不活化技術導入の目的として、既知の病原体のリスク低減化、未知の病原体に対する備え、検査等のコストの削減という3点に整理できるかとの質問に対し、下平氏より、前述の2つについてはメリットと思うが、コストについては最初からの目的ではなく、結果として得られるメリットである。また、使用期限の延長や血漿の有効

利用という点もメリットとして挙げられると思うとの回答があった。

○　不活化技術は、効果がある病原体と効果が不十分な病原体があるので、すべての検査を代替できるものではなく、一つの方策として考えるべき。

○　輸血に伴う感染症には歴史があるが、種々の検査法によって安全性が確立されてきた中、なぜ突然の不活化技術の提示なのかとの質問に対して、下平氏より、99.99%まで安全性は確立されたものだと思うが、世界的な動向を踏まえ、より安全性の高い技術が確立されていれば、それを導入してリスクを限りなくゼロに近づけるよう日本でも検討すべき時期ではないかと考え提言した、との回答があった。

○　提案の順番としては、不活化技術の検討よりも先に、ヘモビジランスの強化、輸血の安全基準の国民への周知が先にあるべきではないか。また、不活化技術はメリットに加えて、ある程度限界があるということも示すべきではないか。

○　素晴らしい提言だが、外国に追従する必要はなく、我が国としてのパラダイムシフトを考えるべきである。不活化技術を承認することと導入することは区別して検討すべき。

○　不活化技術を導入することによって、新たな副作用が出るのではないか。また、凝固因子活性の低下によって、一人当たりの必要量が増えざるを得ないのではないか。

○　不活化技術等、現状の体制に更なる安全対策を導入しても、未知の病原体に対して、必ずしも効果があるとは限らないというのがFDAの考え方だと聞いたがどうか、との質問に対し、下平氏より、デング熱や鳥インフルエンザ等の新興・再興感染症に対しては導入するメリットはあると思う、との回答があった。

○　新しい血液の安全性についての提言と理解したが、不活化が導入されることだけで、社会が先行してしまうのではないか、という危惧が感じられる。日本では全体をカバーできるヘモビジランスを更に確立すべきだと思う。日本とヨーロッパの安全性について、溝が狭まったところで不活化技術導入を議論するのが望ましいのではないか。

○　中国等で導入が検討されている背景には、日本と異なる地域的な問題もあると思うが、日本で不活化技術導入を急がなければいけない根拠は何か、との質問に対して、下平氏より、感染症の状況は国によって違うが、日本では社会的な背景としてHIVが増加していること、鳥インフルエンザウイルスなどのウイルスがブレイク

したときの対策の一つとして検討する必要があること、との回答があった。

○　日本人は因果関係が明らかなものに対してはゼロリスクを求める傾向があるが、不活化によってどこまでリスクが下げられるのか、との質問に対して、下平氏より、献血ドナーを対象とした場合、HBV、HCV、HIV、細菌感染も含めて、確立した技術であれば、限りなくゼロに近い水準に近づけられると思う、との回答があった。

○　フランスのように、日本でもヘモビジランスが更に確立される必要があるが、それと不活化技術の導入は別の話である。輸血の歴史は感染症の歴史と密接に関連しており、新しい病原体が出てくるたびにそれに対応する検査が導入されてきた。なお、不活化が導入されるためには、2つの条件があると思う。1つは核酸を有している全ての病原体に対して効果があること、もう1つは全ての血液製剤に対して適用できる技術であること、である。

○　なぜ今不活化導入が必要と考えるのか、との質問に対し、下平氏より、新しい技術を導入して日本が世界で一番安全な血液製剤を供給できることを目指していただきたい、との回答があった。

○　献血者におけるHIV陽性者の増加率は社会的に増加しているHIV感染者の増加率よりも低い。社会的なHIV感染者の増加率よりも輸血による感染のリスクは低いと考えられる。

○　日本において、献血におけるHIV感染はHIV陽性者が特定の目的で献血に訪れるところに問題がある。蔓延している外国と違って感染数は多くはなく、感染早期での献血は極めて稀と考えられる。

○　新興感染症についてはウイルス量が少ないという保証はなく、不活化技術にも限界があるとのことなので、矛盾点なく説明されたい、との意見に対し、下平氏より、新興感染症のブレイクと血液供給は次元の違う提示だったので、1つの参考としていただきたい、との回答があった。

○　不活化技術は全ての製剤に適用するのか、それともユーザーが選択できるように従来の製剤も残すのか、との質問に対し、下平氏より、諸外国のように、段階を踏んで必要に応じて需給調整していくことになるであろうとの回答があった。

○　不活化技術導入は、新興感染症が起こったときに対処できる体制を構築するという提案なのか、全面導入を求めているのかが不明瞭ではないか。

○　欧米といっても、亜熱帯地域を領土として有している国がその地域での導入を検討しているものなので、一つの国としてまとめて説明すると誤解が生じるのではないか。

○　ヘモビジランスは重要であり、輸血後を観察して副反応が起こったときに具体的にエビデンスに基づいて対処することが重要だが、エビデンスのない状態で不安をあおるのは避けた方がよいと思う。

○　不活化技術を導入することで現状の検査の何を廃止できるのか、との質問に対し、下平氏より、GVHDのための放射線照射や白血球除去フィルターは不要になるであろうと考えられるが、検査の廃止については別の検討で安全の基準を示す必要があるとの回答があった。

○　GVHD予防のための血液照射が不要になるといっても、40年近く種々の検討を行った末、血液照射でGVHDが予防できることが分かってきたものである。原理的に可能であっても、照射の廃止はエビデンスを確認しながら慎重に行わないといけない。白血球除去についても同様である。また、危惧されるデメリットに対して説明が少ないので追加で説明されたい、との意見に対し、下平氏より、ここでは総論的な部分を示したが、提示できない詳細な内容は査察・視察等で入手していただきたい、また欧州のデータで非溶血性の副作用が低減化されているという記述もあった、との回答があった。

○　安全性の高い技術の導入に反対意見はないと思うが、不活化による新たな副作用の発生という懸念が払拭されない限り、導入に対しては慎重にならざるを得ない。EUでは16か国が導入しているといっても、不活化が導入された製剤の供給量は1か国当たり平均1万製剤にも満たなく、各国ともに慎重に使用しているのだろうと思う、との意見に対し、下平氏より、やはり化合物と光線照射を組み合わせた技術なので、毒性試験の専門家による評価や臨床試験での安全性の評価は今後の課題だと思う、との回答があった。

○　輸血の安全性と輸血製剤の安全性は必ずしも同じではない。輸血には感染症以外のリスクもあり、それらを監視するシステムは重要である。

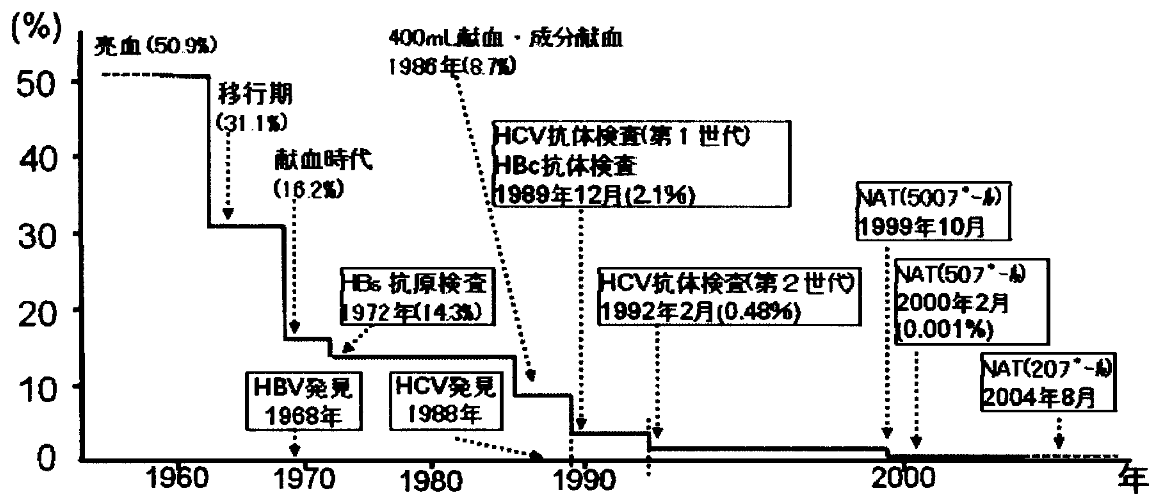
以上

平成20年2月27日開催血液事業部会運営委員会・安全技術調査会　合同委員会配布資料

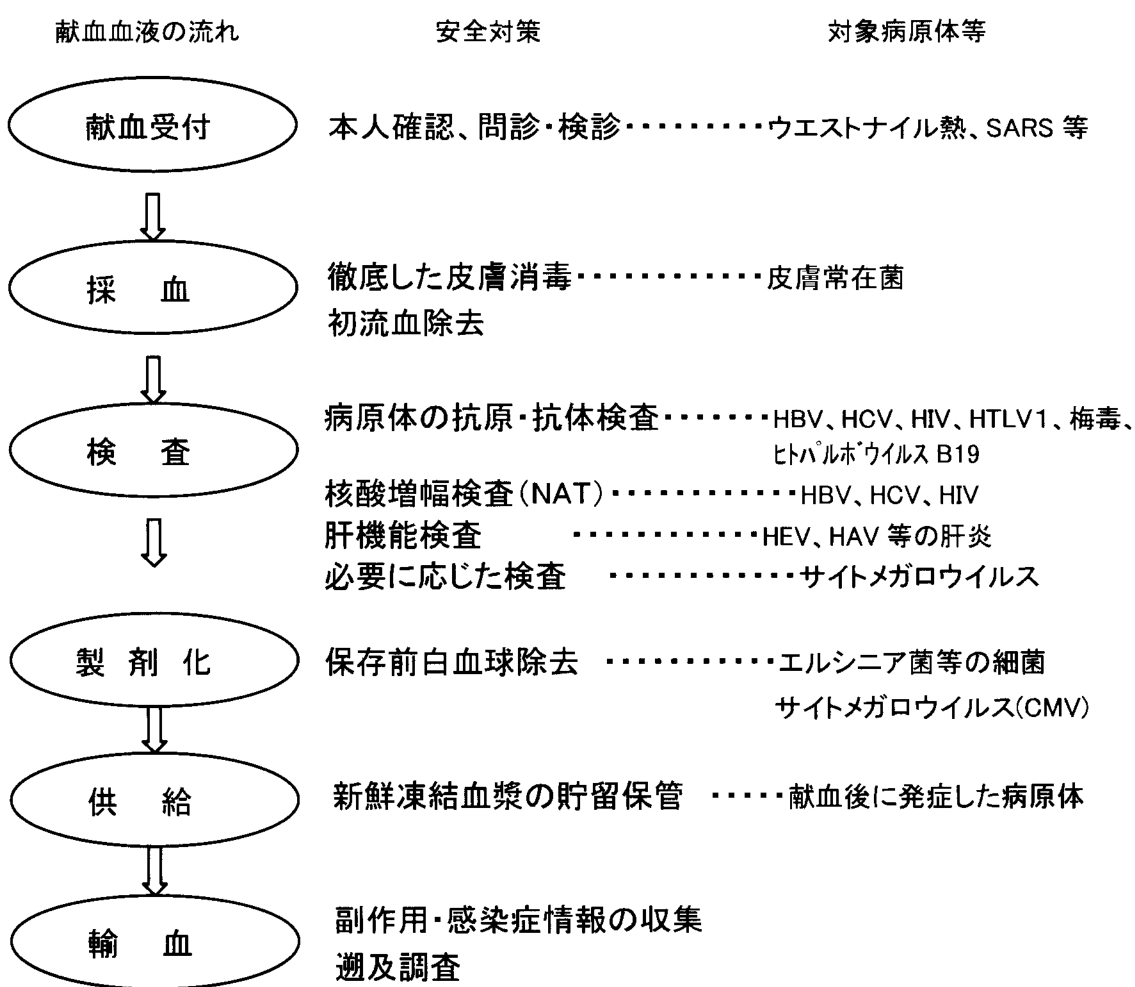
参考資料2

輸血用血液製剤の更なる安全性向上に向けて

1. 輸血後肝炎発症率の推移



2. 輸血用血液製剤の安全対策



3. 安全対策の変遷（平成 11 年 10 月以降）

	受付・問診	検 査		製 剤		その他	
	海外渡航歴等による献血制限	スクリーニング NAT	その他	保存前白血球除去	初流血除去	遡及調査	貯留保管＊
‘00年以前		HBV、HCV、HIV の NAT（500 検体プール）（1999 年～）	ALT検査の実施				
		NAT の精度向上（50 検体）（2000 年～）					
‘03~	帰国後 3 週間					遡及調査	
‘04~	帰国後 4 週間	NAT の精度向上（20 検体）		成分献血由来血小板			
‘05~	英国滞在歴者		北海道での HEV-NAT				新鮮凍結血漿の貯留保管
‘06~	プラセンタ注射剤投与者			成分献血由来新鮮凍結血漿	成分献血由来血小板		
‘07~				全血献血由来の輸血用血液製剤	全血献血由来の輸血用血液製剤		
‘08~		次世代 NAT への移行	CLEIA 法の感染症検査への順次移行		成分献血由来新鮮凍結血漿		
	対象とする病原体等						
	新興・再興感染症の病原体（ウエストナイル熱、SARS 等）異常プリオン	HBV、HCV、HIV	HBV、HCV、HIV、HTLV-1、梅毒ｽﾋﾟﾛﾍｰﾀ、ﾋﾄﾊﾟﾙﾎﾞｳｲﾙｽ B19、その他肝炎ウイルス	エルシニア菌等の細菌、CMV 等 免疫学的副作用（主に発熱性副作用）	皮膚常在菌	HBV、HCV、HIV	感染性病原因子等

＊貯留保管：有効期間が採血後1年間の新鮮凍結血漿を対象とし、180 日間保管した後に医療機関に供給することにより、期間中に得られる遡及調査等の感染症情報に基づく感染リスクの高い血液製剤を除外する安全対策をいう。

4. 日本と諸外国の安全対策と輸血後感染の残存リスク

1）肝炎ウイルス等

運営主体	NAT 実施項目					プール数	残存リスク	確認症例
	HBV	HCV	HIV	WNV	B19			
日本赤十字社	○	○	○	—	—	20	HBV 7.69 : 1,000,000 HCV 0.09 : 1,000,000 HIV 0.09 : 1,000,000	2000～2006 年の 7 年間 HBV 70(10.00/1 年間) HCV 2(0.29/1 年間) HIV 1(0.14/1 年間)
アメリカ赤十字	—	○	○	○	—	16	HBV 4.88 : 1,000,000[※3] HCV 0.56 : 1,000,000[※4] HIV 0.43 : 1,000,000[※4] WNV 2.86 : 1,000,000[※10]	2005 年 HCV 2 HIV 1
英国血液サービス	—	○	○[*1]	○	—	96	HBV 2.20 : 1,000,000[※5] HCV 0.05 : 1,000,000[※5] HIV 0.22 : 1,000,000[※5]	2006 年 ウイルス感染 確認例なし
ドイツ赤十字	○	○	○	—	○	96	HBV 1.00 : 1,000,000[※6] HCV 0.05 : 1,000,000[※6] HIV 0.05 : 1,000,000[※6]	2002～2003 年 HBV 7
フランス血液機構	○[*2] （海外県）	○	○	—	—	8/24	HBV 1.00 : 1,000,000[※7] HCV 0.17 : 1,000,000[※7] HIV 0.26 : 1,000,000[※7]	2004 年 HBV 1 HCV 1 CMV 1

HBV：B 型肝炎ウイルス、 HCV：C 型肝炎ウイルス、 HIV：ヒト免疫不全ウイルス、 WNV：ウエストナイル熱ウイルス、 B19：ﾋﾄﾊﾟﾙﾎﾞｳｲﾙｽ B19
日赤データは遡及調査及び感染症報告（2000.2～2003.1 の約 4 年間）から 50 プール NAT スクリーニング陰性で個別 NAT 陽性の推計値から算出した。

2）細菌等

運営主体	細菌培養 （血小板製剤の有効期間（日数））	残存リスク	確認症例
日本赤十字社[*8]	— (3)	症例が僅かであるため、リスクの推定は困難 RBC　7年間の供給本数　約 2, 356 万本 PLT　7年間の供給本数　約 492 万本	(2000～2006 年の 7 年間) 細菌感染　5　（内　死亡例　2）：0.71/年 マラリア　1（1994 年）、バベシア　1（1999 年）
アメリカ赤十字[*9]	○ (5)	米国[*10]　RBC　細菌感染　1: 40,000～1: 5,000,000 PLT　敗血症　1: 59,000 (single donor) マラリア　1: 1,000,000～5,000,000	(2005 年　アメリカ赤十字) 細菌感染　8（内　死亡例　2）、バベシア　2
英国血液サービス[*11]	○ (5 / 7)	欧州（英国、フランス等であるが詳細不明）[*10] PLT　敗血症　1:11,000（プール） 参考） マラリア　11 件　/　10 年	(2006 年) 細菌感染　2
ドイツ赤十字[*12]	— (5)		(2002～2003 年) 細菌感染 27
フランス血液機構[*13]	— (5)		(2004 年) 細菌感染 10

血小板期限については、採血日を day = 0　として表記した。　RBC　赤血球製剤、　PLT　血小板製剤
血小板は 20～24℃で振とうしながら貯蔵するため、細菌が増殖しやすく、有効期間の短いほど、細菌感染事故の危険性は低くなる。.

5. スクリーニング検査を実施していない病原体

1）肝炎ウイルス

HAV、HEV（ただし、北海道地域限定で調査中）

2）その他ウイルス

WNV（都道府県単位規模での NAT スクリーニングを準備中）
SARS、デング熱ウイルス、麻しんウイルス、
鳥インフルエンザ等

3）細菌

皮膚常在菌（初流血除去で感染リスク低減）
エルシニア菌（保存前白血球除去で感染リスク低減）等の細菌

4）その他病原体等

プリオン、マラリア、バベシア、トリパノソーマ（シャーガス病）、
リーシュマニア

注）下線の病原体は不活化効果がある程度、期待できると思われるもの

【参考文献】

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＊ 4　Busch MP, Glynn SA, Stramer SL, Strong DM, Caglioti S, Wright DJ, Pappalardo B,Kleinman SH; NHLBI-REDS NAT Study Group. A new strategy for estimating risks of transfusion-transmitted viral infections based on rates of detection of recently infected donors. Transfusion. 2005 feb;45(2):254-64
＊ 5　Handbook of Transfusion Medicine 4th edition（英国血液サービス）
＊ 6　hämotherapie Ausgabe 1/2003（ドイツ赤十字社）
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＊ 8　日本赤十字社社内資料
＊ 9　ABC Newsletter. 2007 Apr 13.
アメリカ赤十字社内資料
＊10　Harvey G. klein, David Anderson, Marie-Josee Bernardi, Ritchard Cable, William Carey, Jeffrey S. Hoch, Nancy Robitaille, Marco L.A. Sivilotti, and Fiona Smaill ; Pathogen inactivation : making decision about new technologies , Report of a consensus conference. Transfusion. 2007,dec;47(12): 2338-2347,
＊11　SHOT(serious hazards of transfusion), ANNUAL REPORT 2006
＊12　Haemovigilance in France : annual report (2004)
＊13　Vox Sanguinis Volume 90 Issue 3 Page 207-241, April 2006

1. 感染性因子の不活化技術評価

輸血用血液製剤の不活化技術について

化学物質を用いた感染性因子（ウイルス・細菌・原虫等）の不活化技術とは、化学物質に一定波長の光を照射する時に発生する活性酸素による感染性因子の核酸の破壊、または感染性因子の核酸に化学物質が直接結合することにより、感染性因子の複製を阻害し、死滅させる技術をいう。薬剤を用いずに遠紫外線（UVC）照射のみで病原体を不活化する技術が開発されつつある。

感染性因子不活化技術のうち、一部の諸外国で製造承認されているのは3種類（メチレンブルー、アモトサレン（S-59)、リボフラビン）であり、それぞれの特性により、血漿又は血小板製剤の不活化が可能である。赤血球製剤に対する不活化技術は開発途上にあり、臨床に応用できるものはない。しかし、どの技術も一つの方法であらゆる感染性因子を不活化できるものではない。

1）不活化技術の概要

<table>
<tr><th>不活化技術</th><th>基本仕様</th><th>血漿製剤</th><th>血小板製剤</th><th>赤血球製剤</th></tr>
<tr><td rowspan="5">メチレンブルー</td><td>作用機序</td><td>核酸破壊</td><td rowspan="5"></td><td rowspan="5"></td></tr>
<tr><td>照射光の波長</td><td>可視光</td></tr>
<tr><td>不活化が有効とされる病原体</td><td>ｴﾝﾍﾞﾛｰﾌﾟｳｲﾙｽ、一部原虫等</td></tr>
<tr><td>開発メーカー</td><td>ﾏｺﾌｧﾙﾏ社（仏）</td></tr>
<tr><td>容量規格(mL)</td><td>200～315</td></tr>
<tr><td rowspan="5">リボフラビン</td><td>作用機序</td><td colspan="2">核酸破壊</td><td rowspan="5"></td></tr>
<tr><td>照射光の波長</td><td colspan="2">近紫外線</td></tr>
<tr><td>不活化が有効とされる病原体</td><td colspan="2">ｴﾝﾍﾞﾛｰﾌﾟｳｲﾙｽ、一部細菌、原虫等</td></tr>
<tr><td>開発メーカー</td><td colspan="2">ﾅｳﾞｨｶﾞﾝﾄ社（米）</td></tr>
<tr><td>容量規格(mL)</td><td>170～360</td><td>170～360(10 単位以上)</td></tr>
<tr><td rowspan="5">アモトサレン</td><td>作用機序</td><td colspan="2">核酸との結合</td><td rowspan="5"></td></tr>
<tr><td>照射光の波長</td><td colspan="2">近紫外線</td></tr>
<tr><td>不活化が有効とされる病原体</td><td colspan="2">ｴﾝﾍﾞﾛｰﾌﾟｳｲﾙｽ、一部細菌、原虫等</td></tr>
<tr><td>開発メーカー</td><td colspan="2">ｼｰﾗｽ社（米）</td></tr>
<tr><td>容量規格(mL)</td><td>400～650</td><td>255～325(15～20 単位以上)</td></tr>
<tr><td>イナクチン
S303 等</td><td>開発状況</td><td></td><td></td><td>前臨床開発段階</td></tr>
</table>

2）その他の血小板製剤の不活化技術

ドイツで開発中のUVC照射のみによる不活化技術について情報収集中であり、CE-Mark取得後に日本赤十字社による評価予定
同法により1分間のUVC照射で広範囲の病原体を不活化できると公表されている。

3）感染性因子不活化効果

開発メーカー及び日本赤十字社による評価　（　別添1　）

4）凝固因子活性及び血小板等に及ぼす影響

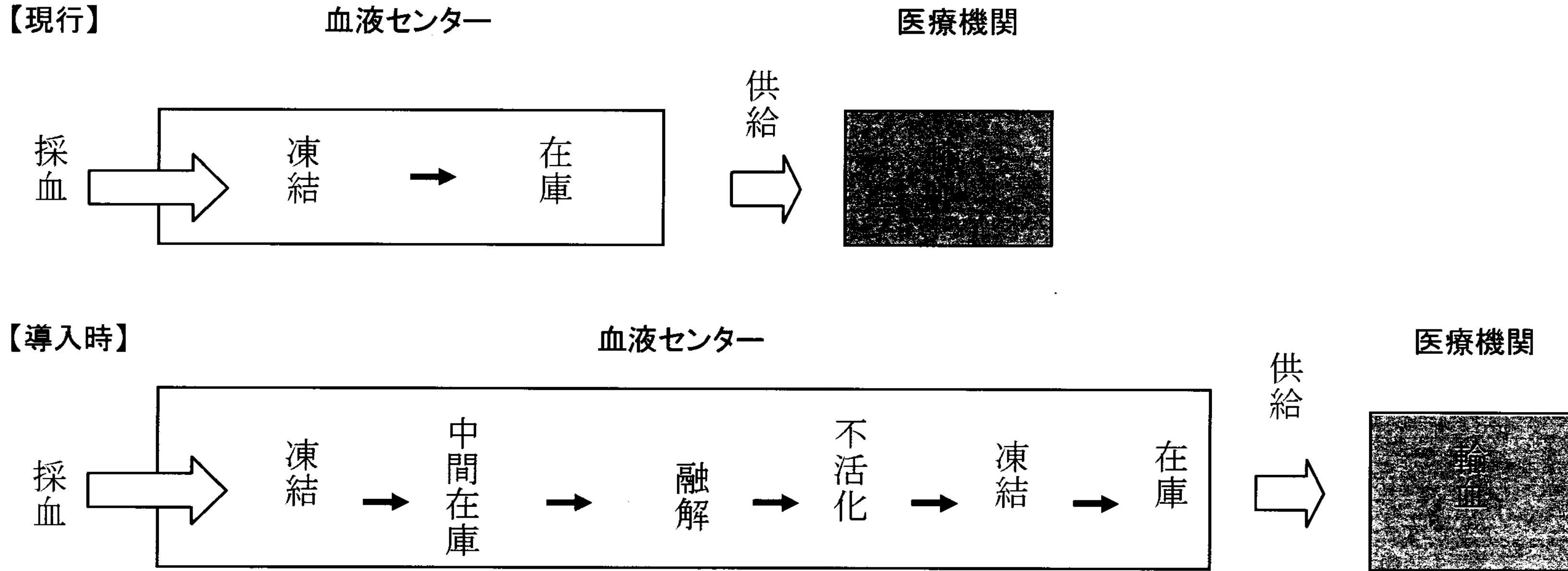
開発メーカー及び日本赤十字社による評価　（　別添2　）

5）感染性因子が不活化された製剤の安全性　　（　別添3　）

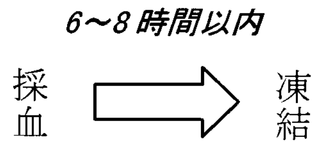
開発メーカーからの情報

6）諸外国における不活化技術の導入状況　　（　別添4　）

7）血漿製剤への不活化技術導入時における実作業について



【導入時の製造体制】　年間新鮮凍結血漿製造量　20万L（95万本）、1本の容量　210mL、一日あたりの製造本数　約4,000本



製造規模	処理能力：　8本／時間／台
500本／日の血液センター	9台
	処理時間　：　7時間
1,000本／日の血液センター	18台
	処理時間　：　7時間

2．不活化技術導入に際しての論点の整理

（1）不活化効果
（2）製剤への影響
（3）製剤の安全性
（4）実作業への影響
（5）全国一律導入と段階的導入

別添1

1. 感染性因子不活化効果

1）論文報告（各開発メーカー資料）による評価の概要

感染性因子 ＼ 不活化技術		メチレンブルー	リボフラビン	アモトサレン
		血漿	血小板	血小板
ウイルス	HIV	＞5.5	＞4.4	＞6.0
	HBV	＞4.9	－	＞5.5
	HCV	＞6.2（BVDV）	－	＞4.5
	HPV　B19	＞4.0	－	－
	WNV	＞6.5	＞5.1	－
	SARS	－	－	－
	HAV	0.0	－	－
細菌	*S.epidermidis*	－	＞4.1	＞6.6
	S.aureus	－	＞3.5	＞6.5
	MRSA	－	＞4.9	－
	Y.enterocolitica	－	－	＞5.9
原虫	*T.Pallidum*	－	－	＞6.8
	Leishmania	－	>5.0	＞5.2
	P.falciparum	－	－	＞7.0
	T.cruzi.	－	－	＞5.3

－ ： データなし

別添2

1. 凝固因子活性及び血小板等に及ぼす影響

1)凝固因子活性

凝固因子活性の低下率　20%以下(青色)
30%以下(黄色)
40%以下(桃色)

① メチレンブルー

パラメーター	正常血漿凝固因子活性参考値	A(n=10) 未処理	B(n=10) MB処理血漿	C(n=10) MB処理血漿 -30℃ 6ヶ月保存	D(n=10) 未処理血漿 -30℃ 6ヶ月保存
Fibrinogen(g/L)	2-4	3.03	2.36	2.04	2.94
Factor V(%)	70-120	105.7	96.4	83.2	89.5
FvWAg(%)	60-150	143.6	122.2	128.0	130.3
activity(%)	60-150	143.6	138.6	84.0	87.4
Factor Ⅷ(%)	60-150	114.0	86.1	76.1	101.1
Factor Ⅸ(%)	60-150	99.2	85.7	86.0	98.6
Factor ＸⅠ(%)	60-140	85.5	65.7	64.4	86.7
Protein C(%)	70-140	120.7	112.6	103.0	108.4
Protein S(%)	70-140	83.8	81.4	71.2	81.6
ATⅢ(%)	80-120	105.8	93.9	101.9	104.4
C3a(mg/L)	100-400	124.2	124.0	143.1	146.8
C5a(μg/L)	0.9-15.4	7.5	10.9	23.9	15.1

マコファルマ社資料による

別添 2

②リボフラビン

パラメーター	正常血漿凝固因子活性参考値	コントロール新鮮血漿平均(Min.−Max.)	コントロール新鮮凍結血漿平均(Min.−Max.)	リボフラビン処理新鮮凍結血漿平均(Min.−Max.)	リボフラビン処理新鮮凍結血漿平均(Min.−Max.)
Fibrinogen (mg/dL)	145−385	304 (217−349)	315 (213−401)	229 (162−276) (76%)	236 (164−284) (75%)
Fibrinogen (mg/dL)	145−385	364 (302−426)	362 (298−432)	**315 (266−374) (87%)**	**316 (266−368) (87%)**
F II (I.U./mL)	0.65−1.54	0.93 (0.78−1.00)	0.99 (0.81−1.17)	**0.78 (0.69−0.84) (84%)**	**0.79 (0.68−0.85) (80%)**
F V (I.U./mL)	0.54−1.45	1.06 (0.97−1.24)	1.14 (0.96−1.38)	0.79 (0.65−0.94) (74%)	0.84 (0.76−0.92) (74%)
F VII (I.U./mL)	0.62−1.65	1.00 (0.70−1.19)	1.05 (0.72−1.32)	**0.91 (0.62−1.08) (91%)**	**0.92 (0.62−1.09) (88%)**
F VIII (I.U./mL)	0.45−1.68	1.01 (0.58−1.69)	0.94 (0.54−1.70)	0.65 (0.32−1.15) (64%)	0.60 (0.27−1.13) (64%)
F IX (I.U./mL)	0.45−1.48	0.94 (0.69−1.12)	0.91 (0.63−1.16)	0.73 (0.51−0.89) (78%)	**0.76 (0.53−0.88) (83%)**
F X (I.U./mL)	0.68−1.48	0.97 (0.78−1.20)	1.03 (0.79−1.20)	**0.78 (0.64−0.94) (81%)**	0.81 (0.67−0.96) (78%)
F VIII (I.U./mL)	0.45−1.68	1.02 (0.58−1.69)	0.94 (0.54−1.70)	0.65 (0.32−1.15) (64%)	0.60 (0.27−1.13) (64%)

ガンブロ社資料による

別添 2

③ アモトサレン(商品名:インターセプト)

パラメータ	正常血漿 凝固因子 活性参考値	アモトサレン未処理血漿	アモトサレン処理血漿	アモトサレン処理前後の活性比較
PT(n=14)	11.1-13.5 秒	11.2±0.3 秒	11.6±0.3 秒	1.0±0.1 秒
APTT(n=14)	23.0-35.0 秒	26.8±1.4 秒	29.1±1.7 秒	4.3±1.8 秒
Fibrinogen (n=91)	167-379mg/dL	290±40 mg/dL	209±36 mg/dL	72±5%
FⅡ(n=59)	71-127U/ dL	96±11IU/ dL	85±11IU/ dL	88±4%
FⅤ(n=91)	77-153U/ dL	130±23IU/ dL	119±19IU/ dL	92±7%
FⅦ(n=91)	58-166U/ dL	123±32IU/ dL	95±20IU/ dL	78±6%
FⅧ(n=91)	67-235U/ dL	157±35IU/ dL	115±28IU/ dL	73±7%
FⅨ(n=91)	63-143U/ dL	108±21IU/ dL	88±16IU/ dL	82±4%
FⅩ(n=59)	66-134U/ dL	100±13IU/ dL	86±11IU/ dL	86±3%
FⅩⅠ(n=91)	62-142U/ dL	130±22IU/ dL	87±18IU/ dL	86±5%
FⅩⅢ(n=26)	—	110±11IU/ dL	102±10IU/ dL	93±3%
vWF(n=12)	—	114±44IU/ dL	111±41IU/ dL	97±8%

Y.Singh, et al ; Transfusion 46;1168:2006

別添 2

2.血小板機能に対する影響

1)リボフラビン

パラメーター	単位	コントロール 血小板 (N = 20)	リボフラビン処理 血小板 (N = 30)
pH (22℃)	NA	7.48 ± 0.06	7.13 ± 0.13
乳酸発生率	mmol/10^{12} cells/hr	0.032 ± 0.006	0.056 ± 0.012
グルコース消費率	mmol/10^{12} cells/hr	0.019 ± 0.004	0.033 ± 0.007
pO_2	mm Hg	54 ± 15	38 ± 2
pCO_2	mm Hg	26 ± 3	28 ± 2
P-セレクチン	%	17.9 ± 7.0	41.7 ± 15.1
スワーリング	−	3 ± 0	2.9 ± 0.6
%HSR	%	72.3 ± 10.9	72.3 ± 8.3
Morphology score	−	254 ± 20	270 ± 27
血小板濃度	$10^3/\mu l$	1662 ± 107	1395 ± 106
総血小板数	$\times 10^{11}$	4.5 ± 0.2	3.9 ± 0.3

ガンブロ社資料による

2)アモトサレン　*:被検試料と対照との有意差 $p \leqq 0.05$ Student paired t-test (桃色)

パラメーター	保存 5 日目 (平均±標準偏差)	
	コントロール対照群 (N = 6)	アモトサレン処理群 (N = 6)
pH (37℃)	6.94±0.12*	6.80±0.07
乳酸(mM)	10.5±2.1	11.3±1.7
グルコース(mM)	3.6±1.7	2.5±0.8
pO_2 (mm Hg)	40.9±11.2*	69.9±22.5
pCO_2 (mm Hg)	29.9±2.9*	24.2±3.3
P-セレクチン (発現率 %)	31.0±4.9*	51.7±7.0
%HSR	58.5±5.6	58.8±10.1
形状変化の程度(ESC %)	14.6±3.8*	9.7±2.4
ATP(nmol/10^8 血小板)	5.2±1.1*	4.6±0.8
LDH 放出(融解率%)	3.0±0.6*	7.0±2.1
Morphology score(0-400)	299±14*	286±17
HCO_3^-(mM)	6.3±1.0*	3.7±0.6
総血小板数($\times 10^{11}$/単位)	4.1±0.5*	3.7±0.5

シーラス社申請資料による

被検血小板:アフェレーシス採血・16 時間 CAD 処理済

別添 3

安全性試験(前臨床試験)の結果

不活化技術名 / 試験項目	メチレンブルー[1]	リボフラビン[1]	アモトサレン[1][2]
急性毒性	陰性	陰性	陰性
慢性毒性	陰性	陰性	陰性
遺伝毒性	陰性	陰性	陰性
細胞毒性	陰性	陰性	陰性
生殖毒性	陰性	陰性	陰性
発がん性試験	陰性	陰性[3]	陰性
Neoantigenicity	—[4]	陰性	陰性

1)メーカー承認申請資料による

2)Toxicity Profile Riboflavin & its derivatives (2nd.ed)BIBRA 1990

3)Lily Lin et al , Transfusion 45 ;1610 :2005

4)現時点で neoantigenicity に関する情報はない。

諸外国における感染性因子不活化技術（S/D処理、メチレンブルー・リボフラビン・アモトサレン）の製造承認及び導入の状況

血漿の不活化については、欧州においてメチレンブルーを中心として、導入が進んでいる国もあるが、全ての血漿製剤に不活化を実施しているのは、ごく一部の国である。また、これらの国においては、有償採血であることや、輸血用血漿製剤の使用量が我が国と比較して、1/3～2/3と少ないなど、実施しやすい状況がある。

一方、血小板の不活化については、感染症が蔓延している地域における導入や国によっては一部試行的に導入しているところもあるが、様々な技術が開発されているところであり、一つの技術を全国的に導入すると決定している国は今のところないと聞いている。

また、多くの感染症が蔓延している国においては、NATなど高額な検査を実施できない場合もあり、広範な病原体に対して有効な不活化技術のみ導入しようとする場合もある。

	製造承認の有無	導入状況	備　　考
米国	不活化技術に対する承認はない	導入を検討中	様々な血液銀行による有償採血であるので、採血量の増加にも対応が可能。血漿に対する不活化技術の導入の動向はない。 新興・輸入感染症と血小板製剤に多発する細菌感染の対策として、血小板の不活化の導入を検討中。不活化血小板の承認申請審査中。 千人当たりの血漿使用量は日本の三分の二程度。
フランス	メチレンブルーによる血漿の不活化 アモトサレンによる血小板の不活化	血漿に対しては、60%がプールした後にS/D処理、40%がメチレンブルーにより不活化処理をされている。 血小板に対する不活化については、インド洋、カリブ諸島、南米の三つの海外県や本国の5センターでアモトサレンやリボフラビンによる処理を導入している。	フランス血液機構は、献血により採血している。 熱帯地域の海外県における感染症発生のリスクがあり、その影響で本国においても、血漿や血小板の不活化対策に取り組む必要性が高い。 千人当たりの血漿使用量は日本の三分の一程度。
ドイツ	メチレンブルーによる血漿の不活化 アモトサレンによる血小板の不活化	血漿に対しては、今年1月からメチレンブルーによる不活化製剤を順次導入する方針。 血小板の不活化については、未導入。	ドイツ赤十字が輸血の8割を実施。 血小板の不活化として、ドイツ赤十字はアモトサレンの使用はしておらず、薬剤を用いない不活化技術（UVC）を開発中。アモトサレンを評価する計画もある。 ドイツの血漿は、有償採血のため、採血量の増加にも対応が可能。 千人当たりの血漿使用量は日本とほぼ同じ。
イギリス	メチレンブルーによる血漿の不活化	血漿については、小児を対象に、メチレンブルーによる不活化製剤を供給。 血小板に対する導入は行っていない。	英国の国営血液サービスは、米国で有償で採血された血漿を輸入している。 感染症のリスクを考慮して、1996年以降に誕生した子供の輸血に使用する際には、メチレンブルーによる不活化を実施している。 千人当たりの血漿使用量は日本の二分の一程度。
ベルギー	メチレンブルーによる血漿の不活化	2004年メチレンブルーによる血漿の不活化を導入	アモトサレン及びリボフラビンによる血小板の評価試験中 アモトサレン承認申請中

別添4

	製造承認の有無	導入状況	備　　考
ルクセンブルク	メチレンブルーによる血漿の不活化	メチレンブルーによる血漿の不活化を導入	
カナダ	不活化技術に対する承認はない	未導入	メチレンブルー不活化血漿の導入を検討中
スイス	不活化技術に対する承認はない	今年から、25%の血漿に対してSD処理をして供給 血小板ついては未導入	アモトサレンによる血小板不活化承認申請中
オランダ	不活化技術に対する承認はない	未導入	
ノルウェー	アモトサレンによる血小板の不活化	一部の血液センター・院内血液銀行でのみ導入	
スペイン	メチレンブルーによる血漿の不活化 アモトサレンによる血小板の不活化	一部の血液センターでのみ導入	
イタリア	メチレンブルーによる血漿の不活化 アモトサレンによる血小板の不活化	一部の血液センターでのみ導入	
ギリシア	メチレンブルーによる血漿の不活化	一部の血液センターでのみ導入	
ロシア	メチレンブルーによる血漿の不活化	一部の血液センターでのみ導入	モスクワ市内の血液センターで導入
マレーシア	アモトサレンによる血小板の不活化	一部センターで小児対象に導入	
シンガポール	不活化技術に対する承認はない	未導入	メチレンブルーによる血漿不活化承認申請中 アモトサレン評価試験中
韓国	不活化技術に対する承認はない	未導入	

＊EU各国では、リボフラビンによる血小板不活化は原則導入可能　　以上、日本赤十字社・血液製剤調査機構が知り得た情報を基に作成　　2008年1月現在

参考資料3

マコファルマ社
提出資料（再配布）

参考資料3（マコファルマ社）

SAFETY OF BLOOD PRODUCTS

THERAFLEX MB Plasma

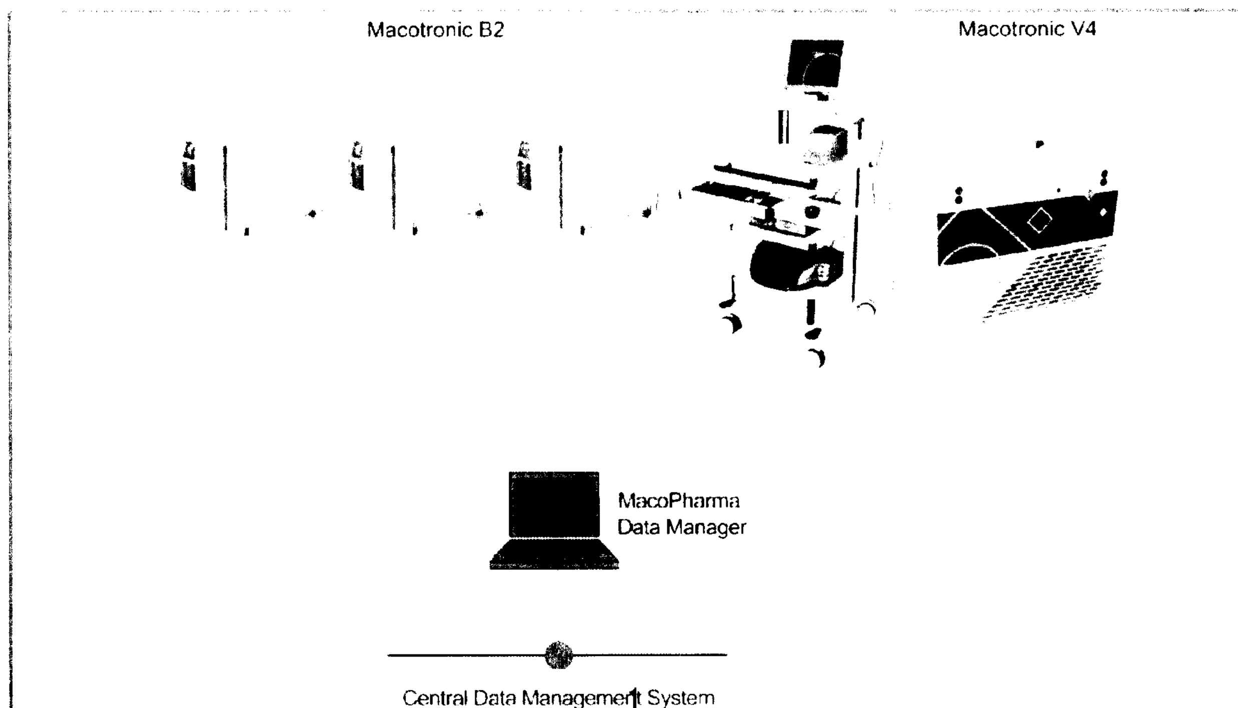
PATHOGEN INACTIVATION SYSTEM FOR PLASMA

Contributing to 10 years of safer Pathogen Inactivated Plasma

- Routine use worlwide: over 4 million MB Plasma units transfused
- Proven clinical efficacy
- In-house processing
- User friendly technology

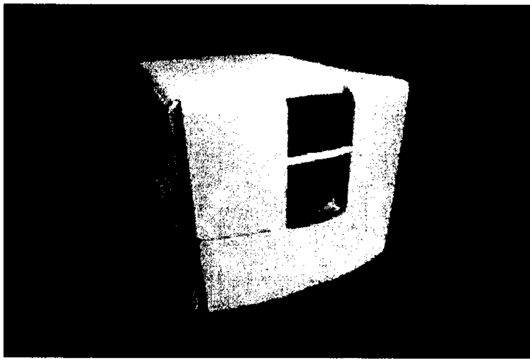
New Developments:
Macotronic B2, next generation of illumination equipment

- New light source: LED (Light-Emitting Diode)
- Optimal wavelenght (630nm)
- Reduced illumination time (15mn)
- Touchscreen operating system
- Integrated RFID technology
- Full GMP-Procedure
- CE Marked by December 2007



MacoPharma

MacoTronic B2



Plasma illumination device for the THERAFLEX-MB Plasma pathogen reduction process

Ref. 9MB2000

Specifications :

Dimensions :	47 x 68 x 44 cm (width x depth x height).
Weight :	~ 35 kg
Power supply :	110-240V, 50-60Hz
Lighting system :	96 light-emitting diodes (LED), 4 modules of 24 LED each, 2 modules per bag (double-side exposure)
Wave length :	627 +/-10 nm
Connectivity :	4 USB ports (rear panel : 3, front panel : 1), 1 Ethernet port (network connection)
Screen :	5,5 inch VGA colour touch screen
Cooling system :	Ventilation of illumination chamber by laminar air flow

- **Operating mode :**

Delivered energy :	Preset as per THERAFLEX-MB Plasma procedure
Bag loading mode :	Manual opening of the drawer
Capacity per cycle :	2 bags per cycle
Temperature of use :	Air-conditioned room (20-22°C)

- **Operating controls :**

Light sensors :	4 control photo-diodes (1 per light module)
Temperature sensors :	2 pyrometers for direct measurement on each bag surface and for ambiant temperature in the illumination chamber
Alarms :	Flashing logo for operating status, sound alarm for errors

- **Process control & traceability :**

Barcode reading :	Bag ID and batch, product code, operator, post-labelling barcode control
Report printing :	Cycle illumination report providing energy, intensity and temperature records
Cycle record :	Up to 8000 illuminations files stored in the internal memory
Backup :	Transfer of illumination files by USB key or MacoTrace (Data Manager)
Network connection :	TCP/IP protocol, assignable IP adress through MacoTrace
Import/export :	Connection to the IT Management System (LIS) through MacoTrace

- **Accessories :** USB barcode laser reader, thermal transfer label printer, report printer, MacoTrace licence, ethernet cable, RFID module

- **Regulations :**

CE Marking :	Conformity with MDD 93/42/EEC expected for end Q1 2008
Electrical safety :	Conformity with EN 61010-1 expected for end Q1 2008
Electromagnetic certification :	Conformity with EN 61326-1 expected for end Q1 2008

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Certified ISO 9001 Certified ISO 13485

MacoPharma
F - 59420 MOUVAUX

First investigations on a newly developed LED illumination device for the treatment of MB-plasma

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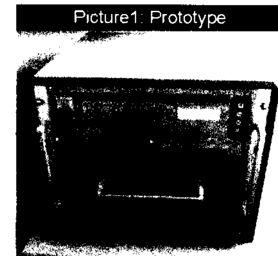
[1]Blood Center of the German Red Cross Chapters of NSTOB, Institute Springe, Germany
[2]Maco Pharma S.A., Tourcoing, France [3]Maco Pharma International, Langen, Germany

Purpose

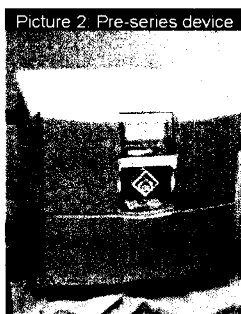
Picture1: Prototype



Treatment of fresh frozen plasma (FFP) with MB (methylene blue) and light is a procedure used for the inactivation of blood-borne viruses for more than 15 years MB plasma is currently produced in several European countries using the MacoPharma Theraflex MB plasma system High-intensity long-lived light-emitting diodes (LEDs) are now available on the market which might replace the sodium vapour lamps currently used in the Macotronic device (MacoPharma) The purpose of this study was the first evaluation of a newly developed LED illumination device (picture 1) with respect to virus inactivation capacity and plasma quality

Methods

Picture 2: Pre-series device



Treatment was done in the Theraflex MB plasma bag system (MacoPharma) containing leucocyte-depletion filter Plasmaflex MB pill (85 µg MB) illumination bag, MB depletion filter Blueflex and plasma storage bag For investigating plasma quality 3 plasma pools were each prepared from 3 different single donor units Plasma was divided into three illumination bags and illuminated on the LED device Samples were taken at different time points of illumination and factor VIII and fibrinogen (Clauss) were determined

For investigating virus inactivation capacity Pseudorabies virus (PRV) was spiked into FFP (n = 4) resulting in a titer of approximately 10^6 tissue culture infectious doses/ml ($TCID_{50}$/ml) The viral titer was determined from samples taken after 5 10 15 and 20 minutes of illumination on the LED device Infectivity was determined by endpoint titration using a Vero cell CPE (cytopathic effect) assay In a preliminary run the currently used Macotronic was compared with the newly developed LED device

Results

Virus inactivation was investigated using the PRV an enveloped double-stranded DNA virus which is used as model virus for HBV MB/light treatment using the LED-based illumination device resulted in an inactivation of > 4 log steps of PRV after 10 – 15 min of illumination (diagram 1) The device is at least as effective as the Macotronic device routinely used at present

Table 1: Inactivation kinetics of PRV (n = 4)

sampling time [min]	0	5	10	15	20
log_{10} $TCID_{50}$/ml (mean ± SD)	6.16 ± 0.23	4.64 ± 0.68	≤ 2.41 ± 1.65	≤ 1.75 ± 0.38	≤ 1.54 ± 0.00
log_{10} reduction factor		1.52	≥ 3.75	≥ 4.41	≥ 4.62

Plasma quality was only slightly affected by illumination Factor VIII was decreased by 17% and fibrinogen (Clauss) by 14% during an illumination of 20 min (Diagram 2) Results are at least comparable to or might even be better than those for the Macotronic device

Diagram 1: Inactivation of PRV by LED device (n=4) and Macotronic (n=1)

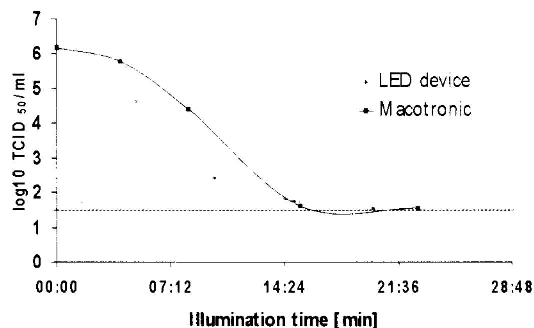
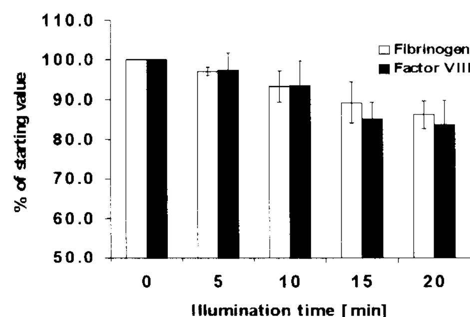


Diagram 2: Influence on coagulation factors (n = 3)



Conclusions

A new, compact illumination device based on long-lived LEDs was developed The preliminary data suggest that this LED device is comparable to the Macotronic device with respect to virus inactivation capacity and preservation of plasma quality. Illumination time might even be shortened by using this high intensity illumination device.

40th Annual Meeting of the German Society for Transfusion Medicine and Immunohaematology (DGTI) 2007

Three years' haemovigilance of methylene blue-treated fresh frozen plasma : no increase in transfusion reaction incidence

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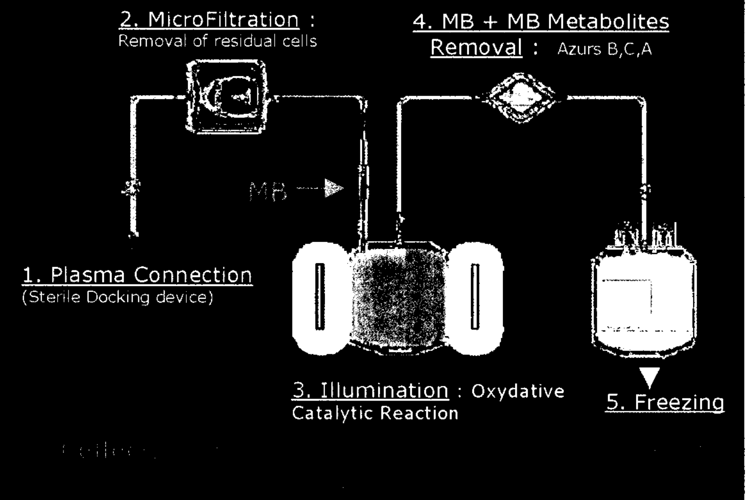
[2] Service Francophone du Sang, Belgian Red Cross, BELGIUM

Background

Due to stringent donor selection, laboratory testing and pathogen inactivation procedures, fresh frozen plasma (FFP) offers a high degree of viral safety. In Belgium, only non remunerated volunteers are recruited as plasma, platelets and blood donors. Pathogen inactivation is a proactive strategy designed to inactivate a pathogen before it enters the blood supply. Methylene blue-photoinactivation was choosen by the Belgian Red Cross two years ago (Theraflex MB plasma - MacoPharma)

MB-viroinactivation (Theraflex-MB plasma MacoPharma)



Methods

Until mid 2004, only solvent-detergent FFP (SD-FFP) was used in our teaching hospital. BM-FFP began to be transfused to our patients thereafter. Since nearly 10 years, all transfusions of labile blood products are checked for the appearance or not of an adverse event.
In this study, the incidence and the seriousness of adverse event after BM-FFP transfusion were compared with those observed with SD-FFP.

Results

(a) : benign allergic reaction (n=4)
moderate allergic reaction (n=1)
NHFR (n=1)
(b) : benign allergic reaction (n=5)
moderate allergic reaction (n=3)
No TRALI episode (any previous pregnancy / transfusion = contra-indication for plasma donation)

	SD-FFP	BM-FFP
Period	2003 - 2004	mid 2004 - 2005
Number of FFP units transfused	5101	5660
Number of adverse reactions after FFP Tf°	6 (a)	8 (b)
Incidence of reaction	0.12 %	0.14 %

Discussion and conclusion

No significant increase in adverse event incidence was observed after BM-FFP transfusion (odds ratio 1.2) and the seriousness of these events was comparable. The clinical efficacy of both FFP was similar : both procedures have limited effects on coagulation factors (especially on fibrinogen and factor VIII for MB, on protein S and alpha 2-antiplasmin for SD).
Finally, plasma pooling may have the undesirable effect of increasing the risk of transmitting viruses that are either resistant to the process or have escaped the virucidal process.
The hallmarkof MB technology is that it allows the viral inactivation of single donor units of FFP, offering reassurance that no increased infectious risks are added due to pooling.
This a crucial point in term of public health safety.

Safety Of Methylene Blue Treated Plasma

Pohler P[1], Leuschner J[2], Gravemann U[1], Reichenberg S[3], Walker W H[3], Mohr H[1]

[1]DRK NSTOB, Springe, Germany, [2]LPT KG, Hamburg, Germany, [3]Maco Pharma International GmbH, Langen, Germany

AABB Annual Meeting 2007, Anaheim, USA

OBJECTIVES

The procedure using methylene blue (MB) to inactivate viruses in therapeutic plasma is well established worldwide. It includes membrane filtration (Plasmaflex, 0.65 µm pore size), addition of MB (dry pill, 85 µg, resulting in 1 µmole/L at 266 ml), illumination (approx. 20 min, 590 nm), and filtration of MB and photoproducts (Blueflex). More than 4 million units of plasma were transfused without any unusual adverse event reported.

Aim of this study was to prove the toxicological safety of MB, its photoproducts *azure A, B and C, and that of MB-treated plasma.*

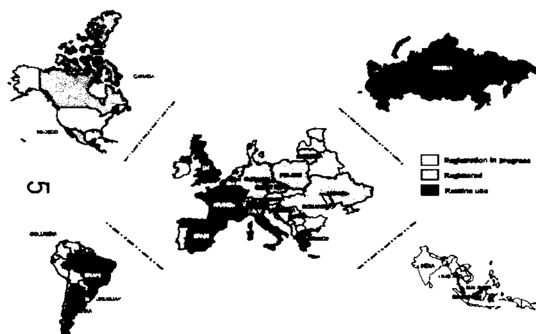


Figure 1: Global status of Theraflex MB-Plasma use

METHODS

Adsorption, distribution and excretion of 14C-labeled MB following 24 h *infusion* were investigated at a dose level of 20 mg/kg body weight (b.w.) in rats. Observation time was 96 hours.

Studies on teratogenic effects were done by intravenous bolus injection of MB into rats and Beagle dogs. MB was administered daily to the dams at 4, 12, 36 mg/kg b.w. (rat) and 2, 6, 18 mg/kg b.w. (rabbit).

In a tolerance test 5 ml/kg b.w. of autologous light-treated plasma (1 or 10 µM MB) was administered to 5 male Beagles per group by intravenous administration. After 21 d 3 dogs/group were treated again and sacrificed 24 h later. Hematology, clinical biochemistry, and electrocardiogram were examined. A complete histopathology was done.

MB and Azure A/B/C were tested in: bacterial reverse mutation test (Ames test), in vitro mammalian cell gene mutation test, in vitro mammalian chromosome aberration test with human lymphocytes, in vivo micronucleus test with rat bone marrow and peripheral blood cells (20 mg/kg b.w., 24 h infusion), in vivo unscheduled DNA synthesis test in rats (20 mg/kg b.w., bolus infusion).

RESULTS

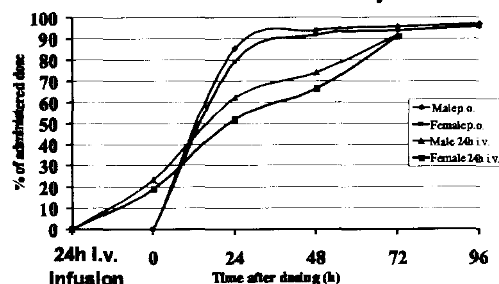


Fig. 2 **Recovery of ^{14}C-labelled Methylene blue (MB).** The recovery was examined in Sprague-Dawley rats following oral administration (p.o.) and 24 h i.v. infusion at a dose level of 20 mg MB/kg body weight. Urine, faeces, organs, expired air, rinse water and infusion site were analysed. The radioactivity recovery rate in organs and at the infusion.

1. Pharmacokinetics of ^{14}C-labeled MB after 24 h infusion were determined in T_{max}, $T_{1/2}$ α and $T_{1/2}$ β It indicated:

- biphasic elimination of MB with an initial half-life of 3 min and a longer terminal half-life of 12.6 h (male) and 16.0 h (female)

- less than 1 % radioactivity in plasma and examined organs

- Excretion *of radioactivity was almost complete after* 96 *h*

- no accumulation or storage of MB

2. The no observed effect level (NOEL) for the fetal organism was 4 mg and 6 mg/kg b.w./day in rats and rabbits.

3. Clastogenic effects of MB and Azure B were found in vitro.

4. No genotoxic effects on bone marrow, peripheral blood cells and hepatocytes after application of 20 mg/kg b.w. MB and Azure B.

5. No signs of intolerance or sensitization after infusion of 1 µM or 10 µM MB *light-treated plasma before removal of MB and photoproducts were observed.*

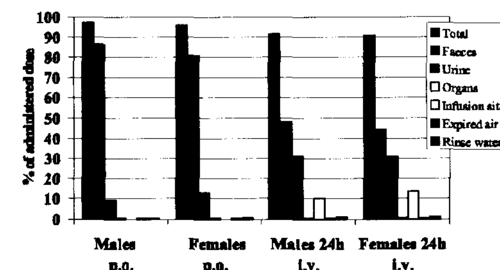


Fig. 3 Mean recovery of radioactivity after oral (gavage) application and 24 h i.v. infusion of 20 mg MB/kg body weight in rats.

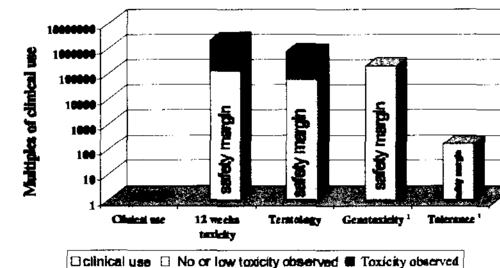


Fig. 4 Safety margins for toxicity from in vivo studies with emthylene blue. Calculations are based on the treshold NOEL: no observed effect level (slight methaemoglobinemia) (12 weeks toxicity) and a normal clinical exposure of 0,1 µg MB/kg body weight (≈6 units MB plasma). [1] no toxicity occured; therefore the safety margins are based on the highest dose tested.

CONCLUSIONS

Thresholds for no or low toxic properties which occurred after administration of MB in preclinical studies varied depending on the amount of MB applicable in the specific test system. They are > 160 to 200,000 fold higher than the estimated clinical exposure of MB after infusion of 6 units MB-light treated plasma.

REFERENCES

1 Williamson et al. 2003 Transfusion 43:1322-1329
2 Pohler et al. 2004 Transfus Med Hemother 31 (suppl 3):1-84, PS305

CHARACTERISTICS OF MACO PHARMA THERAFLEX MB-PLASMA

S. Reichenberg[1], U. Gravemann[2], P. Pohler[2], W. H. Walker[1]
[1]Maco Pharma, Langen, Germany
[2]Blood Center of the German Red Cross Chapters of NSTOB, Institute Springe

ISBT Congress, Madrid, June 2007

Background: During the last 15 years the method using methylene blue (MB) to inactivate viruses in plasma was constantly improved. Invented by the Blood Center of the German Red Cross, chapters of NSTOB, Institute Springe, the initial procedure included: Freezing and thawing to release intracellular viruses from leucocytes, addition of a proportional amount of a MB stem solution to a final concentration of 1 µM, and subsequent one-side illumination for one hour with fluorescent tubes.

Aim: The aim was to improve the original method to facilitate the implementation in the blood bank.

Results

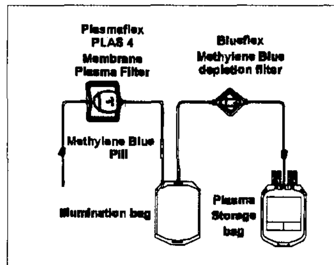


Figure 1: bag system

Figure 2: Illumination device Macotronic

Plasma quality

Test	Unit	Range	Init. value	after prep.	3 month	9 month	18 month	27 month
Thrombin time	[s]	14 - 21	15,2 ± 0,3	17,4 ± 0,9	15,9 ± 1,1	16,2 ± 1,1	18,5 ± 0,5	20,2 ± 0,9
Fibrinogen (Clauss)	[mg/dl]	200 - 450	262,3 ± 8,5	190,5 ± 12,6	194,0 ± 14,0	190,8 ± 11,3	254,5 ± 13,4	227,0 ± 11,7
Factor II	[%]	70 - 130	104,8 ± 2,1	101,9 ± 2,6	96,6 ± 1,4	103,0 ± 5,3	109,3 ± 5,2	104,5 ± 2,9
Factor V	[%]	60 - 130	87,1 ± 6,0	105,6 ± 8,8	108,1 ± 3,8	105,0 ± 8,8	99,4 ± 6,4	107,6 ± 3,8
Factor VIII	[%]	60 - 150	88,6 ± 17,9	72,5 ± 15,7	82,3 ± 17,6	72,9 ± 13,8	73,5 ± 14,8	81,5 ± 13,1
Factor IX	[%]	60 - 130	100,4 ± 5,8	92,8 ± 3,1	90,9 ± 6,8	97,8 ± 4,8	77,5 ± 5,3	99,6 ± 8,1
Factor XI	[%]	60 - 130	98,6 ± 5,4	78,4 ± 7,1	80,1 ± 4,5	79,3 ± 5,9	71,5 ± 4,3	87,5 ± 3,4
vWF:RCo	[%]	60 - 150	96,5 ± 5,3	100,5 ± 15,5	110,3 ± 20,7	112,8 ± 22,2	101,8 ± 15,8	110,8 ± 19,8
free Protein S	[%]	55 - 130	104,3 ± 6,4	103,5 ± 7,0	81,8 ± 7,2	98,8 ± 10,5	99,0 ± 5,9	98,8 ± 7,0
Protein C	[%]	70 - 140	97,8 ± 7,7	89,5 ± 5,9	85,0 ± 7,8	81,0 ± 18,5	114,0 ± 10,3	97,0 ± 6,5
AT III	[%]	80 - 120	91,3 ± 3,3	90,5 ± 3,3	95,5 ± 2,6	91,8 ± 3,8	110,8 ± 4,1	102,0 ± 6,8
a_1-Antitrypsin	[mg/dl]	90 - 200	98,5 ± 1,3	97,5 ± 2,5	98,3 ± 1,5	99,3 ± 2,1	106,5 ± 2,4	100,8 ± 3,4
a_2-Antiplasmin	[%]	80 - 120	95,0 ± 2,8	94,0 ± 2,6	92,8 ± 4,4	84,8 ± 2,1	96,0 ± 4,2	100,5 ± 4,4
Factor XIIa	[mU/ml]	< 50	31,3 ± 4,1	33,3 ± 3,9	33,4 ± 6,2	35,1 ± 6,4	36,3 ± 6,8	36,3 ± 4,7
CH100	[U/ml]	392 - 1019	689,6 ± 157,4	579,1 ± 31,7	949,3 ± 85,7	771,3 ± 161,5	798,8 ± 179,7	978,0 ± 74,1

Figure 3: Plasma quality after treatment during storage for 27 month

Virus reduction capacity

Sensitivity of enveloped viruses		
HIV-1	Retro	5,45 [2]
WNV	Flavi	5,78 [*2]
BVDV	Flavi	5,44 [*2]
Hog cholera	Flavi	5,92 [*1]
PRV	Herpes	5,48 [*2]
Herpes Simplex	Herpes	5,50 [*1]
Bovine herpes	Herpes	8,11 [*1]
Semliki Forest	Toga	7,00 [*1]
Sindbis	Toga	9,73 [1]
Influenza	Orthomyxo	5,1 [1]
HBV (Duck model)	Hepadna	> 6 [3]
Vesicular Stomatitis	Rhabdo	4,89 [*1]
Sensitivity of non-enveloped viruses		
Adeno	Adeno	4 [1]
Calici	Calici	3,9 [*1]
SV 40	Papova	4 [1]
Parvo B 19	Parvo	5 [1]

[1] Mohr et al. Immunological Investigations 1995, 24(1&2);73-85
[2] tested by Analysis Biomedizinische Test GmbH
[3] tested by Prof. Christian TREPO et al., INSERM Unit 271, Lyon, France
[*] tested under production conditions
Reduction below the limit of detection

Figure 4. Virus reduction capacity

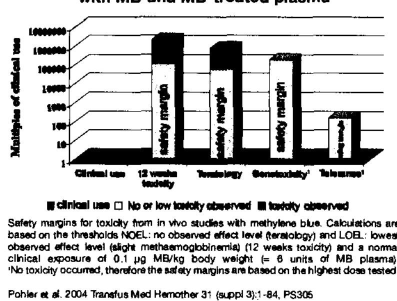


Figure 5: Safety margin for toxicity of Methylene Blue

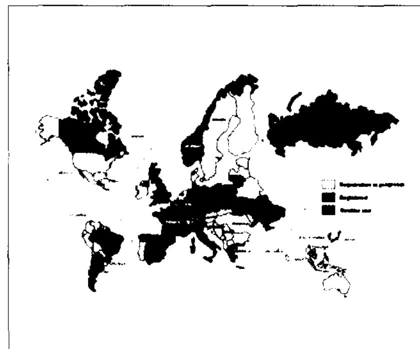


Figure 6: Global status of Theraflex MB-Plasma use

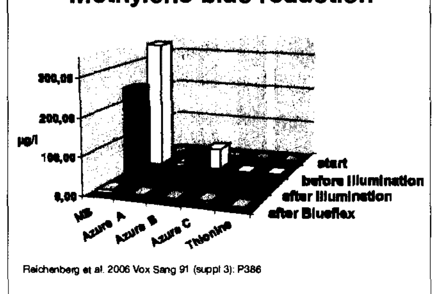


Figure 7: Methylene Blue reduction by Blueflex filtration

Results: With the current Theraflex MB-Plasma procedure provided by MacoPharma the procedure is markedly improved. The elimination of leucocytes is realized by membrane filtration, MB is added as an integrated dry pill, and residual MB and photoproducts are removed by a special Blueflex filter. The specially designed illumination device (Macotronic) ensures treatment under GMP conditions. Illumination dose and intensity are constantly monitored and temperature is controlled. The use of sodium low pressure lamps as improved light sources allowed the reduction of the illumination time to about 20 min.

The characteristic features of the system are:

1. Virus inactivation of enveloped viruses shows a reduction rate of at least 5 log10 steps. (Figure 4)
2. Plasma quality: Only fibrinogen and factor VIII are reduced by about 20-25%. (Figure 3)
3. Clinical use: More than 4 million MB-treated plasmas were transfused with excellent tolerance and efficacy in several countries all over the world. (Figure 6)
4. MB and photoproducts are eliminated by more than 90% using the Blueflex filter. (Figure 7)
5. Toxicology: Investigation on toxicology of MB and photoproducts showed a high safety margin for the concentration used. (Figure 5)

Conclusions

Conclusions: The MacoPharma Theraflex MB-Plasma represents an efficient, safe, and easy to use system which generates virus-safe plasma of high quality.

THE EFFECT OF METHYLENE BLUE PATHOGEN REDUCTION SYSTEM ON Fc VIIIc IN PLASMA DERIVED FROM WHOLE BLOOD DURING STORAGE

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[1] Dienst voor het Bloed, Rode Kruis-Vlaanderen, Belgium.
[2] Service du Sang, Croix Rouge de Belgique, Belgium.

BACKGROUND

The virucidal properties of methylene blue have been documented since 1930. In 1991, the Springe Institute developed a photodynamic method to inactivate pathogens, particularly viruses, in human plasma using methylene blue in combination with visible light. MacoPharma has improved this method and developed the THERAFLEX MB-Plasma system consisting of the Macotronic illumination machine, together with an appropriate disposable set for pathogen reduction and removal of residual methylene blue to a level less than 4µg/unit.

This method is known to be effective on viruses as well as other documented pathogens, although reducing slightly the activity of clotting factors such as factor VIII (Fc VIIIc) and Fibrinogen. According to Belgian legislation, plasma should be frozen within 18 hours after blood collection. In the case of pathogen reduced plasma, a level of at least 0.5 IU / ml for Fc VIIIc should be attained.

The aim of this study was to investigate the effect on Fc VIIIc recovery of various time delays, between donation and the photodynamic treatment of plasma derived from whole blood.

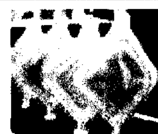
MATERIALS AND METHODS

In centre 1, 143 units of whole blood were selected from volunteer male A+ donors. These units were divided into 3 groups. Plasma from group A was separated at 4 hours and treated at 5.5 hours, group B plasma at 4 and 16.5 hours, and group C plasma at 15 and 16.5 hours, respectively.

In centre 2, 120 units of whole blood were selected from volunteer male or female donors of any blood group. These units were also divided into 3 groups. Plasma from group D was separated at 3.5 hours and treated at 8 hours, group E plasma at 3.5 and 11 hours, and group F plasma at 12.5 and 16.5, respectively.

Pathogen reduction was performed using the THERAFLEX MB-Plasma system (disposable with leucodepletion filter and methylene blue removal filter, Macotronic illumination machine). Samples for Fc VIIIc activity assay were taken immediately after separation and after treatment. Fc VIIIc measurement was done using a one-stage aPTT clotting assay with Fc VIIIc deficient plasma. Results of Fc VIIIc recovery are expressed in percentage of activity.

Prior to separation and photo treatment, the whole blood and plasma were stored on eutectic plates to keep the products at a temperature of 20 °C. Results were analysed using repeated measures Anova for general comparison, student t-test for group comparison and paired student t-test when appropriate.



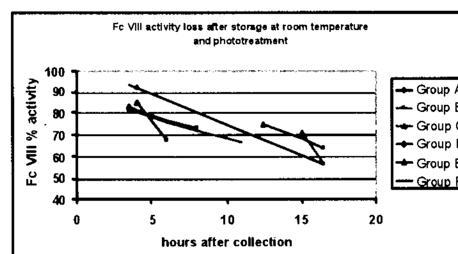
RESULTS

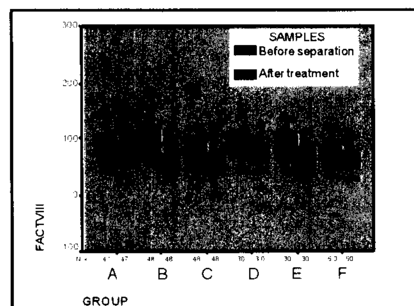
Fc VIIIc pre- and post-photo treatment mean results are presented in table 1.

	Time before separation (h)	Time before treatment (h)	Pre treatment [1] Fc VIIIc (%)	Post treatment [2] Fc VIIIc (%)
Group A	4	5.5	85	67
Group B	4	16.5	92	56
Group C	15	16.5	71	56
Group D	3.5	8	83	73
Group E	3.5	11	81	66
Group F	12.5	16.5	75	64

Table 1 : [1] Sample taken after separation [2] Sample taken after treatment

- The difference between the two groups was statistically significant ($p<0.001$).
- A significant decline in Fc VIIIc activity was measured in all groups following the photo treatment process ($p<0.001$).
- No significant difference between group B and groups C and F after photo treatment was observed ($p=0.87$, $p=0.10$); this suggests no significant difference in the loss of Fc VIIIc activity between the time of separation of whole blood into plasma and photo treatment 16.5 h.
- No significant difference between Fc VIIIc activity loss in group A and C ($p=0.66$) and in group D and F ($p=0.53$) suggesting that the time interval between blood collection and separation does not influence the loss of Fc VIIIc activity post photo treatment.





CONCLUSION

Regarding the Fc VIIIc activity of plasma, Methylene blue pathogen reduction has to be completed within a limited time interval after whole blood donation. The processing / separation of whole blood can be performed at any time between donation and the photo treatment of plasma. Following the attainment of these results, methylene blue pathogen reduction of plasma has been implemented in both centres.

XE7RA01A 07/04

Quality Control Evaluation of Methylene Blue Light Treated Plasma.

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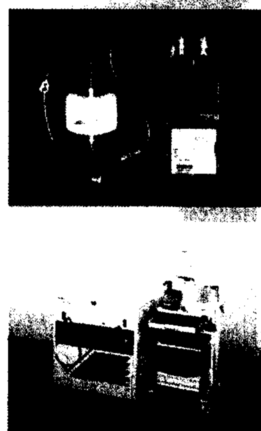
[1]Centro De Transfusion De La Comunidad Valenciana, Valencia, Spain;
2H General Universitario, Valencia, Spain.

INTRODUCTION

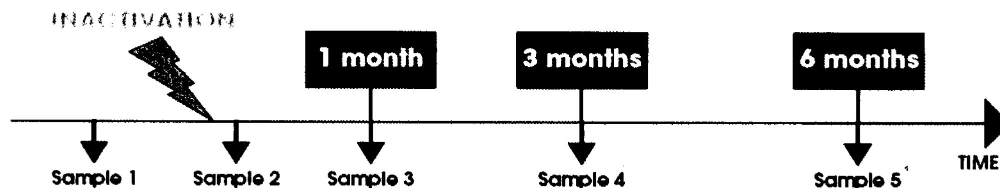
Methylene blue light treated plasma has been used in Spain from 1998. Since then slight variations of the technique have been implemented such as plasma leukoreduction instead of freezing and thawing.
Studies on plasma quality have been published analysing initial methods. We have tested several plasma units to evaluate plasma quality with the current technology.

MATERIAL AND METHODS

Briefly speaking, 450±50 mL whole blood units were collected in automatic scales using top & bottom blood bags.
After collecting, units were cooled with 1.4- butanodiol plates and later stored at 22±2°C. Then, whole blood was centrifuged at high speed to obtain a concentrate of red blood cells and plasma, while maintaining the buffy coat in the initial blood bag. For plasma inactivation the Springe modified method was used (Theraflex-MB-Plasma system: Macopharma®). Plasma was joined to the MB system by means of sterile docking and, simultaneously, gravity filtered. In batches of four plasma was illuminated for 20 min. Units for storage were frozen after inactivating before 24 hour postdonation. For the study we inactivated 30 plasma units (10 A, 10 O, 5 B and 5 AB). We took several samples: before inactivation (sample1), just after inactivation (sample2), after 1 month of storage at -30°C (sample3), after 3 months of storage at -30°C (sample4) and after 6 months of storage at -30°C (sample5). After each moment samples were stored at -80°C till the tests were performed.
In every sample we performed the following tests: PT, APTT, FV, FVIII and fibrinogen.



The process of the 5 plasma samples



RESULTS

As published before most affected parameters by the inactivation procedure were fibrinogen and FVIII (18 and 16% respectively decrease from sample 1 to 2). FV was scarcely affected (a 3% decrease from sample 1 to 2). PT an APTT were prolonged only in 2.74 and 5.26% respectively from sample 1 to 2. Results may be seen with more detail in the attached table.

Results of the tests (PT, APTT, Fib, FV and FVIII) for the 5 plasma sample

Sample parameter	(%)↑PT	(%)↑APTT	(%)↓Fib	(%)↓FV	(%)↓FVIII
Λ					
Λ					
Λ					
Λ					

PT: Prothrombin rate **APTT:** Activated Partial Thromboplastin Time test **Fib:** Fibrinogen
FV: Factor V **FVIII:** Factor VIII

CONCLUSIONS

The Methylene blue inactivation methodology is very easy to use and the plasma factors after inactivation are preserved. However, during storage there is a certain loss of coagulation factors. If the reason for this is related to the treatment or the storage conditions remains to be evaluated.

THERAFLEX MB-PLASMA PROCEDURE: PLASMA QUALITY AFTER 15 MONTH STORAGE

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ISBT Congress, Cape Town, September 2006

Introduction

Background: Although in the last decades thanks to the implementation of several methods like donor selection and testing procedures the risk of virus transmission from plasma has decreased, infection of patients still exists. Additionally new viruses like West Nile Virus enter the transfusion chain. Therefore, the treatment of therapeutic plasma with methylene blue (MB) is a technique used in several European countries for virus inactivation. MacoPharma has developed the proprietary photodynamic Theraflex MB-Plasma bag system including a MB pill, an illumination system (Maocotronic) with visible light, and a final MB filtration step with the Blueflex filter (Williamson et al. Transfusion 2003;43:1322-1329).

Aims: Aim of this study was to show the reduction of MB and photoproducts due to the Blueflex filter and to prove the reproducibility of the filtration efficiency. Additionally the quality of the plasma after 15 month storage was checked.

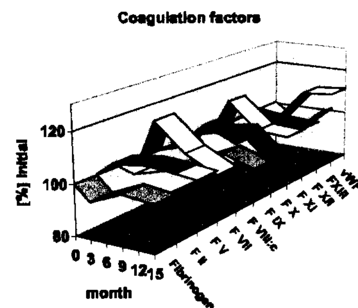
Materials & Methods

18 plasmas were treated at three different days. At different steps of the Theraflex MB-Plasma procedure the MB and photoproduct content was measured by HPLC, which was described previously (Verpoort et al. 2003; ISBT Istanbul P246). Measurement was done after dissolution of the MB pill, after illumination, and after filtration (Fig. 2).

At each day plasma was pooled and divided into several storage bags (storage temperature <-30 °C). At different time points a palette of plasma factors was measured.
1. global tests (Quick, INR. aPTT, thrombin time)
2. coagulation factors (Fibrinogen, F II, F V, F VII, F VIII:c, F IX, F X, F XI, F XII, FXIII, vWF Ristocetin Co-Factor)
3. Inhibitors (AT III, Protein C, Protein S)
4. Fibrinolysis (Plasmin inhibitor, alpha-1-Antitrypsin, Plasminogen)
5. Complement (CH50, C3a)
6. Activation (TAT, F XIIa, D-Dimer)

Results

Test	Limits	Unit	0 month	6 month	9 month	15 month
1. Gobal tests						
Quick	80 -130	%	98 ± 2	105 ± 3	94 ± 2	93 ± 3
INR			1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
aPTT	30 - 60	sec	34 ± 1	35 ± 1	34 ± 1	35 ± 1
Thrombin time	14 - 21	sec	22.7 ± 0.7	23 ± 1.0	23 ± 1.0	22 ± 1.0
2. Coagulation factors						
Fibrinogen	1.8 - 3.5	g / l	2.4 ± 0.1	2.3 ± 0.2	2.4 ± 0.2	2.4 ± 0.1
F II	70 - 130	%	99 ± 4	104 ± 4	106 ± 7	105 ± 12
F V	65 - 150	%	101 ± 3	99 ± 6	103 ± 9	101 ± 10
F VII	70 - 130	%	103 ± 8	119 ± 9	108 ± 3	102 ± 4
F VIII:c	050 - 2.00	I.U. / ml	0.81 ± 0.15	0.83 ± 0.12	0.92 ± 0.16	0.87 ± 0.11
F IX	0.70 - 1.30	I.U. / ml	1.00 ± 0.05	0.92 ± 0.04	0.98 ± 0.04	0.94 ± 0.04
F X	70 - 130	%	106 ± 6	108 ± 5	111 ± 7	98 ± 3
F XI	50- 130	%	82 ± 2	92 ± 3	83 ± 3	83 ± 4
F XII	70 - 130	%	97 ± 10	101 ± 11	99 ± 10	98 ± 11
F XIII	70 - 130	%	81 ± 11	77 ± 7	75 ± 4	85 ± 9
vWF (Ristoc. Co-F.)	50 - 140	%	85 ± 8	81 ± 19	94 ± 13	95 ± 7
3. Inhibitors						
AT III	0.80 - 1.30	I.U. / ml	1.12 ± 0.08	1.02 ± 0.08	1.06 ± 0.07	0.99 ± 0.06
Protein C	70 - 150	%	110 ± 7	109 ± 6	116 ± 6	110 ± 3
Protein S	70 - 140	%	75 ± 7	70 ± 2	76 ± 2	81 ± 2
4. Fibrinolysis						
Plasmin inhibitor	80 - 120	%	103 ± 4	101 ± 3	103 ± 6	105 ± 10
alpha-1-Antirypsin	0.70 - 1.50	I.U. / ml	1.14 ± 0.03	1.12 ± 0.07	1.24 ± 0.09	1.20 ± 0.08
Plasminogen	75 - 140	%	102 ± 7	105 ± 8	12± ± 11	104 ± 10
5. Complement						
CH50	70 - 100	%	116 ± 14	110 ± 11	108 ± 5	114 ± 8
C3a	123-2228	ng / ml	1029 ± 323	1106 ± 596	898 ± 332	921 ± 217
6. Activation						
TAT	1 - 4.1	µg / l	2.1 ± 0.2	2.4 ± 0.5	2.0 ± 0.0	2.2 ± 0.7
F XIIa	< 3.0	ng / ml	1.0 ± 0.2	0.9 ± 0.1	1.3 ± 0.6	1.0 ± 0.0
D-Dimer	64 - 246	µg / l	241 ± 120	239 ± 131	199 ± 97	225 ± 122



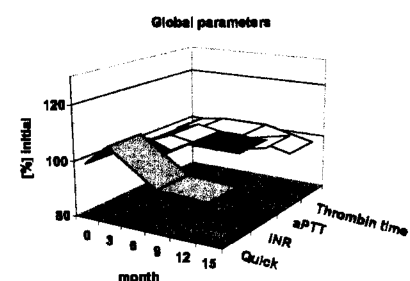


Fig. 1: Storage stability of Theraflex MB-Plasma

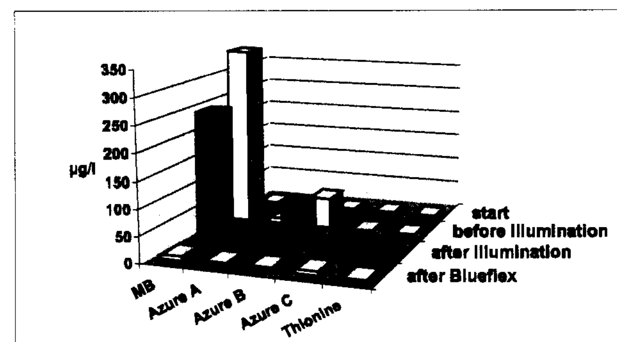


Fig. 2: Methylene blue and photoproduct reduction due to Bluflex filtration

Illumination of MB-containing plasma with visible light using the Macotronic illumination device resulted in the generation of photoproducts as described previously. Mean reduction of the total phenothiazine content was 94.5%. Every single filtration yielded in a filtration efficiency of minimum 91%. The mean reduction capacity for MB was above 99.9%.

There was no significant change in the plasma factor content after treatment during the whole 15 month storage period. Slight variations are within the error of measurement. The only difference between the plasma parameters resulted from the treatment itself. Here, an increase in the INR and aPTT (14.3 %; 18.2 %), decrease of fibrinogen (-19.3 %), factor V (-25.3 %), factor VIII (-21.8 %), factor IX (-25.6 %), factor X (-23.4 %), and factor XI (-16.8 %) was observed. Despite this variations the values were within the ranges found in non-treated plasma.

Conclusions

The filtration of plasma with the Blueflex filter is a reliable method to reduce the amount of MB and photoproducts substantially.

QUALITY OF THERAFLEX® MB-PLASMA DURING STORAGE AND TREATMENT

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ISBT Congress, Athens, July 2005

Introduction

Background: Although in the last decades thanks to the implementation of several methods like donor selection and testing procedures the risk of virus transmission from plasma has decreased, infection of patients still exists. Additionally new viruses like West Nile Virus enter the transfusion chain [1]. Therefore, the photodynamic treatment of therapeutic plasma with methylene blue (MB) is a technique used in several European countries for pathogen inactivation [2]. MacoPharma has developed the proprietary Theraflex® MB-Plasma bag system including a MB pill and a final MB filtration step.

Aim: Aim of the study is to show the quality of the MB plasma during the preparation procedure and during storage using the Theraflex® system (see Figure 1).

Materials & Methods

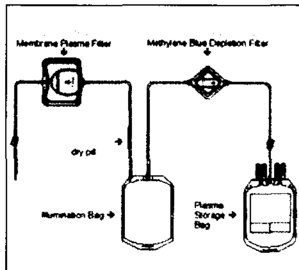

Figure 1: Theraflex® MB-Plasma bag system

Preparation Process

For the preparation process every single step was evaluated using 18 single donor plasma units. For the evaluation of the plasma factors 5 ml were drawn at different stages (see Figure 2). The samples were pooled after drawing and measured for the specified factors. Six samples of each stage were pooled at three days. A whole panel of plasma factors was measured for the resulting three pools (see Figure 3).

Stability

Stability data were generated using three plasma pools. Six plasmas were pooled and afterwards divided into six aliquots. Each was treated as single unit and then each was divided into six storage samples. The same plasma factors as for the manufacturing process were evaluated.

MB and photoproduct content was below the detection limit as previously described [3].

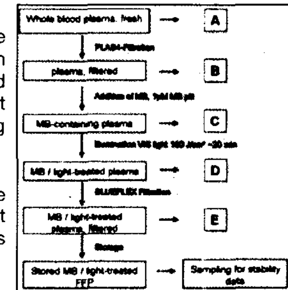

Figure 2: Sampling scheme

Results

In-process control | **Storage stability**

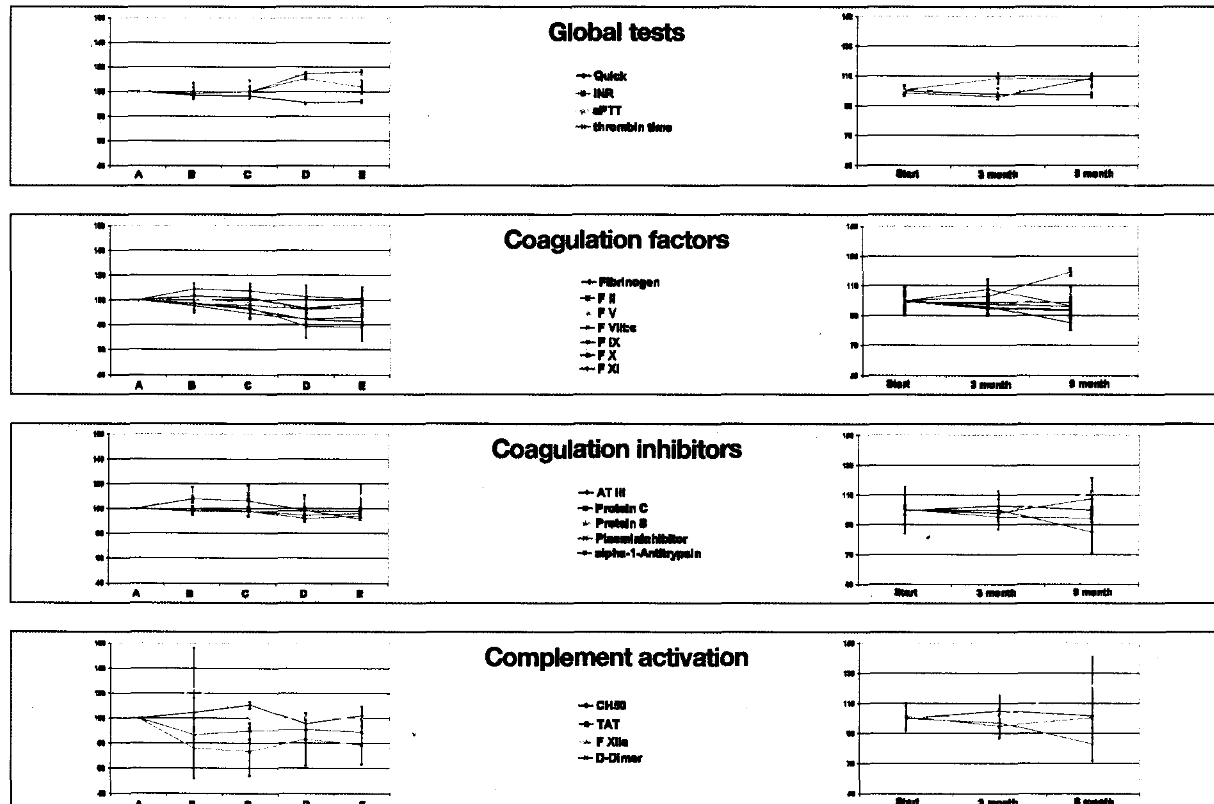

Figure 3: Percentage of deviation from the source plasma for different plasma factors during storage and treatment A: before treatment; B: after PLAS4 filtration; D: after MB addition; D: after illumination; E: after Blueflex filtration

All investigated plasma factors remained stable during the investigated storage time. A moderate reduction for some coagulation factors during the preparation was found in the illumination step but not in the other preparation stages. This was mainly fibrinogen (17,5 %), factor VIII (22,2 %), and factor X (13,4 %). Despite this reduction the values were within the ranges found in non-treated plasma.

Conclusions

Plasma treated with the Theraflex procedure showed slight reduction during treatment and no reduction during storage. All plasma factors remained within the threshold values. The treatment of therapeutic plasma with MB is a valid technique of pathogen inactivation.

[1] **West Nile virus in plasma is highly sensitive to methylene blue-light treatment** Mohr et al, **Transfusion** 2004;44:886-890

[2] **Methylene blue-treated fresh-frozen plasma: what is its contribution to blood safety?** LM Williamson et al, **Transfusion** 2003;43:1322-1329

[3] **Filtration of Methylene Blue and Photoproducts after Photodynamic Treatment of Plasma using BLUEFLEX** T Verpoort et al, 2003 VIII European ISBT Congress P245

PREPARATION OF METHYLENE BLUE –TREATED PLASMA UNDER WORST-CASE CONDITIONS - INFLUENCE ON QUALITY AND STABILITY-

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DGTI Congress, Erfurt, 2005

INTRODUCTION

Treatment with methylene blue (MB) and light is a well-known procedure for the inactivation of blood-borne viruses in Fresh Frozen Plasma (FFP). The purpose of this study was to assess the quality and stability of MB/light-treated plasma (MB plasma) processed by the MacoPharma Theraflex MB-Plasma® system. Preparation was done under worst case conditions for routine processing to evaluate the worst plasma quality to be expected during production.

METHODS

12 single donor units of MB/light treated plasma were prepared using the MacoPharma Theraflex MB-Plasma® system. Preparation included leukocyte depletion (Plasmaflex-filter), addition of methylene blue (MB-pill) prior to illumination and depletion of MB and photoproducts (Blueflex-filter) after treatment. Samples were taken before treatment and from the final product. For the assessment of stability, plasma from four different plasma pools was photodynamically treated and stored for up to 9 months. Treatment was done under worst-case conditions for the preservation of product quality: maximum MB concentration during illumination (1.15 µmol/l), maximum storage time of whole blood before separation (4°C, 17 h), maximum storage time of MB plasma before freezing (1 h).

RESULTS

Thrombin time, fibrinogen (Clauss), factors V, VIII, XI and protein C were significantly altered by MB/light treatment, while anti-thrombin III (AT III), vWF:RCo, vWF cleaving protease (vWF CP), plasmin inhibitor and α_1-antitrypsin remained unchanged (Fig. 1). There was no activation of the coagulation markers (F 1+2, D-dimers) attributed to the virus inactivation procedure including the filtration steps for leukocyte depletion and MB and photoproduct depletion. The influence of each manufacturing step on the activity of coagulation factors was investigated using three plasma pools. Most of the activity was lost during illumination (Fig. 2). After illumination MB and its photoproducts (azure A, azure B, azure C) were depleted by Blueflex filtration (Fig 3) to a final concentration of <0.1 µmol/l (MB + sum of photoproducts). Stability of MB-Plasma was tested during storage at -30°C for up to 9 months (Fig 4). Stability testing will be continued for a total of 27 months.

Parameter		Before treatment	After treatment	Percentage of loss (-) or increase (+)
Thrombin time	[s]	15.8 ± [illegible]	19.1 ± [illegible]	+20.6
Fibrinogen (Clauss)	[mg/dl]	279.9 ± [illegible]	222.8 ± [illegible]	20.3
Factor V	[%]	126.7 ± [illegible]	[illegible]	16.4
Factor VIII	[%]	[illegible]	[illegible]	22.2
Factor XI	[%]	94.6 ± [illegible]	82.1 ± 18.7	[illegible]
Antithrombin III	[%]	[illegible]	[illegible]	[illegible]
Protein C	[%]	[illegible]	[illegible]	9.8
Protein S, free	[%]	94.2 ± [illegible]	94 ± [illegible]	[illegible]
vWF:RCo	[%]	[illegible]	[illegible]	[illegible]
vWF CP	[%]	[illegible]	[illegible]	[illegible]
Plasmin inhibitor	[%]	[illegible]	[illegible]	[illegible]
α_1-Antitrypsin	mg/dl	[illegible]	[illegible]	[illegible]
F 1+2	nmol/l	[illegible]	[illegible]	[illegible]
D-Dimers	mg/l	[illegible]	[illegible]	[illegible]
CH 100	U/ml	[illegible]	[illegible]	[illegible]

n = 12 [illegible]

Fig.1 Influence of the MB/light treatment on plasma quality (data from 12 single donor units, treatment under worst-case conditions)

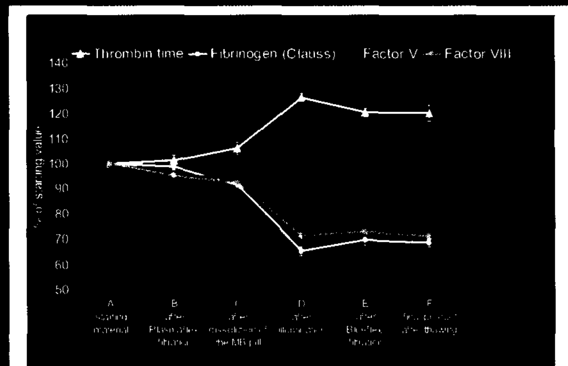


Fig.2 Influence of the individual manufacturing steps on plasma quality (data from three pools)

Methylene Blue	µmol/l	Depletion (C / A)
A (before illumination)	0.97 +/- 0.06	
B (after illumination)	0.65 +/- 0.15	
C (after Blueflex filtration)	0.01 +/- 0.01	98.8 %
Azure A	**µmol/l**	
A	0.01 +/- 0.01	
B	0.06 +/- 0.03	
C	0.01 +/- 0.01	
Azure B	**µmol/l**	
A	0.11 +/- 0.01	
B	0.24 +/- 0.04	
C	0.01 +/- 0.01	
Azure C	**µmol/l**	
A	0.00 +/- 0.00	
B	0.03 +/- 0.03	
C	0.01 +/- 0.02	
Phenothiazine total	**µmol/l**	
A	1.09 +/- 0.07	
B	0.99 +/- 0.08	
C	0.04 +/- 0.03	96.4 %

Fig.3 Depletion efficacy of the Blueflex filter (data from 12 single donor units)

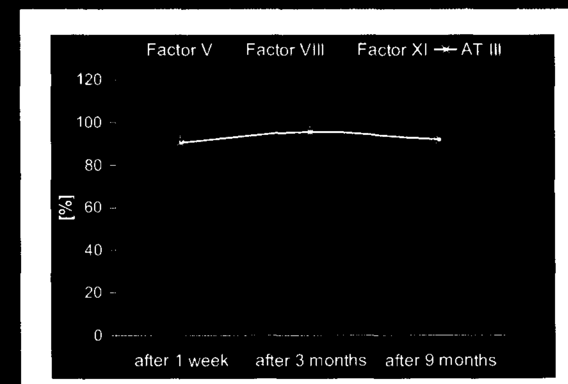


Fig.4 Stability of MB/light-treated plasma (data from 4 pools, , treatment under worst-case conditions)

CONCLUSIONS

Even under worst-case conditions, photodynamic treatment of FFP using the Theraflex MB-Plasma® system only moderately affects the activities of coagulation factors. The Blueflex-filter depletes MB and its photoproducts by over 90% after photodynamic treatment. Storage of MB plasma for up to 9 months had no effect on coagulation factors.

THERAFLEX – MB PLASMA

Coagulation factors and activation parameters

For each stage in the preparation

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Prothrombin rate (%)	70 - 130	80.8	71.8	87.7	76.6	73.5	68.2	65.7
INR	1	1.21	1.33	1.11	1.24	1.3	1.39	1.43
Activated partial thromboplastin time test (ratio)		1.12	1.26	1.05	1.18	1.13	1.22	1.27
Fibrinogen	2 - 4	3.11	2.29	2.94	2.04	3.03	2.28	2.36
Factor II (%)	70 - 120	102	98	98	95.1	95.1	86.7	92.3
Factor V (%)	70 - 120	102.2	94.1	89.5	83.2	105.7	98.1	96.4
Factor VII (%)	70 - 130	113.1	101.9	106.1	92.1	107.6	100.6	104.2
Factor VIII (%)	60 - 150	100.6	73.5	101.1	76.1	114	89.5	86.1
Factor IX (%)	60 - 150	96.5	78.8	98.6	86	99.2	78.9	85.7
Factor X (%)	70 - 120	105.9	95.3	106.9	92.6	97.3	91.7	92.3
Factor XI (%)	60 - 140	90.9	75.3	86.7	64.4	85.5	70.8	65.7
Factor XII (%)	60 - 140	103.7	92.6	110.5	99.9	98.4	96	93.9
Antithrombin III (%)	80 - 120	104.8	89.9	104.4	101.9	105.8	103.7	93.9
Protein C (%)	70 - 140	110.9	105.3	108.4	103	120.7	112.9	112.6
Protein S (%)	70 - 140	82.6	78.6	81.6	71.2	83.8	77.6	81.4
V Willebrand Factor CoF ristocetin (%)	60 - 150	97.6	92.9	87.4	84	143.6	137.4	138.6
Von Willebrand Factor Ag (%)	60 - 150	134.6	118.2	130.3	128	143.6	121.8	122.2
Plasminogen (%)	80 - 120	102.7	100.9	100.8	96.9	103.6	101.6	101.6
α2-antiplasmin (%)	80 - 120	111.9	107.7	106.2	104.2	112.5	106.5	107.7
C3a (mg/l)	100 - 400	134.8	134.1	146.8	143.1	124.2	125.5	124.0
C5a (μg/l)	0.9 - 15.4	10.9	14.1	15.1	23.9	7.5	8.3	10.9
Factor XIIa (ng/ml)	< 3.0	2.39	2.22	2.87	2.63	2.57	1.79	2.49
F1 + 2 prothrombin (nmol/l)	0.4 - 1.1	0.99	1.20	0.88	0.87	0.79	0.72	0.78
Platelet factor 4 (UI/ml)	56 - 805	317.2	301.1	413.9	375.4	186.9	197.2	208

A : Plasma before contact with methylene blue

Ab : Plasma after visible light (plasmas 31 to 40)

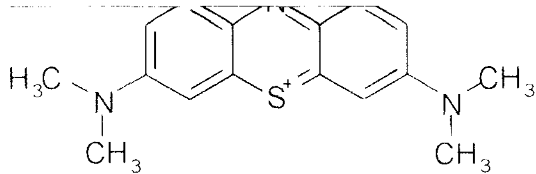
B : MB-removed Plasma before freezing

C : MB Plasma after 6 months at - 30° C

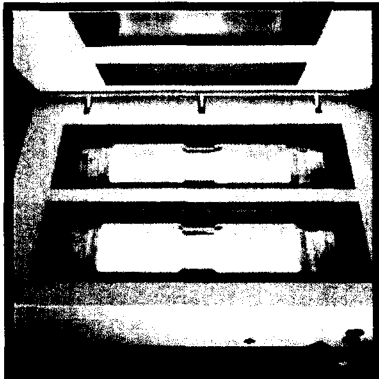
D : MB-removed Plasma after 6 months at - 30° C

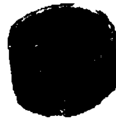
THERAFLEX-MB Plasma

Processing principle



Methylene Blue molecule





MacoPharma Methylene Blue Pill
(85µg / unit of plasma)

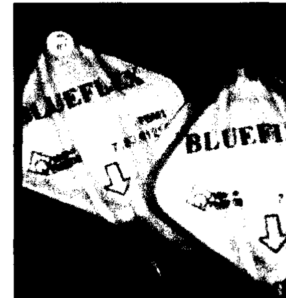
Illumination of plasma + Methylene Blue
(590 nm, 180J/cm^2)

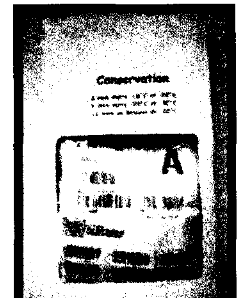
- Intercalation of MB into nucleic acids
- Excitation of MB by visible light
- Oxidation of Guanosine
- Degradation of nucleic acids

The combined action of Methylene Blue and light is a photodynamic process which blocks transcription and replication of viral RNA and DNA.

Photo-inactivation procedure of THERAFLEX-MB PLASMA

200-315ml of plasma (Aphaeresis or Whole Blood)	Filtration of plasma and dissolution of the MB Pill	Illumination of plasma+MB with the Macotronic V4	MB removal by filtration with Blueflex	Plasma freezing





THERAFLEX-MB Plasma

Examples of systems and treatments

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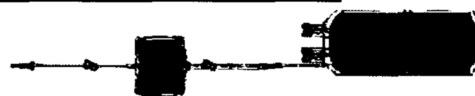
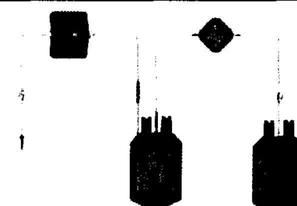
Min 250[illegible]0 mL*

Whole Blood Plasma with MB treatment



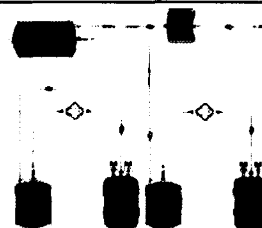
BSV system : plasma filtration with Plasmaflex > MB Pill dissolution > illumination

Whole Blood Plasma with MB treatment and MB removal

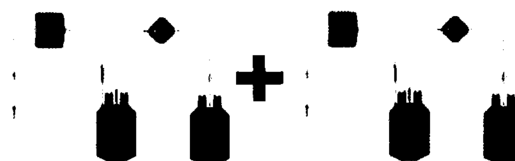


SDV system : plasma filtration with Plasmaflex > MB Pill dissolution > illumination > MB removal with Blueflex

Aphaeresis Plasma : distribution in 2 units

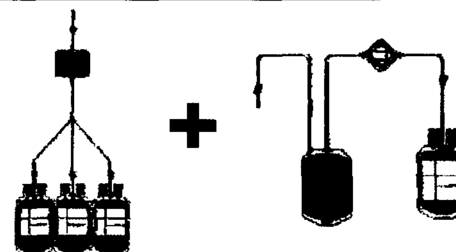


SPV system : filtration of the aphaeresis unit with Plasmaflex MB Pill dissolution and distribution in 2 units > illumination > MB removal with Blueflex



SDV system : plasma filtration with Plasmaflex > MB Pill dissolution > illumination > MB removal with Blueflex

Aphaeresis Plasma : distribution in 3 units



BSV + ZDV system : filtration of the aphaeresis unit with Plasmaflex and distribution in 3 units with the BSV system. Connection of each unit to a ZDV system : MB Pill dissolution > illumination > MB removal with Blueflex

* *Volume ranges for plasmas to be treated with THERAFLEX-MB Plasma, based on process requirements.*

Methylene blue-treated fresh-frozen plasma: what is its contribution to blood safety?

Lorna M. Williamson, Rebecca Cardigan, and Chris V. Prowse

With current donor-selection criteria and virus genome testing, fresh-frozen plasma (FFP) in the developed world is probably safer than it ever has been. In the UK, where FFP is not manufactured from first-time or lapsed donors, it has been estimated that the residual virus risks from a single unit of FFP are 1 in 10 million for HIV, 1 in 50 million for HCV, and 1 in 1.2 million for HBV (Eglin R, written communication, January 2003). Against these levels of risk, it has been questioned whether pathogen reduction of FFP is a necessary strategy and/or the best use of healthcare resources.[1] However, the appearance of West Nile virus in blood components in the US in 2002, with fatal transmissions in immunocompromised recipients,[2] reminds us that sometimes viruses move ahead of our ability to test for them. Also, background viral incidence in a population can change, as is currently observed in Scotland, with HIV levels showing an increase to three per million population (Soldan K, written communication, February 2003). It is now over 10 years since a photodynamic system using methylene blue (MB) and visible light was developed in Springe, Germany, for virucidal treatment of FFP. The method has been used at various times since then in Germany, Denmark, Portugal, Spain, and the UK, so it is timely to review its potential contribution to overall FFP safety.

MB is a phenothiazine compound (Fig. 1), which was first used clinically by Paul Ehrlich in the 1890s and has been used to kill viruses since work at the Walter Reed Hospital in the 1950s.[3] When activated by visible light, MB generates reactive oxygen species, mainly singlet oxygen, through a Type II photodynamic reaction, and it is these that are responsible for its pathogen inactivating properties.[3-5] The original system developed in Springe, Germany, used an initial freeze-thaw step to disrupt intact WBCs, then added an amount of MB solution calibrated to the weight of the plasma pack, to achieve precisely the same MB concentration in every pack. Later systems (Baxter and Macopharma) developed for small-scale use in blood centers involve sterile connection of the plasma pack (before or after freezing) to a pack with a WBC-reduction filter upstream of a liquid pouch or a dry pellet containing 85 to 95 μg of MB (Fig. 2). To achieve the desired final MB concentration of 1 μ*M*, the input plasma volume has to be within a 200-to-300-mL range, so 600-mL apheresis units require splitting. In both the Springe and commercial systems, the MBFFP packs are then exposed to visible wavelengths of light to activate the MB. Because it is not possible to use the equivalent of radiation-sensitive labels to confirm illumination, the light-exposure system must be designed to ensure good manufacturing practice (GMP)-compliant control of both light intensity and duration. Radio-frequency chips for this purpose are in development. During illumination, MB is converted to its bleached leuko- form and to demethylated components (azure A, B, and C, and thionine; Fig. 1). A recent feature has been the development of commercial filters for post-treatment MB removal, which reduce the residual MB concentration to 0.1 to 0.3 μ*M*. The plasma is then ready for freezing or refreezing.

One of the attractions of the technique is that it is applied to single units of FFP, without the need for pooling. Commercial systems are available that can be set up in standard blood center GMP conditions, without the need to install specialized plant, and it is this model that is in operation in the UK. Plasma is frozen locally, sent to one of three central MB-treatment points, then returned for distribution to hospitals.

ABBREVIATIONS: APTT = activated partial thromboplastin time; FFP = fresh-frozen plasma; MB = methylene blue; MBFFP = methylene blue-treated fresh-frozen plasma; PT = prothrombin time; TTP = thrombotic thrombocytopenic purpura.

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PATHOGEN-REDUCTION SPECTRUM

The ability of MB to inactivate viruses is dependent on its binding to nucleic acid, being greater for double stranded

than single stranded, although viruses containing genomes of either type may be efficiently inactivated (see below). Activation results in a mixture of strand cross-linking, guanosine oxidation, and depurination. MB may also modify proteins and lipids, the relative rates depending on the MB and local oxygen concentrations. For virus-infected cells, this may be influenced by the reducing and detoxifying mechanisms present inside the cell. MB is not considered useful for inactivation of intracellular viruses or to attain bacterial or protozoal reduction, although it does enter cells.[5-7] Its only application in transfusion has been to achieve virus inactivation of plasma, with prior cell removal by filtration or freeze-thaw lysis[8-10] (Flament J, Mohr H, and Walker W, written communication, 2000).

Photodynamic treatment with MB results in efficient virus inactivation for all lipid-enveloped viruses tested to date, including all those for which the UK and US currently routinely screen blood donations, as well as West Nile virus.[3-5,10] The extent of removal for such viruses is usually at least 5 logs, this being true for both double- and single- stranded RNA and DNA viruses (Table 1). Nonlipid-enveloped viruses show a more diverse spectrum of susceptibility, some being totally unaffected (EMC, polio, HAV, porcine parvovirus), whereas others (SV40, HEV models, human parvovirus B19) show reduction factors of 4 logs or more (Table 1). More recently, testing using PCR methods has shown direct removal of HIV, HBV, HCV, and parvovirus B19 reactivity from infected donations,[11-14] the last of these demonstrating 4-log reduction by a newly developed B19 bioassay on the KU 812 EP 6 cell line (Flament J, Mohr H, and Walker W, written communication, 2000).

Methylene blue homologues

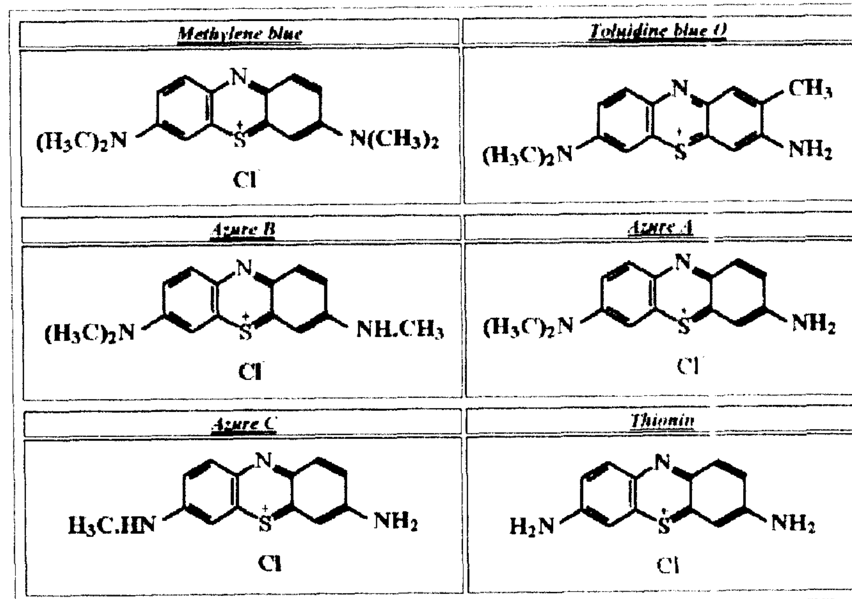

Fig. 1. MB and its photodegradation products.

TABLE 1. Virus reduction

Lipid enveloped		Non-lipid enveloped	
Virus	log reduction factor	Virus	log reduction factor
HIV	>5.5	HAV	0.0
Bovine viral diarrhea	>6.2	Encephalomyocarditis	0.0
Duck HBV	3.9	Porcine parvovirus	0.0
Influenza	5.1	Polio	0.0
Pseudorabies	5.4	SV40	4.3
Herpes simplex	>6.5	Adenovirus	4.0
Vesicular stomatitis	>4.9	Human parvovirus B19	≥4.0
West Nile virus	>6.5	Calicivirus (HEV)	>3.9

Are such reduction factors sufficient to assure that a single plasma donation, taken during the peak of viremia, is rendered noninfectious? The answer will depend on whether the donation is also subjected to NAT or serologic testing and on the level of viremia. For most viruses, we know that the answer is almost certainly yes, but in a few cases such as parvovirus B19, in which the peak of viremia is around 10^7 genome equivalents per mL, this conclusion is more dubious. However, for viruses of major concern, peak viremia levels are either within the clearance range of the system, or screening with assays of high sensitivity will have ensured that only donations with lower levels of viremia enter the processing laboratory (handling errors excepted). In the pregenome testing era, there was a possible HCV exposure from a unit of MBFFP taken from a donor in the sero-negative window period (Flament J, written communication, March 1998). The patient seroconverted for HCV but remained genome negative. The precise events remain unproven, but it is possible that the patient generated an antibody response against inactivated virus.

Although MB and other phenothiazine dyes have been suggested as having inhibitory action against transmissible spongiform encephalopathies,[15] there is no evidence of in vitro inactivation of infectivity at the concentrations used in the transfusion setting.

The MACO PHARMA Plasma Membrane filtration Methylene Blue Illumination and MB Depletion Set

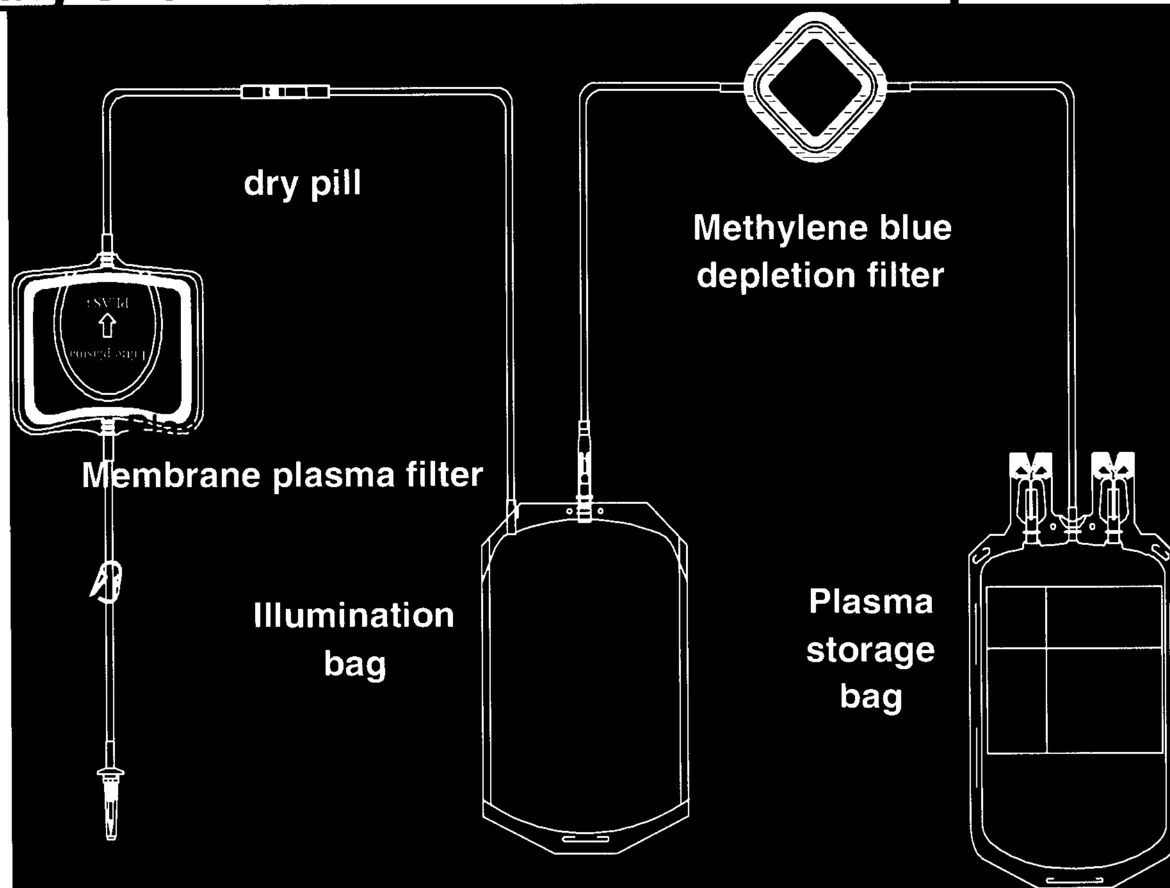

Fig. 2. Schematic representation of the closed bag system for MB treatment of fresh-frozen plasma.

EFFECT OF MB TREATMENT ON COAGULATION PROTEINS

It is well established that MB treatment of plasma affects the functional activity of various coagulation proteins and inhibitors (Table 2). The proteins most severely affected by MB treatment of plasma are FVIII and fibrinogen, where activity is reduced by 20 to 35 percent. The decrease in fibrinogen is seen when assayed by the method of Clauss, but not in antigenic assays,[16] suggesting that MB treatment effects the biologic activity but not concentration of fibrinogen. It has been suggested that this is due to the photo-oxidation of fibrinogen inhibiting polymerization of fibrin monomers.[17] The effects on fibrinogen are probably due to an interaction of MB with histidine residues and may result in a modified in vivo clearance.[16,18-20] However, fibrinogen isolated from MB-treated plasma retains normal ability to bind to glycoprotein IIb/IIIa receptors on platelets,[21] an important mechanism in platelet activation and aggregation. The inhibitory effects are ameliorated by the presence of ascorbate[22] but do not appear to result in the formation of any neoantigens[16,18,19] or positivity in tests for the formation of IgE antibodies (Flament J, Mohr H, and Walker W, personal communication, 2000).

Unsurprisingly, the changes in coagulation proteins observed in MB-treated plasma are associated with a prolongation of the prothrombin time (PT) and activated partial thromboplastin time (APTT).[16,23]

Original studies on MB inactivation were reported on plasma freeze-thawed before treatment, but later work on the Baxter Pathinact and Maco Pharma Theraflex systems was performed on fresh plasma (Table 2). However, we have recently shown that the major cause of coagulation factor loss is the MB treatment itself and not the freeze-thawing.[16,24] Fortunately, changes in coagulation proteins induced by WBC-reduction and MB-removal filters appear to be negligible compared to the effect of the MB process itself. Filtration of plasma using a filter (Hemasure)

TABLE 2. Changes in coagulation factor proteins and inhibitors in MBFFP

Parameter*	Percent change due to MB treatment†‡§	Mean residual levels‡§
Fibrinogen (Clauss) g/L	↓ 24,[10] 24,[23] 39[29]	1.65,[10] 1.80,[16] 2.01,[23] 1.97,[28] 2.05[29]
Fibrinogen (antigen) g/L		2.74[16]
Prothrombin (FII) (U/mL)	↓ 8,[10] 8,[16] 18,[23]	1.15,[10] 1.05,[16] 1.00[23]
FV (U/mL)	↓ 4.5,[10] 21,[16] 32,[23] 10,[28]	0.84,[10] 0.73,[16] 0.79,[23] 0.76[28]
FVII (U/mL)	↓ 8,[10] 9,[16] 7,[23]	1.10,[10] 0.90,[16] 0.90[23]
FVIII (U/mL)	↓ 13,[10] 33,[16] 28,[23] 26,[28] 29[29]	0.78,[10] 0.58,[16] 0.58,[23] 0.83[28]
FIX (U/mL)	↓ 17,[10] 23,[23] 11[28]	1.00,[10] 0.72,[23] 0.88[28]
FX (U/mL)	↓ 13,[10] 7[23]	1.05,[10] 0.90[23]
FXI (U/mL)	↓ 17,[10] 27,[23] 13[28]	1.00,[10] 0.73,[23] 0.84[28]
FXII (U/mL)	↓ 17[10]	1.20[10]
FXIII (U/mL)	↓ 7,[23] 16[29]	1.02,[23] 1.12[29]
vWF antigen (U/mL)	↓ 7,[23] 5[29] →[28]	0.94,[23] 0.83,[29] 1.00[28]
vWF:ristocetin cofactor(U/mL)	↓ 8,[23] 18[29]	0.92,[23] 0.79[29]
C1-inhibitor (U/mL)	↓ 23,[10] →[16]	0.88,[10] 1.03[16]
Antithrombin (U/mL)	↓ 8,[10] 3[23] →[16,23]	0.78,[10] 0.95,[16] 1.00,[23] 0.96[28]
Protein C (U/mL)	→[16,28]	1.03,[16] 0.89[28]
Protein S (U/mL)	→[16]	1.11[16]
α_1-antitrypsin (U/mL)	→[16]	155 mg/dL
Plasminogen (U/mL)	→[10,16]	0.90,[10] 0.98[16]
α_2-antiplasmin (U/mL)	→[16]	0.96[16]

* Results given as U/mL because not all studies were calibrated against international standards. Assays are functional unless otherwise stated.
† Arrows indicate direction of change, with horizontal arrow indicating no change.
‡ [10,16,23]Studies used frozen-thawed plasma.
§ [28,29]Studies used fresh plasma (<8 hr from collection).

designed to remove both WBCs and MB simultaneously results in a prolongation of the APTT but has no effect on the PT or fibrinogen when measured by manual techniques.[25] Filters to remove residual MB in plasma developed more recently by Pall and Maco Pharma are reported to result in a small increase in the APTT but minimal loss of coagulation factor activity.[26,27] It has been suggested that the increase in the APTT in the latter studies may be a result of some activation of the contact system of coagulation after contact of plasma with the artificial surface of the filter.[26]

Levels of thrombin-antithrombin complexes are not elevated in MB-treated plasma,[16] indicating that MB treatment is also not associated with excessive thrombin generation. Functional measurements of the naturally occurring anticoagulants protein C & S and antithrombin also appear to be relatively unaltered in MB-treated plasma.[10,16,23,28] MB treatment is reported to have little effect on levels of plasminogen, alpha-2-antiplasmin (the main inhibitor of plasmin), fibrin monomer, and D-dimers,[16] suggesting that the use of MBFFP is unlikely to result in enhanced fibrinolysis. vWF activity in plasma, as measured by ristocetin-induced agglutination of platelets, is reduced by 10 to 20 percent,[23,29] but vWF multimeric distribution and cleaving protease activity are reported to be unaffected.[23,28-30]

After transfusion of MB-treated plasma to healthy adults, there was no significant difference from baseline values in APTT, PT, TT, FVIII, FXI, Clauss fibrinogen, fibrin degradation components, or platelet aggregation induced by collagen or ADP, suggesting no major influence on coagulation or fibrinolytic systems.[31]

There have been relatively few studies examining cryoprecipitate and cryosupernatant produced from MB plasma. Levels of FVIII and fibrinogen activity in cryoprecipitate are 20 to 40 percent lower than untreated units[23,32] but remain within Council of Europe Guidelines. The effect on levels of vWF antigen and activity seem more variable: one study reports no significant difference,[23] whereas in a two-center study, one center also reported no change, while the other saw 15 to 20 percent lower values in MB units.[32] These differences might be explained by variation in the methodology used to prepare the cryoprecipitate. However, both studies show that the multimeric distribution of vWF is unaltered. Cryoprecipitate produced from MBFFP has not yet been introduced in any country that provides MBFFP, but work is ongoing in the UK to optimize fibrinogen concentration.[33]

Cryosupernatant produced from standard or MB-treated plasma lacks the largest molecular weight forms of vWF.[23] The main clinical indication for cryosupernatant is for the treatment of thrombotic thrombocytopenic purpura (TTP). Patients with TTP tend to have unusually large molecular weight vWF multimers,[34] which are known to promote platelet aggregation, and some believe that treatment with a plasma component that lacks the high molecular weight forms of vWF may be beneficial. However, no clinical data are available to answer this question. Levels of vWF cleaving protease have not been measured in cryosupernatant produced from MB-treated plasma, but given

that levels appear to be relatively unaltered in the source plasma,[30] one would not expect them to differ significantly. It would thus appear that MB-treated cryosupernatant would be suitable for the treatment of TTP, but it has yet not been manufactured for clinical use.

If MB plasma is used to suspend single-donor platelets, there is no significant effect on platelet numbers, morphology scores, osmotic recovery, or levels of LDH, CD62P expression, lacate, pH, and glucose compared to standard plasma.[35] Similarly, if MB-treated plasma is added to RBCs, there appears to be no appreciable effect on leakage of potassium, hemolysis, or osmotic fragility during 28 days of storage.[35] This is in contrast to direct treatment of RBCs with MB and light, which results in membrane leakage and enhanced surface binding of IgG.[5-7]

PHARMACOLOGY AND TOXICOLOGY

The major clinical application of MB in the past has been as a redox reagent in the reversal of methemoglobinemia and cyanide poisoning using intravenous doses of 1 to 5 mg per kg. It has also been used at higher oral doses for the treatment of manic depression (300 mg/day) and renal calculus disease (195 mg/day). Intravenous doses of 2 to 5 mg per kg have also been used for heparin neutralization and for perioperative staining of the parathyroid gland.[3,4,9,10,20] For comparison, the plasma pathogen-reduction systems described here result in a MB concentration of 1 μM in the FFP, equivalent to an intravenous dose per 250 mL FFP unit of 0.0012 mg per kg. If MB-removal filters are used during processing,[25,36] this level is reduced approximately ×10, to a final concentration of 0.1 to 0.3 μM. For a 70-kg adult receiving the recommended 15 mL per kg of FFP, this equates to a total MB dose of approximately 33 µg, or less than 1 µg in a 2-kg premature infant. Infused MB is rapidly cleared from the circulation and marrow (half-lives in rats are 7 and 18 min, respectively) to an extent that its presence in blood (half-life in man approx. 60 min) is difficult to detect after infusion of MBFFP. There is some tissue uptake, but the majority of MB is excreted via the gastrointestinal tract and in urine within 2 or 3 days[3] (Flament J, Mohr H, and Walker W, written communication, 2000).

A US toxicologic report summarizes its use to assess membrane rupture during amniocentesis, noting mild and transient side effects at most.[37] In mammals, the half lethal dose for MB is of the order of 100 mg per kg, with photo-illumination products having similar, or lesser, toxicity profiles to the parent compound.[3,5] Chronic dosing of animals with MB at doses up to 0.2 g per kg day for 13 weeks are nontoxic. Chronic exposure of rats to a diet containing 4 percent MB had no carcinogenic or cirrhotic effects, while testing in both rodents and Drosophila revealed no genotoxic effects at near lethal doses. Testing for induction of birth defects at doses up to 5 mg per kg per day has also given negative results,[3,5] although recently higher doses have been reported as inducing fetal growth retardation.[38] In contrast to this, in vitro tests, such as the Ames test for mutagenic effect in selected bacteria, have yielded some mutagenic and genotoxic data, particularly in the presence of a liver microsomal (S9) fraction. Testing on human lymphocytes and the mammalian V79 cell line has been reported by some to show no mutagenicity, although in the presence of the microsomal S9 fraction, some chromosomal aberrations were seen in lymphocytes at 1 to 2 µg per mL (Flament J, Mohr H, and Walker W, written communication, 2000). Wagner et al.[39] has reported genotoxic effects in mouse lymphoma cells at 30 µg per mL of MB, which was enhanced by S9 addition, but failed to detect any activity in vivo in a mouse micronucleus assay.

Between 1992 and 1998, more than a million units of MBFFP were used in Germany, Switzerland, Austria, and Denmark. Use has continued in the UK, Portugal, and Spain using the Grifols, Baxter, and Macopharma versions of the technology. The latter two systems have a European Medical Devices licence (CE mark), granting of which includes a toxicologic assessment. Both passive and active surveillance[40] have yielded adverse event rates that do not differ from those for standard FFP. In neonates, where the concern is greater due to the immature detoxification system, there are few reports on surveillance, but data from both Germany and Spain indicate no acute adverse events, even when MBFFP is used for exchange transfusion (Castrillo A, Pohl U, written communication, 1999). Concern over the potential in vitro mutagenic effects of MB and its derivatives, particularly in the presence of the S9 fraction, was the reason for the failure to re-license the product (without MB removal) in Germany in 1998. An opinion has not been reached on whether the system including the MB-removal step will be granted a German license. However, a large amount of clinical usage and in vivo toxicology testing suggest that despite the effects seen in vitro, in vivo side effects are minimal, presumably mainly due to the dilution on infusion and the rapid clearance of the compound. One toxicology expert in the field has suggested the risk is on a par with smoking a pack of cigarettes over a lifetime (Flament J, Mohr H, and Walker W, written communication, 2000).

CLINICAL STUDIES

Most studies in patients have been small and/or have used laboratory rather than clinical endpoints. Despite usage of more than 1 million units in Europe, there have been no full reports of large, randomized trials of MBFFP using relevant endpoints such as blood loss or exposure to other blood components. Early studies described successful use of MBFFP in either single or small groups of patients with

deficiencies of FV or FXI, TTP, and exchange transfusion in neonates.[41,42] One study of 71 patients compared MBFFP with S/D-treated FFP in cardiac surgery and showed better replacement of protein S and alpha$_2$-antiplasmin with MBFFP but no difference in blood loss.[20] However, one hospital in Spain has reported that after a total switch to MBFFP, FFP demand rose by 56 percent, with a two- to three-fold increase in demand for cryoprecipitate, which was not MB treated.[43] The authors suggest that the increase in demand, particularly for cryoprecipitate, may have been required to offset the reduced fibrinogen level in the component. Indeed, after orthopedic surgery, transfusion of MBFFP has been associated with increased reptilase clotting times and ratio of immunologic to functional measured fibrinogen,[44] suggesting that MB may interfere with fibrin polymerization in vivo. However, the data from the Spanish study need to be interpreted with care. In the period studied, which spanned introduction of MBFFP, 2967 patients received no fewer than 27,434 units of plasma, but only 24,607 units of RBCs, with 26 percent of admissions receiving FFP only. The very high FFP to RBC ratio (1.11) contrasts sharply with the recent corresponding figure for the UK Transfusion Services (0.14).[45] This suggests very different prescribing practices for FFP between Spain and the UK, including routine use of FFP in all cardiac surgery procedures in Spain.[43] Nevertheless, their study emphasizes the importance of monitoring clinical demand after any change to MBFFP, to see whether the in vitro effects truly result in a requirement for larger doses.

No specific data are available from studies in neonates, but no specific problems have been found. The only report of MB toxicity in a neonate was a case of severe bullus formation and desquamation was reported in a baby who received phototherapy for hyperbilirubinemia after administration of 10 mL of 1 percent MB to the mother to investigate possible rupture of amniotic membranes.[46] Although neonatal blood levels of MB were not reported, the skin of the baby was visibly stained blue, suggesting blood and tissue levels many times higher than would be achieved after infusion of MBFFP. No problems with MBFFP-treated infants requiring phototherapy have been reported in Europe, and glucose 6 phosphate dehydrogenase deficiency is not a contra-indication to its use (Walker W, written communication, 2002). Similarly, digital capillary measurement of oxygen saturation by colorimetric means is not affected by infusion of MBFFP.

Limited data are available on the use of MBFFP for plasma-exchange procedures for TTP.[47] Although levels of vWF cleaving enzyme in MBFFP are normal,[30] one study of two small cohorts of patients (13 treated with FFP and 7 with MBFFP) reported an increase in the number of plasma-exchange procedures and days in hospital in the MBFFP group.[48] This is of concern, although the small patient numbers make it difficult to draw conclusions; clearly, larger studies are required to establish the role of MBFFP in TTP.

FFP SAFETY: WHERE ARE WE GOING?

Five years ago, an editorial in this journal accompanied the availability in the USA of pooled S/D FFP.[1] Despite the impact of the previous HIV and HCV transmissions on transfusion services in many countries, S/D FFP did not subsequently become a standard of care in the USA, although it has become so in Norway, Belgium and Portugal. Other European countries have chosen quarantining of FFP with donor re-test as their method of minimizing virus risk from FFP. This avoids potential toxicity or loss of activity, but provides no protection against new agents such as West Nile virus. In virus reduction terms, the MB system appears to have acceptable efficacy, and has the advantage of being a single unit system, so that potentially increased risks from new agents unaffected by the system, such as prions, are minimized. The major disadvantage is loss of coagulation factors, such as fibrinogen. The as yet unlicenced single unit psoralen S59 pathogen reduction system for FFP appears to result in much better preservation of fibrinogen, with only 3-13 percent reduction.[49] However, toxicity will be a concern for any pathogen reduction system which interacts with nucleic acids, especially if administered to very young recipients.

In the UK, provision of MBFFP is linked to the most recent Department of Health precautionary decision to minimize the unknown risk of variant CJD from UK blood components. In August 2002, UK Transfusion Services were instructed to seek supplies of US plasma for FFP production for children born after January 1, 1996, a date from which the UK food supply has been considered safe from bovine spongiform encephalopathy. This imported FFP will be subjected to MB treatment, and, in preparation, UK Transfusion Services have already introduced MBFFP for this age group. No immediate problems with side effects or loss of efficacy have been reported, although the number of children treated is still small. Hospitals also have access to S/D FFP from commercial sources.

But to take an overview of FFP safety, 5 years' hemovigilance data in the UK reveal that virus transmission is a much smaller risk than that of TRALI. From 1996 to 2001, there were 15 TRALI cases in which FFP was clearly implicated, and another 4 where FFP was among a range of components transfused. In the same time period, there was not a single proven virus transmission from FFP.[45] Single-unit pathogen-reduction systems by themselves contribute nothing to TRALI prevention, which may be helped by selection of male donors for FFP[50] and/or screening of parous females for WBC antibodies. Interestingly, the pooling of several hundred donations required in the S/D FFP process may provide benefit against TRALI

by diluting out those with high-titer WBC antibodies. The National Blood Service in England has begun a formal option appraisal of TRALI-prevention strategies, beginning with plasma-rich components. The relative cost effectiveness and long-term role of pathogen reduction of FFP in an overall blood safety strategy remain to be elucidated.

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The effect of methylene blue photoinactivation and methylene blue removal on the quality of fresh-frozen plasma

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BACKGROUND: The effects of using fresh or frozen-thawed plasma, WBC reduction of plasma before freezing, and the use of two different methylene blue (MB) removal filters on the quality of MB-treated plasma were compared.

STUDY DESIGN AND METHODS: In a paired study (n = 11/arm) plasma was frozen within 8 hours of collection, thawed, MB photoinactivated, and then filtered using one of two MB removal filters. Fresh plasma (n = 16) and plasma WBC reduced before freezing (n = 19) were MB inactivated.

RESULTS: Freeze-thawing resulted in loss of activity of FXII and VWF of 0.06 and 0.04 units per mL, respectively, but no significant loss of activity of factors II through XI or fibrinogen. Further loss of activity occurred after MB treatment: FII (0.07 IU/mL), FV (0.11 U/mL), FVII (0.08 IU/mL), FVIII (0.28 IU/mL), F IX (0.12 IU/mL), FX (0.16 IU/mL), FXI (0.28 U/mL), FXII (0.15 U/mL), VWF antigen (0.05 IU/mL), VWF activity (0.06 U/mL), and fibrinogen (0.79 g/L). Losses due to this step were significantly (5-10%) lower in fresh plasma compared to frozen-thawed plasma. Neither MB removal filter resulted in significant loss of activity of any factor studied.

CONCLUSION: MB removal, by either of the available filters, has little impact on the coagulation factor content of plasma, but freezing of plasma before MB treatment results in a small additional loss.

Due to stringent donor selection and testing procedures, fresh-frozen plasma (FFP) in the developed world offers a high degree of viral safety. For example, the risk of an infectious FFP donation entering the blood supply in England is estimated to be 1 in 10 million for HIV, 1 in 50 million for HCV, and 1 in 1.2 million for HBV (Eglin R, written communication, 2002). Nevertheless, viral transmission from blood components continues to occur, with 16 cases reported in the UK in the last 6 years.[1] There is, therefore, considerable research activity in pathogen inactivation of single-unit components because methods suitable for single components offer reassurance that no increased infectious risks are added due to pooling. For FFP only, one licensed single-unit system is currently available (methylene blue photoinactivation). It is desirable that there is as much flexibility as possible in the handling conditions for plasma before inactivation, to enable production of FFP from collection centers distant from the processing site. This is particularly relevant because the UK Departments of Health have recently recommended that FFP is imported from North America for neonates and children born after 1995 (after the introduction of relevant food bans to limit BSE transmission) as a precautionary measure against vCJD transmission. Previous studies have

ABBREVIATIONS: APC = allophycocyanin-conjugated; APTT = activated partial thromboplastin time; FFP = fresh-frozen plasma; MB = methylene blue; PMN, = neutrophil; PRP = platelet-rich plasma; PT = prothrombin time; VWF:CB = VWF collagen-binding activity.

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demonstrated vCJD infectivity in plasma of rodents infected with prion diseases,[2,3] and a recent report describes interim results from a study that demonstrate transmissions of bovine spongiform encephalopathy (BSE) and scrapie between sheep by whole-blood transfusion.[4] Because background levels of virus marker positivity in the North American population are significantly higher than in the UK, it has been deemed sensible to subject imported plasma to a pathogen-inactivation step.

The methylene blue (MB) photoinactivation process for viral inactivation of human plasma has been well described[5] as has its effect on the loss of coagulation factor activity of plasma.[5-9] The original Springe MB process, also used by Grifols in Spain, described by Lambrecht,[5] used freeze-thawing of plasma before MB inactivation to expose intracellular viruses to the action of MB. However, recently, blood collection packs that integrate WBC reduction and MB addition before inactivation of plasma (Baxter Pathinact, Baxter Healthcare, Compton Newbury, Berkshire, UK, and Maco Pharma Theraflex, Middlesex, UK) remove the need to freeze-thaw plasma.[10-12] There are also differences between the systems in how MB is added to plasma. With two of the systems (Springe and Baxter), a variable dose of MB solution is added to achieve a standard final concentration of 1 μM MB. The other system (Maco Pharma Theraflex) incorporates an 85-µg pellet of MB hydrochloride per plasma unit, therefore the concentration can vary slightly (0.84-1.13 μM) depending upon the plasma volume (recommended range, 235-315 mL).

For MB photoinactivation of plasma to be centralized, but plasma from remote sites used as a start material, it is essential to be able to freeze and thaw plasma before treatment. Although we have previously evaluated the use of the two systems (Baxter and Maco Pharma) using fresh plasma,[10-12] we have not evaluated freeze-thawing of plasma before MB treatment using such systems. Although it is known that freeze-thawing itself has minimal effect on the coagulation factor activity of plasma,[7] there are no comparative data available on whether the loss of coagulation factor activity due to the MB inactivation step is affected by prior freeze-thawing. Furthermore, in the UK, there was concern that freeze-thawing non-WBC-reduced plasma could potentially increase exposure to vCJD due to fragmentation of platelets and WBCs, which are known to contain normal cellular prion protein (Prp^c)[13] and might therefore host the infective abnormal prion protein Prp^{sc}. We therefore assessed the effect of removing these cells by an additional WBC reduction step before freezing on the quality of MB plasma.

Following concerns over possible side effects of residual MB in plasma, a further recent development is the ability to remove MB by filtration before final component storage. Evaluations of two removal filters (Pall MB1, Pall Biomedical, Portsmouth, UK, and HemaSure LeukoVir, Marlborough, MA) MB have been previously reported,[14,15] but there are no data available on the use of a new MB removal filter (Maco Pharma Blueflex). The aim of this study was therefore to evaluate the combined effect of WBC reduction before freezing, freeze-thawing, MB photoinactivation, and MB removal using two different filters, on coagulation factor activity and activation markers in FFP. We also examined the effect of freeze-thawing and subsequent filtration of non-WBC-reduced plasma on its cellular constituents, to provide assurance that the process is not likely to increase the risk of vCJD transmission after transfusion to patients.

MATERIALS AND METHODS

Blood collection and processing

The experimental design is shown in Fig. 1. Twenty-four units of whole blood (group A, n = 12; group O, n = 12) were collected into "Top and Bottom" configuration blood packs (Pall Medsep 789-94 U, Pall Biomedical). Blood was then centrifuged (Heraeus Cryofuge 6000, Kleinostheim, Germany) at 3300 rpm for 12 minutes at 22°C and processed to RBCs and plasma (Compomat G4 system, Fresenius-Hemocare NPBI, Abingdon, UK). In Experiment A, plasmas were pooled in groups of two units of identical ABO group into 600-mL transfer packs (Baxter FGR2089, Baxter Healthcare). The pools were mixed thoroughly and divided equally between two 300-mL transfer packs (Fresenius Hemocare P4164, Fresenius Hemocare). All units of plasma were frozen within 8 hours of collection in a freezer (Thermogenesis MP1101, Cheshire, UK) to −45°C within 45 minutes and stored frozen at −40°C for 4 days to 4 weeks. The units were then thawed at 37°C and immediately WBC reduced (PLAS 4, Maco Pharma, Middlesex, UK) and MB photoinactivated (Maco Pharma Maco-Tronic system) as previously described.[11] For each pair of plasmas, MB was removed (either Pall MB1 or Maco Pharma Blueflex) according to the manufacturers' instructions. Plasmas were refrozen in a freezer (Thermogenesis MP1101) and stored at −40°C. In addition, 19 units of plasma were WBC reduced before freezing using one of two filters (RZ2000, Baxter Healthcare, or LPS1, Pall Biomedical), MB inactivated, and then MB removed (MB1 filter, Experiment B). These filters were selected because they are known to have minimal effect on coagulation factors in plasma.[16] A further 16 units of plasma (group O, n = 8; group A, n = 8) were MB inactivated without the freeze-thaw step and MB removed (Blueflex filter, Experiment C).

We took 15-mL samples by sterile connection of a sample pouch at four time points: 1) before freezing, 2) after thawing and before WBC reduction and MB addition, 3) after MB treatment before MB removal, and 4) after MB removal. Samples were frozen at −80°C for coagulation assays, and two aliquots were frozen in EDTA for C3a des arg assays.

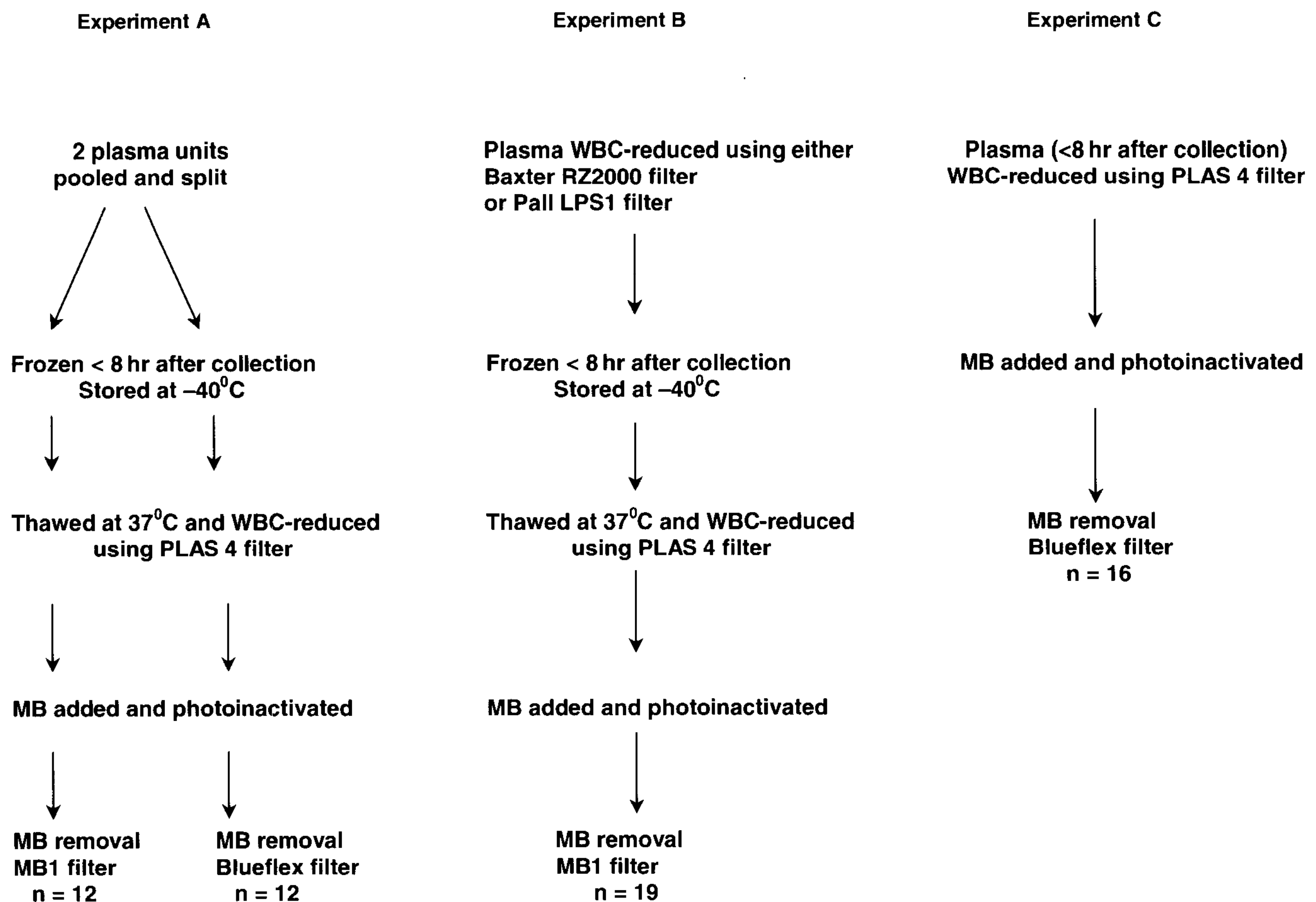

Fig. 1. Study design.

Plasma factors

All coagulation assays were performed using commercially available analyzers (Sysmex CA 1500 analyzer, Sysmex, Milton Keynes, UK; Coagamate X2 analyzer, Organon-Teknika, Cambridge, UK; or Amelung KC 4 A micro analyzer, Sigma Diagnostics, Poole, Dorset, UK). FII, FV, FVII, and FX were assayed by one-stage prothrombin time (PT)-based assays and F IX and FXII using a one-stage activated partial thromboplastin time (APTT)-based assay, using deficient plasma (Dade Behring, Marburg, Germany). The PT and APTT were expressed as a ratio to the geometric mean result of 20 normal citrated plasmas. These types of samples were chosen as "normal" plasma to provide a standard reference point between studies. VWF antigen was measured by latex agglutination (STA Liatest Kit, Diagnostica Stago, Asnieres, France). FVIII and FXI were assayed using one-stage clotting assays with deficient plasma (Diagnostics Scotland, Edinburgh, Scotland; and Sigma-Aldrich Company, Poole, Dorset, UK, respectively). Fibrinogen was measured using a Clauss assay with Fibriquick reagents (Organon-Technika, Cambridge, UK). FVIII assays were standardized using the British plasma standard (NIBSC, South Mimms, UK). All other assays were standardized using Coagulation Reference plasma 100 percent (Technoclone, Dorking, UK). A control plasma of known potency was assayed on each occasion for all coagulation assays.

Commercially available ELISA kits were used to determine levels of prothrombin fragment 1 + 2 (Dade-Behring), FXIIa (Axis-Shield, Dundee, Scotland), and VWF collagen-binding activity (VWF:CB, Immuno, Vienna, Austria). C3a des arg was assayed by radioimmunoassay (Amersham Pharmacia Biotech, Buckshire, UK). VWF cleaving protease activity was measured as previously described[17] and results expressed as a ratio to that of a pooled normal citrated plasma.

Effect of freeze-thawing plasma and filtration steps on cellular content of plasma

Double WBC-reduced plasma (LPS1 filter, Pall Biomedical) was spiked with WBCs (<1-200 × 10^6/L) with or without platelets (<1-100 × 10^9/L), both of which were prepared from fresh whole blood by density gradient centrifugation,

to represent levels of cellular contamination that may be expected to occur in non-WBC-reduced plasma. Plasma was then blast-frozen, thawed, WBC-reduced by sterile connection with the (PLAS 4) filter, MB added, and MB removed using a filter (the Pall MB1 filter). Samples were taken at four stages: before freezing, after thawing, after PLAS4 LD filter, and after MB removal. Samples were analyzed for platelet count by a hematology analyzer (Sysmex SE9000, Sysmex) and WBC count by flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK) using LeucoCount reagents (Becton Dickinson). Release of the neutrophil primary granule marker elastase was measured by ELISA of α_1-proteinase inhibitor: neutrophil (PMN) elastase complexes (Pathway Diagnostics, UK) and release of LDH by enyzymatic assay in supernatant plasma (Vitros DT60II, Axis-Shield, Dundee, Scotland). RBC microparticles were measured by flow cytometry (FACSCalibur) as previously described using antibodies to glycophorin A.[18]

Analysis of platelet microparticles (PMP) was determined as follows: Plasma (5 µL) was incubated for 20 minutes at room temperature with 5 µL allophycocyanin-conjugated anti-CD61 (APC-CD61, Caltag-Medsystems, Towcester, UK), 5 µL rhodophycoerythrin-conjugated anti-CD42b (Caltag-Medsystems), 10 µL FITC annexin V (FITC-AV, Caltag-Medsystems), 5 µL 10 × HBSS (Sigma, Poole, UK) and made up to 50 µL with HEPES-calcium buffer (2.8 m*M* $CaCl_2$, 20 m*M* HEPES). Samples were resuspended in 0.45 mL of 1 × HBSS and transferred to a tube containing a known amount of beads (Trucount, Becton Dickinson), and analyzed using a flow cytometer (FacsCalibur, Becton-Dickinson). Platelet microparticles were defined using forward scatter as events falling in a region, which includes less than 2 percent of platelets in plasma (PRP) from 20 normal donors, and of less than 1 µm as determined by APC fluorescent beads (Spherotec, Libertyville, IL). Platelet-derived events were defined by fluorescence due to APC-CD61 binding above that of an isotype-matched control. Annexin-V-positive events were defined as events binding FITC-AV above a control containing 5 m*M* Na_3 EDTA. In normal subjects ($n = 20$), less than 1 percent of unstimulated platelets bind FITC-AV. To control assay variability, a negative control of unstimulated PRP and a positive control (PRP incubated with 10 µ*M* A23187 [Calibochem-Novabiochem, San Diego, CA] for 15 min) were included for platelet microparticle assays.

Effect of MB on assays

To assess the effect of the presence of MB itself on coagulation factor assays, a MB pellet (from the Maco Pharma pack) was dissolved in each of six units of plasma. Samples were collected before and after addition of MB, and once MB had been added, the plasma was not photoilluminated and was protected from light at all times. All parameters were performed as for the main study with the exception that all coagulation assays were performed using a particular analyzer (Sysmex CA 1500 analyzer) and C3a des arg levels were performed by ELISA (Quidel, San Diego, CA).

Statistical analysis

Since the distribution of some data were non-Gaussian with positive skew, nonparametric tests were applied. The Wilcoxon rank sum test was used for paired data and the Mann-Whitney U-test for unpaired data. A p value less than 0.05 was considered significant. All results are given as median with range.

RESULTS

Plasma processing

From the 24 paired units (Experiment A), one unit of plasma fractured on thawing, therefore data on 11 paired units of plasma are presented, all of which were within the required volume range before MB inactivation. Due to sampling, 2 out of 16 fresh plasma units (Experiment C) were slightly below the lower range limit (233 and 234 mL). Filtration time for one WBC-reduction filter (PLAS 4) was 8 to 15 minutes, with a loss of 20 mL of plasma. Filtration times for two other removal filters (Pall MB1 and Maco Blueflex) were 2 to 5 minutes and 5 to 11 minutes, respectively, with a loss of 10 mL of plasma for each.

Effect of freeze-thawing and MB on loss of coagulation factors

The change in coagulation activity due to freeze-thawing and the MB process is shown in Table 1. There was a significant loss of FXII (2%) and VWF:CB (9%), and a small increase in levels of FVII (1%) and FXI (4%) due to freeze-thawing. This was associated with an increase in both PT ratio (1.06 [0.97-1.12] vs. 1.05 [0.95-1.11], $p < 0.0001$) and APTT ratio (1.04 [0.91-1.20] vs. 1.02 [0.89-1.18], $p < 0.0001$). The degree of loss of coagulation activity due to the MB process varied between factors, the highest losses occurring with FVIII (29%), fibrinogen (28%), and FXI (25%), therefore the evaluation of plasma treated fresh was mainly restricted to these factors. For FV, FVIII, FXI, and fibrinogen, the loss of activity due to the MB process was approximately 8-percent higher in the frozen-thawed plasma units compared with fresh plasma (Table 1). In addition, the increase due to this step in both PT ratio (0.09 [0.04-0.16] fresh vs. 0.14 [0.08-0.28] frozen-thawed, $p < 0.0001$) and APTT ratio (0.11 [0.05-0.15] fresh vs. 0.16 [0.11-0.26] frozen-thawed, $p < 0.0001$) was also higher. However, there was no significant difference in changes in levels of FVII, FXIIa, and C3a due to MB treatment between units which were treated fresh or after freeze-

TABLE 1. Percentage change in coagulation factor activity due to freeze-thawing and MB treatment of plasma

Factor	Due to freeze-thawing median (range)	Due to MB treatment + WBC reduction* (freeze-thawed plasma) median (range)	Due to MB treatment + WBC reduction (fresh plasma) median (range)
Number	22	22	16
Fibrinogen (g/L)	−10 (−27 to 14)	−28 (−51 to −20)	−21 (−38 to −7)†
FII (IU/mL)	0 (−4 to 4)	−8 (−11 to −2)	NA
FV (U/mL)	0 (−5 to 4)	−13 (−20 to 4)	−5 (−11 to 4)†
FVII (IU/mL)	1 (−1 to 4)‖	−7 (−10 to −1)	−4 (−9 to −1)
FVIII (IU/mL)	−3 (−20 to 9)	−29 (−42 to −9)	−24 (−37 to −11)†
F IX (IU/mL)	1 (−3 to 4)§	−13 (−20 to −11)	NA
FX (IU/mL)	0 (−2 to 3)	−15 (−22 to −10)	NA
FXI (U/mL)	4 (−19 to 11)‖	−25 (−35 to −7)	−15 (−23 to −6)†
FXII (U/mL)	−2 (−6 to 1)‖§	−18 (−31 to −14)	NA
VWF:Ag (IU/mL)	−1 (−4 to 3)§	−6 (−11 to −3)	NA
VWF:CB (U/mL)	−9 (−17 to 5)‖§	−8 (−16 to 3)	NA
FXIIa (ng/mL)	0 (−25 to 43)	−20 (−43 to 33)	−14 (−43 to 0)
Prothrombin *F*1 + 2 (n*M*)	−19 (−55 to 32)¶	91 (36 to 160)	27 (−12 to 180)‡
C3a des arg (ng/mL)	0 (−41 to 65)	−10 (−45 to 155)	16 (−27 to 295)

* WBC reduction was performed with an integral PLAS 4 WBC-reduction filter in the Maco Pharma MB pack configuration.
† $p < 0.01$ refers to significance from the Mann-Whitney U-test between fresh and frozen plasma.
‡ $p < 0.05$.
§ n = 11.
¶ $p < 0.01$ refers to significance from the Wilcoxon rank sum test between plasma before freezing and after thawing.
‖ $p < 0.05$.

thaw. The increase in prothrombin *F*1 + 2 levels due to MB treatment was higher in frozen-thawed units compared to fresh.

When the influence of MB on the assays was studied (in the absence of photoinactivation), there was no significant difference between before or after the addition of MB for any parameters, apart from FXIIa, which was significantly lower after MB addition (1.59 [0.76-2.13] ng/mL before, 0.72 [0.56-1.05] ng/mL after, $p < 0.05$ before vs. after).

Effect of MB removal filters on coagulation activity

To assess the difference between the two MB removal filters, pairs of units were pooled and half of each pool MB-treated and processed through each of the removal filters in parallel. Due to logistical problems, it was not possible to process and assay these two sets simultaneously. A small difference was apparent in levels of some coagulation factors between the two arms of the study, probably due to small differences in processing and storage. However, the percentage change in activity due to freeze-thawing and MB treatment was the same for each arm of the study (data not shown), and therefore these data were combined. To evaluate the effect of MB removal filters, a comparison of pre- and postcoagulation activity for each arm of the study was examined (Table 2). There was no apparent decrease in any parameter studied with either removal filter, apart from a reduction in levels of C3a using one of the filters (Pall MB1). There was a slight increase in levels of fibrinogen, FII, FV, FVII, and FX, using the Pall filter. There was an apparent increase in levels of FXIIa after filtration with both removal filters, which was probably due to the influence of MB on the assay and was comparable between filters. There was an extremely small variation in the PT and APTT ratios with both filters (Table 2).

We compared the final levels of coagulation activity in frozen-thawed MB-treated plasma to a reference range based on 66 samples of WBC-reduced plasma that had not been MB treated. Because there was no loss of activity with either MB removal filter, both sets of data were pooled. The reference data was not collected as part of this study but from previous studies carried out by the National Blood Service over the past 4 years. The methodology used in these studies[16] for either plasma processing or assay did not differ significantly from the current study. Over 90 percent of MB units were within our reference range for all coagulation factors, apart from prothrombin *F*1 + 2 and PT ratio, the MB-treated plasma having 23 percent and 50 percent of values above the range, respectively (Table 3). The range of PT ratios observed in reference and MB-treated plasma is shown in Fig. 2.

We did not evaluate MB removal by the filters used in this study, but previous studies have shown that the MB1 filter removes 81 to 95 percent of MB[14] and the Blueflex filter removes more than 95 percent MB.[19]

Effect of WBC reduction before freezing

WBC reduction of plasma before freezing appeared to have little influence on final levels of FVIII (0.67 [0.44-1.23] WBC reduced vs. 0.62 [0.48-0.86 IU/mL] non-WBC reduced) or fibrinogen (1.88 [1.45-3.24] WBC reduced vs.

TABLE 2. Effect of MB-removal filters on plasma coagulation activity using frozen-thawed MB photoinactivated plasma

Factor	MB1 filter median (range)		Blueflex filter median (range)	
	Before MB removal	After MB removal	Before MB removal	After MB removal
Number	11		11	
PT (ratio)	1.17 (1.11-1.34)	1.07 (1.03-1.24)*	1.23 (1.13-1.33)	1.22 (1.13-1.32)*
APTT (ratio)	1.11 (1.05-1.28)	1.13 (1.06-1.30)†	1.25 (1.17-1.39)	1.27 (1.19-1.43)*
Fibrinogen (g/L)	1.88 (1.30-2.13)	1.93 (1.28-2.27)*	2.04 (1.37-2.13)	1.96 (1.28-2.32)
FII (IU/mL)	0.96 (0.77-1.04)	1.00 (0.78-1.04)†	0.95 (0.77-1.04)	0.97 (0.77-1.04)
FV (U/mL)	0.78 (0.55-0.86)	0.80 (0.56-0.91)*	0.76 (0.58-0.97)	0.76 (0.58-0.88)
FVII (IU/mL)	0.99 (0.79-1.43)	1.02 (0.83-1.55)*	1.01 (0.77-1.40)	1.00 (0.78-1.45)
FVIII (IU/mL)	0.63 (0.47-0.89)	0.62 (0.48-0.86)	0.61 (0.48-0.79)	0.61 (0.46-0.76)
F IX (IU/mL)	0.96 (0.78-1.06)	0.96 (0.83-1.11)		
FX (IU/mL)	0.95 (0.70-1.12)	1.02 (0.75-1.15)*	0.95 (0.72-1.09)	0.96 (0.73-1.07)
FXI (U/mL)	0.77 (0.57-0.99)	0.77 (0.59-0.99)	0.75 (0.55-0.99)	0.71 (0.58-0.78)
FXII (U/mL)	0.88 (0.41-1.07)	0.88 (0.40-1.09)		
VWF:Ag (IU/mL)	0.99 (0.74-1.19)	0.98 (0.75-1.18)	0.92 (0.70-1.09)	0.93 (0.70-1.09)
VWF:CB (U/mL)	0.60 (0.47-0.68)	0.73 (0.42-0.85)	0.70 (0.59-0.87)	0.74 (0.56-0.82)
FXIIa (ng/mL)	1.50 (0.40-2.50)	1.80 (0.70-2.90)*	1.25 (0.75-2.00)	1.50 (1.00-2.25)*
Prothrombin *F*1 + 2 (nM)	0.96 (0.61-1.75)	0.88 (0.76-1.83)	0.74 (0.58-1.19)	0.75 (0.52-1.25)
C3a des arg (ng/mL)	438 (207-1928)	304 (107-431)*	337 (225-1909)	302 (226-1713)

* $p < 0.01$ refers to statistical significance from the Wilcoxon rank sum test between plasma before and after MB removal.
† $p < 0.05$.

TABLE 3. Final levels of coagulation factors and activation markers in freeze-thawed MB-photoinactivated plasma after MB removal

Factor	Final level in plasma*	Reference range†	Units in range (%)
Number	22	66	
PT (ratio)	1.16 (1.03-1.32)	1.05 (0.95-1.16)‡	50
APTT (ratio)	1.24 (1.06-1.43)	1.09 (0.86-1.36)‡	95
Fibrinogen (g/L)	1.95 (1.28-2.32)	1.10-4.30	100
FII (IU/mL)	0.98 (0.77-1.04)	0.70-1.20	100
FV (U/mL)	0.79 (0.56-0.91)	0.50-1.40	100
FVII (IU/mL)	1.01 (0.78-1.55)	0.60-1.40	100
FVIII (IU/mL)	0.62 (0.46-0.86)	0.40-1.60	100
F IX (IU/mL)	0.96 (0.83-1.11)§	0.60-1.40	100
FX (IU/mL)	1.01 (0.73-1.15)	0.70-1.30	100
FXI (U/mL)	0.75 (0.58-0.99)	0.60-1.30	91
FXII (U/mL)	0.88 (0.40-1.09)§	0.40-1.50	100
VWF:Ag (IU/mL)	0.96 (0.70-1.18)	0.60-1.65	100
VWF:CB (U/mL)	0.74 (0.42-0.85)	0.50-1.50	91
FXIIa (ng/mL)	1.50 (0.40-2.90)	0.50-5.00	100
Prothrombin *F*1 + 2 (n*M*)	0.85 (0.52-1.83)	0.20-1.10	77
C3a des arg (ng/mL)	303 (107-1713)	1117 (0-12,330)	100

* Data (n = 22) are represented by the median (range) from plasma MB-removed by MB1 filter (n = 11) and Blueflex filter (n = 11).
† Reference range of normal plasmas is defined as the mean ± 2 SD for normally distributed data and the geometric mean with 95-percent CI for skewed data based on WBC-reduced FFP. Percentage of units in range is defined as above the lower limit for coagulation factors and below the upper limit for PT, APTT, and activation markers.
‡ n = 100.
§ n = 11, using MB1 filter only.

1.70 [1.24-2.31 g/L] non-WBC reduced) in plasma subsequently MB treated and removed using the MB1 filter. VWF cleaving protease (VWF:CP) activity was measured in four MB-inactivated plasmas, which were WBC reduced before freezing and MB-removed using the MB1 removal filter. VWF:CP results ranged from 0.81 to 1.00 (normal range, 0.80-1.20 in citrated plasma). We have not assessed VWF:CP activity in plasma MB-depleted using the Blueflex filter. Quality-monitoring data from routinely processed units (n = 225), WBC-reduced before freezing, MB-treated, and MB-removed using the MB1 filters, showed a mean volume of 242 mL (SD = 19) and FVIII content of 0.79 IU per mL (SD = 0.25). These results comply with UK specifications for MB-treated FFP.[20]

Effect on cellular content of plasma

When platelets were spiked into plasma, there was no consistent difference between levels measured before and after freeze-thawing when measured by hematology analyzer, but levels were consistently lower after thawing when measured by flow cytometry (Table 4). When WBCs were spiked into plasma, there appeared to be a trend for lower WBC counts and higher levels of α_1-proteinase inhibitor: PMN elastase complexes in plasma after freezing (Table 4). However, in the absence of platelets, levels of LDH did not increase substantially. Freeze-thawing of plasma resulted in an increase in levels of platelet microparticles as well as microparticles characterized by the binding of purified annexin V (Table 4). Platelet microparticles were reduced to levels observed in fresh WBC-reduced plasma or below after WBC reduction of frozen plasma with the PLAS 4 filter. However, a significant proportion (~30%) of microparticles characterized by annexin V binding were not removed by the WBC-reduction or the MB1 removal step. These also appeared to be derived solely from plate-

lets because levels after WBC reduction in samples spiked with WBCs in the absence of platelets were not different from WBC-reduced plasma alone. However, even at an added platelet count of 30×10^9 per L (Sample D, the current UK specification), the number of annexin V-positive microparticles in frozen-thawed plasma subsequently filtered using the PLAS 4 filter (43×10^9/L) is similar to that seen in our current routine WBC-reduced non-MB-treated plasma product (mean residual platelet count of 3×10^9/L).

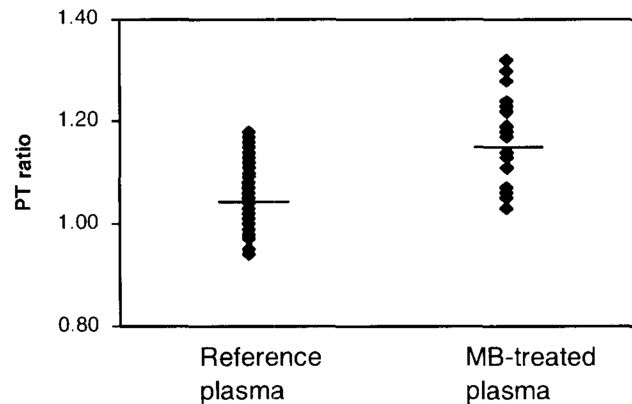

Fig. 2. PT ratio in MB-treated (n = 22) or reference plasma (n = 100). For MB-treated plasma, MB was removed by MB1 filter (n = 11) and Blueflex filter (n = 11). Reference plasma is historical data from WBC-reduced FFP. Horizontal bar represents the median value. The PT is expressed as a ratio to the geometric mean result of 20 normal citrated plasmas.

Before freezing, levels of RBC microparticles were 8 (5-12 × 10^9/L), increasing by 33 percent after freeze-thawing. This was not related to platelet or WBC content, and levels after WBC reduction were below the detection of the assay system used (data not shown).

DISCUSSION

MB treatment of plasma has been shown to inactivate 4 to 6 logs of transfusion-transmitted viruses, including HIV, HBV, parvovirus B19, and West Nile virus.[5,21,22] Original studies on MB inactivation were reported on plasma freeze-thawed before treatment.[5,7] Later, work on other systems (Baxter Pathinact and Maco Pharma Theraflex systems) was performed on fresh plasma.[10-12] However, there are no data available on the difference between using fresh or freeze-thawed plasma as a starting component for MB treatment. In our study, freeze-thawing of plasma resulted in a small loss of FXII and VWF:CB activity

TABLE 4. The effect of freeze-thawing, WBC reduction, and MB removal on cellular components of plasma

	Spike*									
	A	B	C	D	E	F	G	H	I	J
Platelets – HA† (10^9/L)										
Before freeze	<3	<3	10	31	108	<3	<3	<3	<3	<3
After thaw	<3	6	10	32	97	<3	<3	<3	<3	<3
Platelets – FC‡ (10^9/L)										
Before freeze	<0.5	5.9	10.8	32.0	125.1	<0.5	<0.5	<0.5	<0.5	<0.5
After thaw	<0.5	4.8	9.0	24.6	92.2	<0.5	<0.5	<0.5	<0.5	<0.5
WBCs (10^6/L)										
Before freeze	<1	8.9	29.6	90.7	233.8	<1	1.8	14.1	50.6	92.9
After thaw	<1	9.1	28.1	94.0	210.9	<1	1.6	13.2	43.4	89.5
PMN elastase (µg/L)										
Before freeze	46.4	39.7	37.5	46.3	57.0	20.6	23.7	24.0	25.3	25.7
After thaw	37.5	35.8	42.2	53.5	79.8	22.8	23.1	30.4	43.2	58.8
After WBC reduction	34.5	35.3	40.8	57.4	77.4	21.1	20.5	27.0	41.2	53.5
After MB1 filter	35.1	37.0	45.1	58.2	71.9	18.1	21.3	24.8	37.9	57.9
Platelet microparticles (10^9/L)										
Before freeze	0.2	0.2	0.4	0.5	1.0	0.3	0.5	0.4	0.3	0.9
After thaw	0.1	0.5	0.7	2.6	10.1	0.1	0.1	0.0	0.3	0.0
After WBC reduction	0.0	0	0	0.1	0.2	0	0	0	0.1	0
After MB1 filter	0.1	0.0	0.1	0.1	0.2	0.1	0.0	0.1	0.0	0.3
Annexin V +ve microparticles (10^9/L)										
Before freeze	6	7	8	10	13	4	4	4	5	4
After thaw	12	31	54	138	574	9	9	9	13	13
After WBC reduction	3	10	20	46	198	5	2	3	5	5
After MB1 filter	3	8	15	43	196	4	4	4	4	8
LDH (U/mL)										
Before freeze	393	400	423	476	732	390	381	388	376	382
After thaw	384	404	434	523	796	388	386	385	393	395

* WBC-reduced plasma units were spiked with WBCs alone (Samples G-J) or WBCs and platelets (Samples B-E) to the concentrations shown in the before freeze rows. Samples A and F were not spiked. Plasma was frozen-thawed, WBC reduced using the PLAS 4 filer; MB added and MB removed using the MB1 filter. Results are from a single experiment. Platelets and WBCs were not detectable following WBC reduction.
† FC-flow cytometry.
‡ HA-haematology analyser.

as well as a minor prolongation of PT and APTT. Interestingly, there was an apparent increase in activity of FVII and FXI on freeze-thawing, presumably reflecting small changes in the activation status of these factors. However, other factors studied remained unchanged. This agrees with the work of Zeiler et al.,[7] who showed that the loss of coagulation activity in MB-treated plasma was mainly attributable to the MB photoinactivation step rather than freeze-thawing of plasma. The loss of activity observed in frozen-thawed units due to MB photoinactivation in our study was similar to that previously reported.[5,7] For the variables we studied, with the exception of FVII, loss of coagulation factor activity due to the MB-inactivation step (including the WBC reduction filter) was 8-percent higher when frozen-thawed plasma units were used rather than fresh. In addition, the increase in prothrombin *F*1 + 2 levels after MB inactivation and WBC reduction was higher in units frozen-thawed compared with fresh plasma, indicating a higher degree of thrombin generation. This was not associated with an increase in FXIIa. Because our MB process includes an integral WBC-reduction step, we cannot determine whether the differences seen between fresh and frozen-thawed plasma are attributable to the WBC-reduction or MB process. The WBC-reduction filter used in the MB packs has previously been shown to have minimal effect on coagulation activity (unpublished data) using fresh plasma, but this could be different for frozen-thawed plasma.

We also sought to compare the effect of two different types of MB removal filters (Maco Pharma Blueflex or Pall MB1 filter) on plasma factor activity. Neither filter resulted in loss of any variable studied. Both MB removal filters resulted in a small increase in the APTT ratio, which might be attributable to contact activation of plasma with the filter. This is difficult to assess because although both filters increased FXIIa antigen, this assay is influenced by MB. However, levels of FXIIa antigen in the final MB-removed component were not higher than untreated plasma units. The filtration times for one filter (Maco Pharma Blueflex) were longer compared with the other (Pall MB1) (5-11 vs. 2-5 min, respectively), but the loss of plasma was equivalent for both filters. However, there did not appear to be any difference between the two filters in terms of activation of the contact system or thrombin generation as evidenced by the generation of FXIIa or prothrombin *F*1 + 2. Our results using the MB1 filter compare well with that previously published,[11] showing minimal loss of clotting factor activity. However, there was an apparent increase in levels of fibrinogen and PT-derived coagulation factors after filtration with the MB1 filter, which was associated with a small decrease in the PT ratio. We cannot explain these results because these assays do not appear to be influenced by the presence of MB, but they could possibly be a result of small increases in the activation state of coagulation factors. The changes in coagulation factors observed after filtration with either MB-removal filter appear to be clinically insignificant. Neither MB-removal filter resulted in generation of C3a des arg, a marker of complement activation. However, levels after filtration were reduced using the Pall MB1 filter. Whether this has any clinical benefit in terms of acute reactions is unclear.

As well as examining the loss of coagulation factors during MB photoinactivation, we compared residual levels in the final component with reference ranges based on previous studies of nontreated FFP in our laboratories. Despite the observed losses of coagulation factors due to MB treatment, final levels of all coagulation factors in frozen-thawed, MB-treated, and removed FFP were above the lower limit of the reference range in over 90 percent of units. However, over 50 percent of units were above the upper reference range for PT ratio. This presumably reflects the loss of fibrinogen and FII, FV, FVII, and FX because the PT is dependent upon these factors. However, the PT ratio of all units was less than 1.35. In addition, 23 percent of units had levels of prothrombin *F*1 + 2 higher than the upper limit of the reference range, reflecting the increase in prothrombin *F*1 + 2 seen due to the MB process. The clinical significance of increased *F*1 + 2 levels is unclear, but values higher than those observed in our study are seen in S/D-treated plasma.[23] We did not assess the effect of MB treatment or removal on plasma inhibitors of coagulation. However, MB treatment is reported to have minimal effect on levels of antithrombin, α_2-antiplasmin and protein C & S.[5,7,11] After MB removal (Pall MB1 filter), levels of antithrombin and protein C & S are within the normal range.[11]

We also evaluated the addition of a WBC-reduction step before freezing plasma, which did not appear to augment the loss of fibrinogen and FVIII activity. VWF:CP activity in plasma that was WBC reduced, MB inactivated, and removed using the MB1 filter was also within reference ranges established by other laboratories, suggesting that these processing steps do not have a major effect on VWF:CP using the techniques employed. These results are consistent with our previous findings on MB-treated fresh plasma[24] and others results on frozen-thawed plasma.[25] However, we cannot exclude small losses of activity given the relatively small number of samples used in this study.

In the UK, all blood components are WBC-reduced before storage. However, for logistical reasons we also wanted the flexibility to freeze non-WBC-reduced plasma intended for MB treatment, as a subsequent WBC-reduction step is integral to the Maco Pharma MB process. We were concerned that freeze-thawing may result in reduced cell removal by the WBC-reduction filter or cause fragmentation of cells with the potential for increasing the risk of transmission of vCJD. Most cellular prion protein in blood, used here as a surrogate marker for the potentially infective abnormal prion protein, is associated with

plasma (65%), with 26.5 percent, 1.8 percent, and 0.8 percent associated with platelets, RBCs, and polymorphonuclear cells, respectively.[13] Our results indicate that the majority of WBCs can be detected after thawing, and these are removed to undetectable levels after the WBC-reduction step of the MB process. However, the method we employed to detect WBCs predominantly measures WBC nuclei (unpublished data), and therefore provides little information on cellular integrity. The majority of WBCs in freeze-thawed plasma are detectable with PI without prior permeabilization,[26,27] suggesting that freeze-thawing alters WBC membrane integrity. The increase in levels of α_1-proteinase inhibitor: PMN elastase complexes after thawing of plasma spiked with WBCs shows that PMN degranulation is occurring, but the postthaw levels remain below 100 µg per L, which is not suggestive of large-scale PMN disintegration. Furthermore, in the absence of platelets, levels of LDH did not increase substantially after freezing, suggesting that WBCs do not disintegrate. We were unable to assess WBC fragments due to the insufficient sensitivity of available methods.

When platelets were spiked into plasma, there was an increase in platelet-derived microparticles after freeze-thawing of plasma, which probably explains the small decrease in platelet count detected by flow cytometry because these events would not be included in the platelet count. This fall was not detected by hematology analyzer, possibly because cell fragments can be detected as platelets by impedance-based methods. These fragments were reduced to or below the level in WBC-reduced fresh plasma after the WBC-reduction step of the MB process. However, we also analyzed cell microparticles based on the binding of purifed annexin V, which has a high affinity for anionic phospholipids. Freeze-thawing resulted in an increase in annexin-V-positive microparticles, which appear to be mainly derived from platelets and were only partially removed by WBC reduction. The increased detection of microparticles by this method compared with using an antibody against the platelet receptor CD61 is probably attributable to the greater number of molecules per platelet of anionic phospholipid (1×10^6) compared with CD61 ($4\text{-}8 \times 10^4$).[28,29] The presence of RBC and WBC microparticles (which will also bind annexin V) may also help to explain this difference, but this seems unlikely because in the absence of platelets the differences between methods were less pronounced. The number of annexin-V-positive microparticles found in non-WBC-reduced plasma that has been frozen-thawed and then filtered is not appreciably higher than would be found in plasma that we currently produce.

The effect of loss of coagulation factor activity due to MB treatment on the in vivo efficacy of the component is difficult to assess because there are no published randomized, controlled clinical trial data comparing MB to either standard FFP or S/D-treated FFP. However, 2.5 million units of MB FFP have been transfused internationally without obvious clinical sequelae.[30,31] In Spain, the switch from standard to MB-treated FFP has been associated with an increase in demand for FFP and cryoprecipitate,[32] which the authors attribute to loss of coagulation activity. However the increase in use (56%) appears to be disproportionate to the decrease in coagulation factors, suggesting that other factors, such as perception of a safer component, may have been influencing usage. It is also reported that the use of MB FFP is associated with a higher number of plasma exchanges compared with untreated FFP for the treatment of thrombotic thrombocytopenic purpura,[33] although we found no difference in the levels of VWF cleaving activity, the presumed therapeutic moiety in plasma treatment of thrombotic thrombocytopenic purpura, in MB FFP.[21] It is critical that transfusion services introducing pathogen inactivation of components monitor ongoing trends in usage as well as having a system for hazard reporting. At the time of writing, MB-treated and -removed FFP is routinely produced in England and Wales for transfusion to children and neonates born after 1995, with similar arrangements in other parts of the UK. However, in the near future, plasma to be pathogen inactivated for this patient group throughout the UK will be imported from volunteer donors in North America. Processes currently available for the pathogen inactivation of plasma all result in a decrease in coagulation factor activity. Improvements in the safety of blood need to be balanced against some likely reduction in the component potency. Single-unit systems for pathogen inactivation of plasma that have less effect on coagulation factor activity are clearly desirable.

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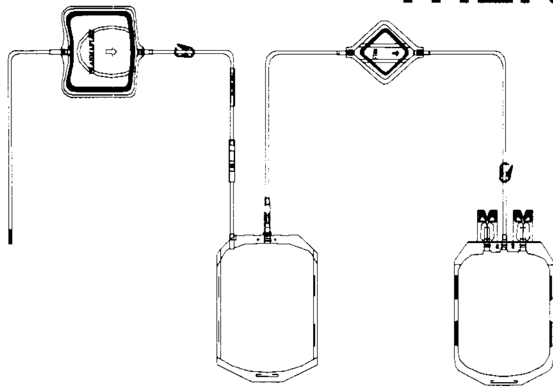
Pathogen Reduction System
THERAFLEX - MB PLASMA



Pathogen Reduction of Leucodepleted Plasma Methylene Blue Removal by Filtration

Ref. SDV0001XQ

Specifications

. **Filters** : Plasmaflex PLAS4, Blueflex filter

. **Bags** : 2 PVC

. **Included Items** : Methylene Blue pill (85µg)

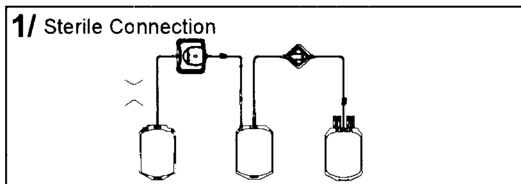
. **Label** : English, French, German, Dutch

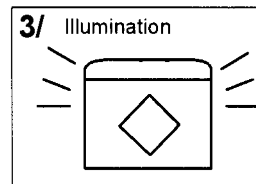
. **Sterilisation** : Steam

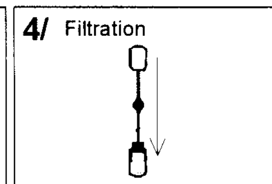
. **Shelf life** : 2 years

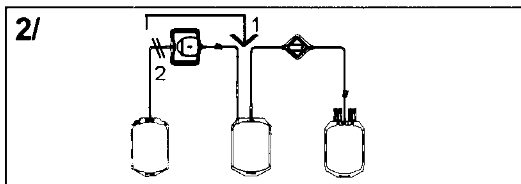
. **Packaging** : 2 packs/peelable sachet - 24 packs/box

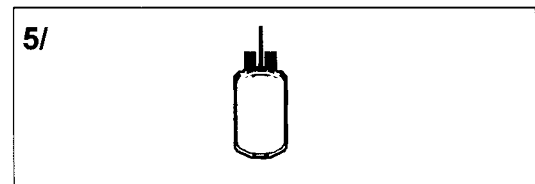
Use











SDV0001XQ_A_A 08/03/2006

■ Whole Blood ■ RCC □ PLASMA □ MB PLASMA □ PRP □ Platelets □ SAG-M PAGGSM □ NaCl ■ Buffy Coat ■ Serum □ Supernatant □ Cryoprecipitate

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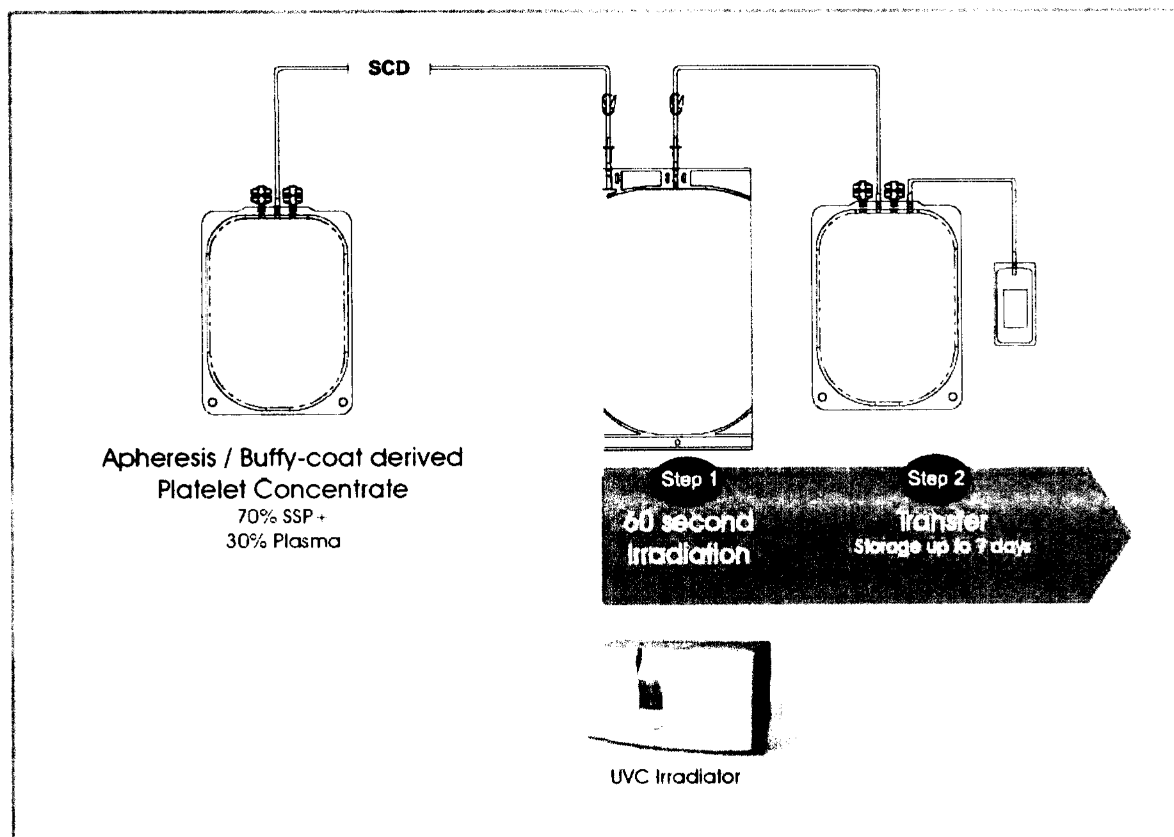
- Storage up to 7 days with SSP+

THERAFLEX UV Platelets: 2 step process



A NOVEL TECHNIQUE FOR PATHOGEN INACTIVATION AND ITS EFFECT ON THE QUALITY OF PLATELET CONCENTRATES: THERAFLEX UV PLATELETS

Walker W H[1], Tolksdorf F[1], Mohr H[2], Gravemann U[2], Müller T H[2],
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[2] German Red Cross Chapters of NSTOB, Springe, Germany

ISBT Regional Congress Asia, Hanoi, November 2007

INTRODUCTION

The use of a pathogen inactivation technology is an option to enhance the safety of platelet transfusions. Current procedures need chemicals to be added to the platelet concentrates (PCs). These compounds are of concern if they remain in the final product. Moreover, treatment may cause deterioration of the platelets. A novel procedure has been developed using only short-wave UV light (UVC, 254 nm) that effectively inactivates pathogens in plasma-reduced PCs. The equipment used consists of an irradiation device with a specific mechanism for agitation. Its capacity is one platelet unit (random donor or apheresis) per treatment cycle. Treatment parameters, e.g. UVC intensity, UVC dose, temperature and agitation, are microprocessor-controlled. Platelets are processed in the THERAFLEX twin-bag kit, which comprises a highly UV-transparent polyolefin acetate bag[1] for irradiation and a platelet storage container (Fig.1, 2). It was investigated to what extent platelet integrity and storage stability of the treated products were influenced by this new inactivation procedure.

MATERIALS & METHODS

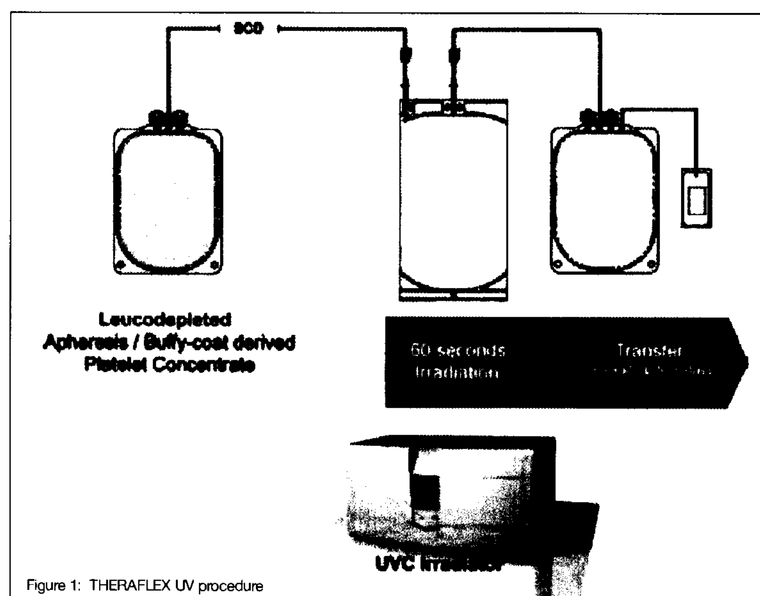

Figure 1: THERAFLEX UV procedure

Plasma-reduced PCs were treated with the THERAFLEX UV procedure (Fig.1) and stored until day 8 after blood donation (day 6 after treatment). The in vitro quality of UVC treated PCs was evaluated in comparison to non-treated control platelets. Control PCs were stored for the same time period. PCs in storage medium SSP+ (MacoPharma) were prepared from pools of 5 buffy coats. The average volume treated was 350 mL. Plasma concentration was approx. 30%. PCs were transferred into irradiation bags (Fig.2) and treated with UVC light at a dose of 0.4 J/cm² (approx. 60 sec) (Fig.3). They were strongly agitated during irradiation.

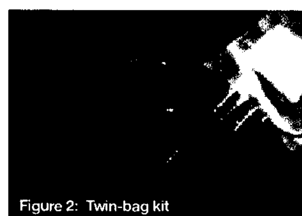
Figure 2: Twin-bag kit

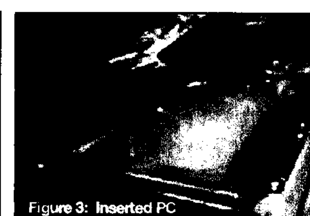
Figure 3: Inserted PC

RESULTS

In vitro characteristics were hardly influenced by the THERAFLEX treatment. HSR reactivity was only slightly reduced whereas collagen induced aggregation was moderately increased. Glucose consumption and lactate formation were found to be marginally higher in the treated PCs. Thus, pH slightly dropped but remained above 7.0 until day 8 after donation. The mean platelet loss due to UVC treatment was 4% (Tab.1).

Day 3	Plts [x10⁹/mL]	HSR [%]	pH	Spontaneous aggregation [%]	Collagen-ind. aggregation 100 µg/mL [%]	Glucose [mg/dL]	Lactate [mmol/L]
Control	10.2 ± 1.6	68 ± 4	7.13 ± 0.05	11 ± 3	94 ± 3	122 ± 8	7.5 ± 1.0
Treated	9.6 ± 1.3	64 ± 5	7.07 ± 0.07	14 ± 2	89 ± 5	118 ± 6	7.7 ± 0.8
Day 6							
Control	9.9 ± 1.0	66 ± 2	7.24 ± 0.13	12 ± 2	74 ± 9	86 ± 10	10.8 ± 1.0
Treated	9.5 ± 1.3	64 ± 8	7.09 ± 0.06	14 ± 3	81 ± 9	68 ± 10	12.8 ± 1.5
Day 8							
Control	9.4 ± 1.6	68 ± 1	7.29 ± 0.12	10 ± 1	62 ± 7	63 ± 9	12.5 ± 0.9
Treated	9.1 ± 1.3	61 ± 8	7.09 ± 0.05	16 ± 4	69 ± 7	41 ± 8	15.2 ± 1.0

Table 1: Platelet parameters of untreated an treated PCs on day 3, 6 and 8 after blood donation (mean +/- SD; n=4)

CONCLUSIONS

Plasma-reduced PCs were only slightly affected when treated with the THERAFLEX UV system for pathogen inactivation. In vitro parameters and storage stability were well preserved until day 8 after blood donation.

[1] Polyolefineacetate bags for pathogen inactivation and for storage of platelet concentrates, H Mohr, TH Müller, F Tolksdorf, WH Walker, Vox Sang 2004; 87 (Suppl. 3): 70

Funded by Forschungsgemeinschaft der Blutspendedienste des Deutschen Roten Kreuzes e.V. and Maco Pharma.

PATHOGEN REDUCTION IN PLATELET CONCENTRATES USING UVC LIGHT IN COMBINATION WITH STRONG AGITATION: EFFECT ON ACTIVATION MARKERS AND STORAGE STABILITY

Tolksdorf F[1], Walker W H[1], Mohr H[2], Gravemann U[2], Mueller T H [2]

[1] Maco Pharma, Langen, Germany, [2] Blood Centre of German Red Cross Chapters of NSTOB, Institute Springe, Germany

AABB Annual Meeting 2007, Anaheim, USA

INTRODUCTION

Pathogen Inactivation technologies are proactive measures to enhance the safety of platelet transfusions. Their use can be effective also against emerging unknown pathogens. Current procedures need chemicals like photosensitizers. We developed a procedure, which allows an efficient reduction of pathogens (e.g. bacteria and viruses) in plasma-reduced platelet concentrates (PCs) using short-wavelength UV light (UVC) in combination with strong agitation, i.e. there is no photoactive compound needed (THERAFLEX UV Platelets technology). The irradiation device developed is emitting UVC light at a wavelength of 254 nm. Moreover a mechanism for orbital agitation is installed. UVC irradiation is microprocessor-controlled. Relevant treatment parameters are monitored throughout the entire treatment thus allowing a fully documented and reproducible process. PCs are treated in a twin-bag kit, which comprises of a highly UV-permeable irradiation bag and a container for extended platelet storage. In the present study we investigated the influence of the THERAFLEX UV treatment on activation parameters and on the storage stability of PCs.

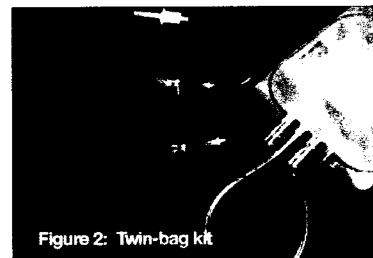

Figure 2: Twin-bag kit

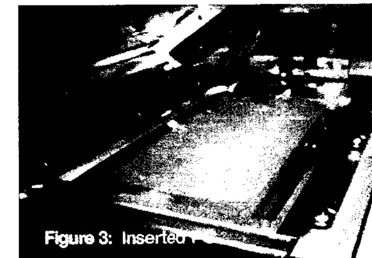

Figure 3: Inserted

MATERIALS & METHODS

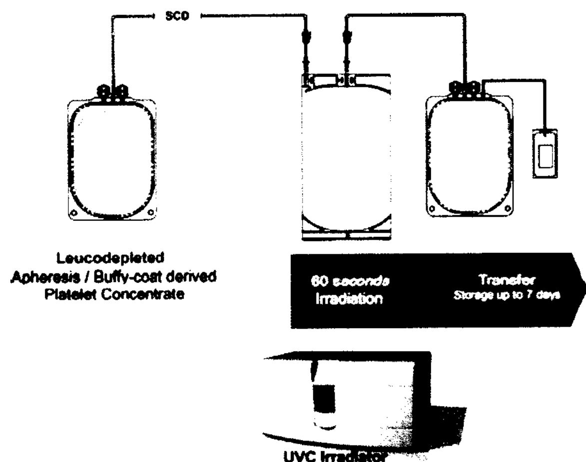

Figure 1: THERAFLEX UV procedure

Plasma-reduced PCs were treated with the THERAFLEX UV procedure (Fig.1) and stored until day 8 after blood donation (day 6 after treatment). The in vitro quality of UVC treated PCs was evaluated in comparison to untreated control platelets. Control PCs were stored for the same time period. PCs in storage medium SSP+/MacoPharma (containing saline, citrate, acetate, phosphate, magnesium and potassium, identical to PAS-IIIM) were prepared from pools of 5 buffy coats. The average volume was 350 mL (platelet concentration approx. 10^9/mL) and the plasma concentration was approx. 35%. PCs were transferred into irradiation bags (Fig.2) for UVC treatment at a dose of 0.4 J/cm^2 (approx. 60 sec) (Fig.3). They were strongly agitated during irradiation. Relevant treatment parameters, e.g. UV dose, UV intensity, temperature and irradiation time, were microprocessor-controlled.

RESULTS

Until day 8 of storage in vitro characteristics were only marginally influenced by the THERAFLEX process. Platelet quality was evaluated by measurement of the hypotonic shock response (HSR) and the expression of the activation marker CD62p. HSR reactivity and CD62p levels were only slightly affected by the Theraflex treatment. Annexin V binding percentage, as a marker for apoptosis, remained almost unchanged. Glucose consumption and lactate formation were found to be marginally higher in the treated PCs. pH remained above 7.0 until day 8 after donation (Tab. 1).

	Plts $[x10^9/mL]$	CD62 [%]	Annexin V [%]	HSR [%]	pH	Glucose [mg/dL]	Lactate [mmol/L]
Day 6*							
Control	9.2 ± 1.1	21 ± 6	5 ± 1	71 ± 5	7.29 ± 0.04	62 ± 17	10.2 ± 2.3
Treated	8.5 ± 0.9	32 ± 5	9 ± 4	68 ± 4	7.22 ± 0.05	52 ± 21	10.8 ± 1.6
Day 7*							
Control	8.9 ± 0.8	24 ± 6	7 ± 1	72 ± 3	7.32 ± 0.05	55 ± 19	10.4 ± 1.6
Treated	8.4 ± 0.9	42 ± 13	10 ± 3	68 ± 3	7.22 ± 0.06	42 ± 20	11.8 ± 1.7
Day 8*							
Control	9.4 ± 1.6	30 ± 3	8 ± 4	71 ± 5	7.34 ± 0.06	45 ± 18	11.5 ± 1.6
Treated	9.1 ± 1.3	52 ± 12	10 ± 3	65 ± 5	7.22 ± 0.09	31 ± 17	13.1 ± 1.7

Table 1: In vitro parameters of untreated and treated PCs on day 6, 7 and 8 after blood donation (N = 6, mean ± SD)

*after blood donation

CONCLUSIONS

THERAFLEX treatment with 0.4 J/cm^2 UVC light has only a minor influence on in vitro parameters of PCs and their storage stability until day 8 after blood donation was maintained.

[1] Polyolefineacetate bags for pathogen inactivation and for storage of platelet concentrates, H Mohr, TH Müller, F Tolksdorf, WH Walker, Vox Sang 2004; 87 (Suppl. 3): 70

Funded by Forschungsgemeinschaft der Blutspendedienste des Deutschen Roten Kreuzes e.V. and Maco Pharma.

IN VITRO QUALITY AND STORAGE STABILITY OF PLATELET CONCENTRATES AFTER THERAFLEX UV TREATMENT

Tolksdorf F[1], Walker W H[1], Mohr H[2], Gravemann U[2], Müller T H[2]
[1]MacoPharma, Langen, Germany
[2]Blood Centre of the German Red Cross Chapters of NSTOB, Institute Springe, Germany

DGTI Congress, Friedrichshafen, September 2007

INTRODUCTION

Pathogen Inactivation technologies are proactive measures to enhance the safety of platelet transfusions. Their use can be effective also against emerging unknown pathogens. Current procedures need chemicals like photosensitizers. We developed a procedure, which allows an efficient reduction of pathogens (e.g. bacteria and viruses) in plasma-reduced platelet concentrates (PCs) using short-wavelength UV light (UVC) in combination with strong agitation, i.e. there is no photoactive compound needed (THERAFLEX UV Platelets technology). The irradiation device developed for this purpose is equipped with a light source emitting UVC light at a wavelength of 254 nm. Moreover a mechanism for orbital agitation is installed. UVC irradiation is microprocessor-controlled. Relevant treatment parameters are monitored throughout the entire treatment thus allowing a well documented and reproducible process. PCs are treated in a twin-bag kit, which comprises of a highly UV-permeable irradiation bag and a container for extended platelet storage. In the present study we investigated the influence of the THERAFLEX UV treatment on in vitro parameters of PCs and on their storage stability.

MATERIALS & METHODS

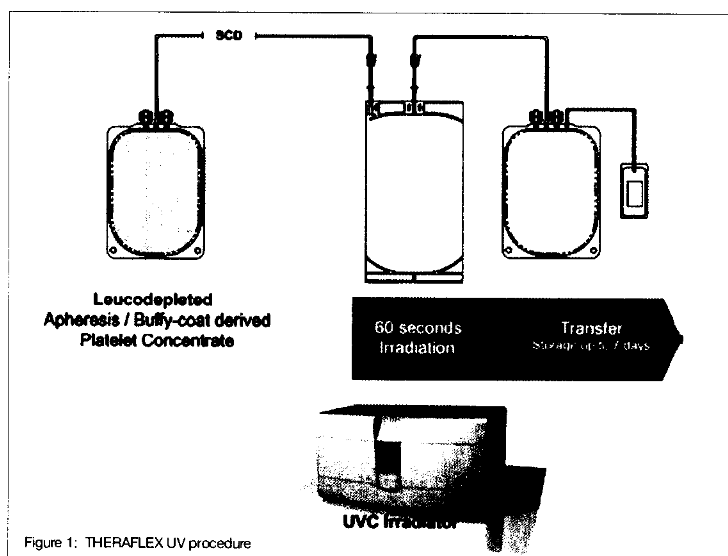

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Figure 2: Twin-bag kit

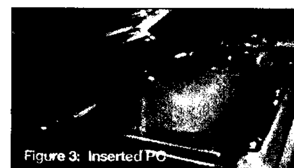


Figure 3: Inserted PC

RESULTS

Until day 8 of storage in vitro characteristics were only marginally influenced by the THERAFLEX process. Platelet activation was evaluated by measurement of the hypotonic shock response (HSR) and the expression of the activation marker CD62p. HSR was only slightly and CD62p levels were moderately affected by Theraflex treatment. Annexin V binding percentage, as a marker for apoptosis, remained almost unchanged. Glucose consumption and lactate formation were found to be marginally higher in the treated PCs. pH remained above 7.0 until day 8 after donation (Tab. 1).

Day 6*	Plts [x10^9/mL]	HSR [%]	pH	CD62 [%]	Annexin V [%]	Glucose [mg/dL]	Lactate [mmol/L]
Control	9.2 ± 1.1	71 ± 5	7.29 ± 0.04	21 ± 6	5 ± 1	62 ± 17	10.2 ± 2.3
Treated	8.5 ± 0.9	68 ± 4	7.22 ± 0.05	32 ± 5	9 ± 4	52 ± 21	10.8 ± 1.6
Day 7*							
Control	8.9 ± 0.8	72 ± 3	7.32 ± 0.05	24 ± 6	7 ± 1	55 ± 19	10.4 ± 1.6
Treated	8.4 ± 0.9	68 ± 3	7.22 ± 0.06	42 ± 13	10 ± 3	42 ± 20	11.8 ± 1.7
Day 8*							
Control	9.4 ± 1.6	71 ± 5	7.34 ± 0.06	30 ± 3	8 ± 4	45 ± 18	11.5 ± 1.6
Treated	9.1 ± 1.3	65 ± 5	7.22 ± 0.09	52 ± 12	10 ± 3	31 ± 17	13.1 ± 1.7

Table 1: In vitro parameters of untreated and treated PCs on day 6, 7 and 8 after blood donation (N=6, mean ± SD)

*after blood donation

CONCLUSIONS

THERAFLEX treatment with 0.4 J/cm^2 UVC light has only a minor influence on in vitro parameters of PCs and on their storage stability until day 8 after blood donation.

Funded by Forschungsgemeinschaft der Blutspendedienste des Deutschen Roten Kreuzes e.V. and Maco Pharma.

THERAFLEX UV PLATELETS: A NOVEL TECHNIQUE FOR PATHOGEN INACTIVATION AND ITS EFFECT ON THE QUALITY OF PLATELET CONCENTRATES

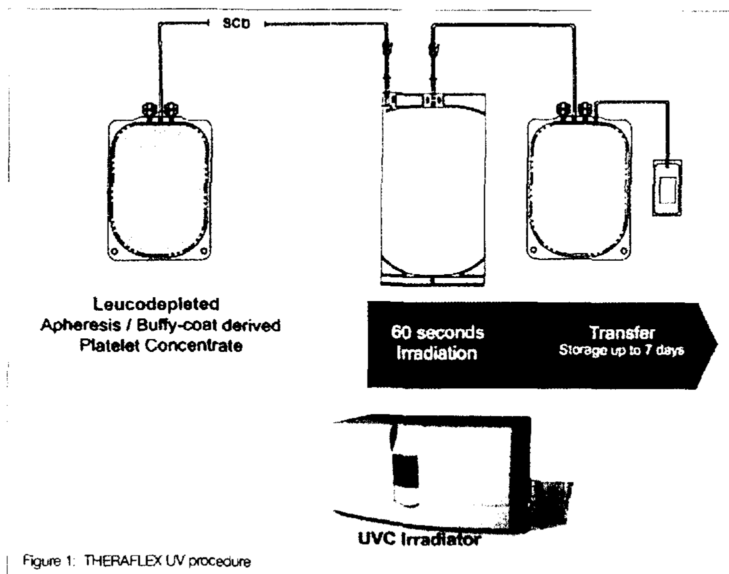
Tolksdorf F[1], Walker W H[1], Mohr H[2], Gravemann U[2], Müller T H[2]
[1]MacoPharma, Langen, Germany
[2]Blood Centre of the German Red Cross Chapters of NSTOB, Institute Springe, Germany

ISBT Congress, Madrid, June 2007

INTRODUCTION

The use of a pathogen inactivation technology is an option to enhance the safety of platelet transfusions. Current procedures need chemicals to be added to the platelet concentrates (PCs). These compounds are of concern if they remain in the final product. Moreover, treatment may cause deterioration of the platelets. A novel procedure has been developed using only short-wave UV light (UVC, 254 nm) that effectively *inactivates pathogens in plasma-reduced PCs. The equipment used consists of an irradiation device with a specific mechanism for agitation.* Its capacity is one platelet unit (random donor or apheresis) per treatment cycle. Treatment parameters, e.g. UVC intensity, UVC dose, temperature and agitation, are microprocessor-controlled. Platelets are processed in the THERAFLEX twin-bag kit, which comprises a highly UV-transparent polyolefin acetate bag[1] for irradiation and a platelet storage container (Fig.1, 2). We investigated to what extent platelet integrity and storage stability of the treated products were influenced by this new inactivation procedure.

MATERIALS & METHODS



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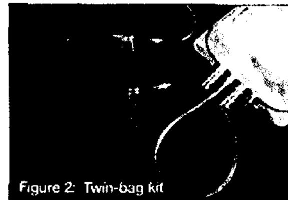

Figure 2: Twin-bag kit

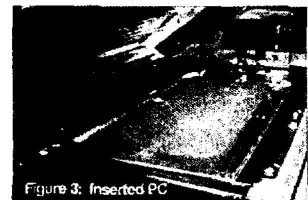

Figure 3: Inserted PC

RESULTS

Until day 8 of storage in vitro characteristics were hardly influenced by the THERAFLEX process. HSR reactivity was only slightly reduced whereas collagen induced aggregation was moderately increased. Glucose consumption and lactate formation were found to be marginally higher in the treated PCs. Thus, pH slightly dropped but remained above 7.0 until day 8 after donation. The mean platelet loss due to UVC treatment was 4%. (Tab. 1)

	Plts [x10⁹/mL]	HSR [%]	pH	Spontaneous aggregation [%]	Collagen-ind. aggregation 100 µg/mL [%]	Glucose [mg/dL]	Lactate [mmol/L]
Day 3							
Control	10.2 ± 1.6	68 ± 4	7.13 ± 0.05	11 ± 3	94 ± 3	122 ± 8	7.5 ± 1.0
Treated	9.6 ± 1.3	64 ± 5	7.07 ± 0.07	14 ± 2	89 ± 5	118 ± 6	7.7 ± 0.8
Day 6							
Control	9.9 ± 1.0	66 ± 2	7.24 ± 0.13	12 ± 2	74 ± 9	86 ± 10	10.8 ± 1.0
Treated	9.5 ± 1.3	64 ± 8	7.09 ± 0.06	14 ± 3	81 ± 9	68 ± 10	12.8 ± 1.5
Day 8							
Control	9.4 ± 1.6	68 ± 1	7.29 ± 0.12	10 ± 1	62 ± 7	63 ± 9	12.5 ± 0.9
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Table 1: Platelet parameters of untreated an treated PCs on day 3, 6 and 8 after blood donation (mean +/- SD; n=4)

CONCLUSIONS

Plasma-reduced PCs were only slightly affected when treated with the THERAFLEX UV system for pathogen inactivation. In vitro parameters and storage stability were well preserved until day 8 after blood donation.

[1] Polyolefineacetate bags for pathogen inactivation and for storage of platelet concentrates, H Mohr, TH Müller, F Tolksdorf, WH Walker, Vox Sang 2004; 87 (Suppl. 3): 70

Funded by Forschungsgemeinschaft der Blutspendedienste des Deutschen Roten Kreuzes e.V. and Maco Pharma.

DEGRADATION OF HUMAN HEPATITIS B VIRUS DNA IN PLATELET CONCENTRATES BY SHORT WAVE ULTRAVIOLET LIGHT AS REVEALED BY REAL-TIME PCR

J. Knüver-Hopf, H. Mohr and T.H. Müller

Blood Center of the German Red Cross Chapters of NSTOB
Institute Springe, Germany
Supported by Forschungsgemeinschaft der Blutspendedienste des Deutschen Roten Kreuzes e.V. & MacoPharma Int.

Conclusion

The present results demonstrate HBV nucleic acid degradation after UVC treatment.
This suggests that HBV in platelet concentrates is sensitive to UVC light.

Introduction

Treatment of platelet concentrates (PC) using short wave ultraviolet light (UVC) has been shown to effectively inactivate several pathogens [1]. Inactivation of human hepatitis B virus (HBV) however could not be proven up to now, because there is no infectivity assay available for HBV. The target structures in UVC treatment of viruses is its nucleic acid. Inactivation of viruses therefore might be determined by PCR.

Material and Methods

PC in storage medium SSP+ (MacoPharma, Langen, Germany) containing approx. 30% plasma were prepared from pools of 5 buffy coats. From each PC 120 ml were transferred to ethylvinyl acetate bags from MacoPharma (Langen, Germany). In this study, treatment with UVC light was performed with different doses on a BS10-illumination device (GROEBEL, Ettlingen, Germany). Routinely UVC treatment was done with a dose of 0.4 J/cm². The QIAamp Blood Mini Kit (QIAGEN, Hilden, Germany) was used for isolation of DNA from all samples. For long-range real-time PCR [LR-PCR] the LightCycler was used (ROCHE, Mannheim, Germany). Primers and hybridization probes were synthesized by TIB MOLBIOL (Berlin, Germany). Primers were selected for the nucleotide positions 44 to 2187 of the circular HBV genome.

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Photodynamic inactivation of human hepatitis B virus in plasma as revealed by long-range PCR
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Photodynamic inactivation of human hepatitis B virus in plasma-reduced platelet concentrates as revealed by long-range real-time pcr
Transfusion Medicine (2006) 33 (Suppl.1) 63

Results and Discussion

From previous investigations it is known that PCR inhibition cannot be shown by a short-range HBV real-time PCR [2]. This is probably due to the short genome region analysed. This finding is in agreement with previous PCR studies with HIV-1 and parvovirus B19. In those studies we found a correlation between the analysed viral genome region and inhibition of PCR in virus-infected samples after photodynamic treatment [3-5]. In the present study the effect of UVC treatment on HBV-DNA was evaluated by the use of LR-PCR. An increased amplicon size of 1090 base pairs was a sufficient target to demonstrate the effect of UVC treatment. As seen in figure 1, the fluorescence curves of treated samples shiftet towards higher cycle number values compared to untreated samples. As shown in figure 2, the PCR signals were lowered from 100% to 2% after treatment with 0.4 J/cm² of UVC, i.e. from approx. 10xE7/ml genome copies to 10xE5/ml.

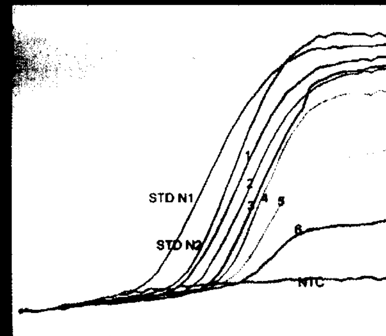

Figure 1: Amplification plot of LR-PCR before and after pathogen inactivation procedure with UVC of HBV in platelet concentrates
1) untreated HBV sample; 2-6) UVC- treated HBV sample;0.1, 0.3, 0.4, 0.5 and 0.6 J/cm²
***STD N1 and N2)** calibration standards; **NTC)** no template control*

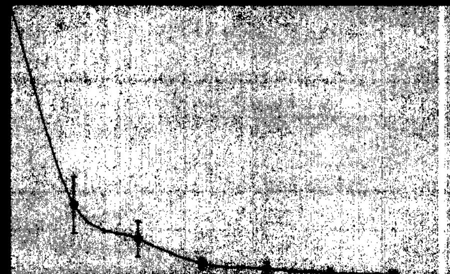

Figure 2: Kinetics of nucleic acid degradation of HBV in PC after UVC treatment detected by LR-PCR
N=6: +/-SD

THERAFLEX UV PLATELETS: NOTHING BUT UVC LIGHT AND STRONG AGITATION

H. Mohr[1], U. Gravemann[1], F. Tolksdorf[2], W.H. Walker[2], T.H. Müller[1]

Purpose

Blood donations may not only be contaminated with viruses, e.g. HBV, HCV or HIV. In addition, they may contain bacteria. This is especially crucial for platelet concentrates (PCs), because they have to be stored at room temperature, at which bacteria can multiply to high levels [1-2].

Short-wave ultraviolet light (UVC, wavelength range: 200-280 nm) is germicidal, but low UV-permeability hampers its use for sterilizing PCs. A simple method was developed which overcomes this limitation.

Materials and Methods

Plasma-reduced PCs in storage medium SSP+ (volume approx. 350 mL, platelet concentration approx. 10^9/mL, plasma content 30-35%) were prepared from pools of 5 buffy coats [3]. PC volume was approx. 350 mL. The PCs were spiked with approx. 10^2-10^6 CFU/mL of different bacteria species or up to 10^7 $TCID_{50}$/mL of lipid-enveloped or nonenveloped viruses. Other PCs were spiked with $5x10^6$/mL peripheral blood mononuclear cells (PBMC). The PCs were filled into UV-transparent plastic bags and irradiated on a device (Fig.1), equipped with mercury vapour tubes emitting monochromatic UVC-light (wavelength: 254 nm). The device was equipped with an orbital agitator. Irradiation was from both sides of the bags. UVC doses applied were up to 0.6 J/cm² (approx. 90 sec). During treatment the PCs were strongly agitated. Bacteria or virus titers, PBMC viability and platelet parameters were determined before and after irradiation. Each experiment was repeated 3-6 times. Results are depicted as mean ± SD.

Results

Pathogen inactivation was enormously enhanced when the PCs were loosely placed on a quartz plate located between the two layers of UVC tubes of the irradiation device and, in addition, strongly agitated during irradiation (Fig. 2).

UVC-light at 0.3-0.4 J/cm² (irradiation time: approx. 1 min) reduced the titers of all bacteria tested by approx. 5-6 $\log_{10}$ steps. PCs spiked with approx. 100 CFU/ml of bacteria were reproducibly sterilized (Tab.1). In one experiment with *B. cereus* the PC was sterile after 3 but unsterile after 6 days storage. This was probably due to spores of *B. cereus* that are more resistant to UVC than vegetative bacteria.

UVC sensitivity of the viruses tested was not so uniform (Table 1): The small single stranded RNA viruses VSV, Sindbis and WNV were completely inactivated at approx. 0.3-0.4 J/cm². Remarkably HIV-1 (also a small single-stranded RNA virus) was only moderately inactivated at UVC doses up to 0.6 J/cm².

The small nonenveloped DNA viruses PPV and EMCV proved to be very sensitive. Complete inactivation was achieved at 0.4-0.5 J/cm².

With the exception of HIV-1, SHV-1 was more resistant than the other viruses tested. This confirms that in general large double stranded DNA viruses are not as susceptible to UVC as smaller single stranded DNA or RNA viruses.

PBMC proved to be extremely sensitive to UVC irradiation: Complete inactivation was found at less than 0.1 J/cm² (Fig. 3)

PC properties remained almost unchanged at doses up to 0.6 J/cm². The storage stability of the treated PCs for up to 6 days after treatment (8 days after blood donation) was maintained (Table 2)

Conclusions

Irradiation with UVC under strong agitation may be used to sterilize platelet concentrates at a light dose that is not harmful to the products. The UVC dose required is 0.4 J/cm². Irradiation time is not more than approx. 1 min.

Parameter	Day 1 after irradiation				Day 6 after irradiation			
	Control	UVC dose (J/cm²)			Control	UVC dose (J/cm²)		
		0.4	0.5	0.6		0.4	0.5	0.6
Pts [x10⁹/mL]	10.8 ± 0.6	10.2 ± 0.6	9.8 ± 0.6	9.1 ± 0.9	10.1 ± 0.8	9.8 ± 0.6	9.3 ± 0.8	9.3 ± 0.9
pH	7.10 ± 0.04	7.04 ± 0.05	7.09 ± 0.05	7.05 ± 0.04	7.27 ± 0.15	7.09 ± 0.06	7.11 ± 0.10	6.98 ± 0.07
Lactate [mmol/L]	7.7 ± 1.0	8.0 ± 0.5	7.7 ± 0.5	8.0 ± 0.7	12.7 ± 1.0	14.9 ± 1.0	14.6 ± 1.4	16.7 ± 1.4
Glucose [mg/dL]	122 ± 9	117 ± 7	117 ± 6	115 ± 7	62 ± 11	43 ± 8	44 ± 11	29 ± 10
Swirling	ok	ok	ok	ok	ok	ok	ok	ok
HSR [%]	69 ± 5	66 ± 2	61 ± 6	62 ± 4	68 ± 2	65 ± 2	62 ± 3	56 ± 5
Collagen-induced aggregation [%]	95 ± 4	90 ± 5	88 ± 3	87 ± 2	62 ± 9	69 ± 8	67 ± 2	69 ± 5
CD62 [%]	36 ± 1	46 ± 3	47 ± 2	49 ± 1	29 ± 1	45 ± 8	50 ± 10	57 ± 8
Annexin V [%]	5 ± 1	6 ± 3	7 ± 4	7 ± 4	9 ± 5	8 ± 2	10 ± 2	12 ± 3

Tab. 3: Treatment of PCs with different UVC doses. Influence on platelet parameters and on storage stability. n=6, mean + SD

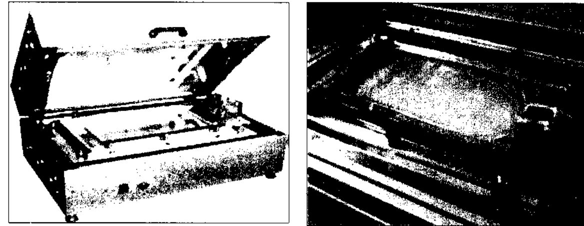

***Fig. 1*: Irradiation device for UVC treatment of PCs**

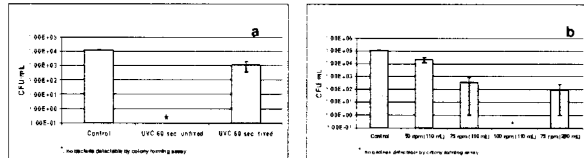

***Fig. 2*: Inactivation of *St. epidermidis* in PC aliquots (110 or 280 mL) by irradiation with UV light: fixed vs. loosely placed irradiation bags (a); dependence of bacteria inactivation in loosely placed irradiation bags on the agitation speed (b). n=3, mean ± SD**

Bacteria species	Characteristics	Gram stain	Number pf experiments	Spike (CFU/mL)	BacT/Alert result*	Remark
B. cereus	fac. anaerobic	pos	12	100-140	11 sterile 1 unsterile**	Spore former
E. coli	aerobic	neg	12	36-65	12 sterile	
K. pneumoniae	fac. anaerobic	neg	12	85-140	12 sterile	
P. acnes	anaerobic	neg	12	61-100	12 sterile	
S. aureus	fac. anaerobic	pos	22	60-110	22 sterile	
S. epidermidis	fac. anaerobic	pos	22	74-210	22 sterile	
Str. pyogenes	fac. anaerobic	pos	12	118-194	12 sterile	

*: Samples (2x10 mL each) were drawn after 3 and 6 days at 22°C
**: sterile after 3 days storage

***Tab 1:* Sterilization of PCs spiked with different bacteria species by irradiation with UVC (0.4 J/cm²)**

Virus	Genome	Lipid Envelope	Model virus for	Log_{10} reduction factor
Vesicular stomatitis (VSV)	ss* RNA	X	-	≥ 6.41
Sindbis (Sindbis)	ss RNA	X	-	5.55
West Nile (WNV)	ss RNA	X	HCV	5.24
Human immunodefiency (HIV-1)	ss RNA	X	-	1.36
Suid Herpes (SHV-1)	ds** DNA	X	HBV/CMV	3.57
Porcine Parvo (PPV)	ss DNA	-	Parvo B 19	≥ 6.42
Encephalomyocarditis (EMCV)	ss DNA	-	HAV	5.73

Tab 2: Inactivation factors of viruses by irradiation with UVC (0.4 J/cm²)

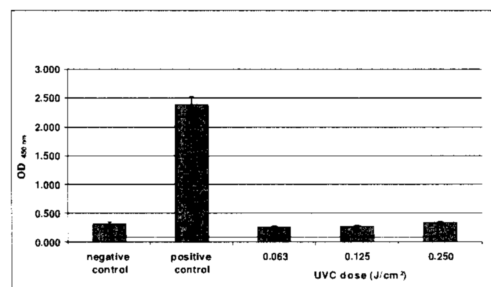

Fig. 3: Inactivation of T-lymphocytes in platelet concentrates by irradiation with UVC. Viability was assayed by mixed lymphocyte culture.

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[1] ✚ **Blood Center of the German Red Cross Chapters of NSTOB, Springe, Germany**
[2] **MacoPharma Int., Langen, Germany**

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INACTIVATION OF SMALL NON-ENVELOPED VIRUSES IN PLASMA REDUCED PLATELET CONCENTRATES BY IRRADIATION WITH SHORT-WAVE ULTRAVIOLET LIGHT

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Aim

To investigate if small nonenveloped viruses in plasma reduced platelet concentrates are sensitive to irradiation with UVC light.

Introduction

Small nonenveloped viruses (e.g. parvovirus B19 and hepatitis A virus) are more resistant than lipid-enveloped viruses to most pathogen reduction procedures used for plasma or for cellular blood products (1-3). We have developed a procedure to decontaminate platelet concentrates (PCs) by irradiation with monochromatic short-wave ultraviolet light (wavelength: 254 nm). It is essential that the products are not fixed and at the same time strongly agitated during treatment (Fig. 1 and 2). We found that the procedure inactivates bacteria and lipid enveloped viruses. As the present data indicate, small nonenveloped viruses are also inactivated.

Materials and Methods

The virological investigations were conducted at NewLab BioQuality, Cologne, Germany. They were carried out according to CPMP/BWP guidelines 268/95 ("Note for guidance on virus validation studies") and 269/95 ("Note for guidance on plasma dreived medicinal products"). The PCR investigations were performed in the own laboratory.

Plasma-reduced PCs (platelet concentration approx. 10^9/mL, plasma content: approx. 30 %) were prepared from pools of 5 buffy coats. The storage medium used was SSP$^+$ (MacoPharma). PCs (volume approx. 350 mL) in UV transparent polyolefine acetate bags (dimensions: 19x38 cm) were spiked with approx. 10^7 up to 10^8 $TCID_{50}$/mL of porcine parvovirus (PPV, strain ATCC CRL-6489 (NADL-2), a model for parvovirus B19) or encephalomyocarditis virus (EMCV, strain ATCC VR 129-B, a model for hepatitis A virus). The thickness of the PC layer was approx. 4-5 mm. Irradiation with UVC light was from both sides of the bags. The UVC dose applied was up to 0.6 J/cm² (irradiation time per 0.1 J/cm² was approx 15 sec). The PC-samples were loosely placed on a quartz plate located in the middle between two layers of mercury vapor tubes emitting monochromatic UVC light (wavelength: 254 nm). During irradiation they were intensively agitated using an orbital agitator. Agitation speed was approx. 100 rpm. Before and after irradiation virus titers (expressed as log_{10} of tissue culture infectious doses ($log_{10}TCID_{50}$)) were determined.

The influence of UVC on the DNA of parvovirus B19 was investigated by long-range RT-PCR, using a LightCycler from Roche, Mannheim, Germany. The primer pair used spanned a region of 1028 bases (approx. one fifth of the genome of the virus). In these experiments PC aliquots of 110 mL were treated; dimensions of the irradiation bags were 12.5x14.5 cm.

Parameter	Day 1 after irradiation				Day 4 after irradiation				Day 6 after irradiation			
	Control	UVC dose (J/cm²)			Control	UVC dose (J/cm²)			Control	UVC dose (J/cm²)		
		0.4	0.5	0.6		0.4	0.5	0.6		0.4	0.5	0.6
Plt-concentration [x10⁹/mL]	10.8 ± 0.6	10.2 ± 0.6	9.8 ± 0.6	9.1 ± 0.9	10.3 ± 0.6	10.1 ± 0.8	9.6 ± 0.8	9.5 ± 0.9	10.1 ± 0.8	9.8 ± 0.6	9.3 ± 0.8	9.3 ± 0.9
pH	7.10 ± 0.04	7.04 ± 0.05	7.09 ± 0.05	7.05 ± 0.04	7.21 ± 0.14	7.06 ± 0.04	7.10 ± 0.06	7.06 ± 0.20	7.27 ± 0.15	7.09 ± 0.06	7.11 ± 0.10	6.98 ± 0.07
Lactate [mmol/L]	7.7 ± 1.0	8.0 ± 0.5	7.7 ± 0.5	8.0 ± 0.7	10.7 ± 1.2	12.3 ± 1.3	11.7 ± 1.3	13.8 ± 2.0	12.7 ± 1.0	14.9 ± 1.0	14.6 ± 1.4	16.7 ± 1.4
Glucose [mg/dL]	122 ± 9	117 ± 7	117 ± 6	115 ± 7	86 ± 12	70 ± 10	75 ± 10	58 ± 15	62 ± 11	43 ± 8	44 ± 11	29 ± 10
Swirling	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok
Hypotonic shock response [%]	69 ± 5	66 ± 2	61 ± 6	62 ± 4	66 ± 3	67 ± 4	65 ± 3	61 ± 3	68 ± 2	65 ± 2	62 ± 3	56 ± 5
Spontaneous aggregation [%]	11 ± 2	14 ± 2	18 ± 3	18 ± 3	12 ± 2	15 ± 2	15 ± 1	17 ± 6	10 ± 2	16 ± 4	13 ± 2	14 ± 1
Collagen-induced aggregation [%] 100 µg/mL	95 ± 4	90 ± 5	88 ± 3	87 ± 2	77 ± 9	83 ± 10	80 ± 6	83 ± 5	62 ± 9	69 ± 8	67 ± 2	69 ± 5
Collagen-induced aggregation [%] 20 µg/mL	54 ± 13	77 ± 8	78 ± 6	74 ± 8	15 ± 7	35 ± 12	33 ± 9	39 ± 8	9 ± 1	16 ± 4	19 ± 1	22 ± 2
CD62 [%]	36 ± 1	46 ± 3	47 ± 2	49 ± 1	27 ± 3	36 ± 5	42 ± 6	48 ± 5	29 ± 1	45 ± 8	50 ± 10	57 ± 8
Annexin V [%]	5 ± 1	6 ± 3	7 ± 4	7 ± 4	6 ± 3	10 ± 5	9 ± 2	9 ± 2	9 ± 5	8 ± 2	10 ± 2	12 ± 3

Tab. 1: Irradiation of PCs with different UVC doses. Influence on platelet parameters and on storage stability. (n= 6, mean ± SD).

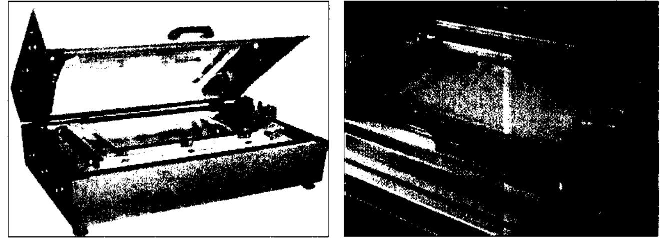


Fig. 1 and 2: Irradiation device for UVC treatment of PCs

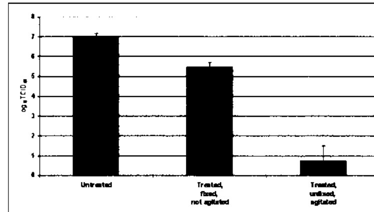
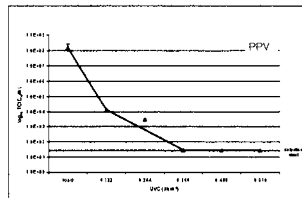


Fig. 2: Inactivation of vesicular stomatitis virus by irradiation with UVC (n=.3). Comparison of two treatment modes: Fixed bags (champed between 2 quartz plates) vs. unfixed bags (loosely put on a quartz plate), both with agitation (100 rpm)



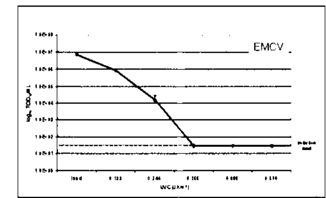


Fig. 3: Inactivation kinetics of PPV and EMCV in plasma-reduced PCs irradiated with UVC. (n= 2).

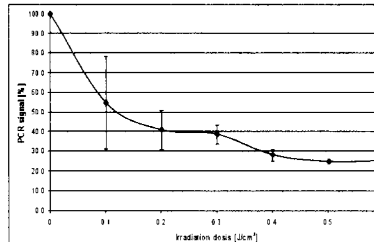


Fig. 4: Degradation of the DNA of parvovirus B19 spiked into plasma-reduced PCs by irradiation with UVC. (n= 3, mean ± SD).

Results

PPV as well as EMCV in plasma-reduced PCs were rapidly inactivated by irradiation with UVC light: no infective virus was detectable at doses higher than 0.366 J/cm² (Fig. 3). The log_{10} reduction factors determined exceeded 6.4 and 5.5 , respectively.

The PCR investigations revealed that the genome of parvovirus B19 was degraded by UVC treatment: at light doses between 0.4 and 0.6 J/cm² the PCR signal was reduced by approx. 75 % (Fig. 4). It remains to be established if this is indicative of complete inactivation of that virus.

At UVC doses up to 0.6 J/cm² platelet functions were only moderately influenced, and the storage stability of the treated products for up to 6 days after treatment (8 days after blood donation) was maintained (Table 1).

Conclusions

Irradiation with UVC light under strong agitation is a unique procedure to inactivate small nonenveloped viruses in PCs at conditions at which platelet functions and the storage stability of the PCs are not impaired.

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Methylene Blue-Treated Plasma: Toxicological Profile of Methylene Blue and Its Photoproducts

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参考資料3 マコファルマ社

Purpose: The MacoPharma Theraflex ®System uses Methylene blue (MB) and visible light for virus inactivation of plasma for transfusion (MB plasma). MB is added at a concentration of 1µM. After illumination most of the photosensitizer and its photoproducts are removed by an integrated depletion filter. A considerable number of toxicological data on MB are available in the literature. However, long-term studies are lacking. They are necessary because in certain indications MB plasma is administered for several weeks, for example in the treatment of thrombotic thrombocytopenic (TTP) patients. Recent studies with MB were conducted by the American National Toxicological Program (NTP). They focused on the endpoints: Short-term toxicity (4 and 13 weeks), conventional teratology and long-term carcinogenicity (2 years). However, in these studies MB was administered orally and therefore this application route differs from the mode of application used for MB plasma (intravenous route). It was the aim of the present investigation to elucidate whether toxicological data from the NTP studies can be used to assess the toxicological properties of MB after intravenous administration.

Furthermore, as part of a preclinical testing program, the toxicological profile of MB and its photoproducts was investigated.

Methods

The adsorption, distribution and excretion (ADE) of ^{14}C-labeled MB. The ADE following oral and 24h infusion, respectively, were investigated in rats. A nominal dose level of 20 mg/kg body weight was administered by gavage or by 24h infusion. The observation time was 96 hours

13 week-gavage toxicity study. The study was conducted by the NTP in male and female F344 rats and B6C3F$_1$ mice. 10 animals/sex/species/group were administered Methylene blue in a suspension with 0.5% aqueous methylcellulose at dose levels of 0 (vehicle only), 25, 50, 100 and 200 mg/kg b.w./day on 5 days/week. In addition, 20 males and 20 females per group were used for interim sacrifices.

Teratology: Teratogenic effects were carried out by intravenous bolus injection of Methylene blue to rats and rabbits. Methylene blue was administered daily to the dams at 4, 12, 36 mg/kg b.w. (rat) and 2, 6, 18 mg/kg b.w. (rabbit).

Tolerance test in Beagles. In a tolerance test 5 ml/kg b.w. of autologous light-treated plasma (1 or 10 µM Methylene blue) was administered to 5 male Beagles per group by intravenous administration. After 3 weeks 3 dogs/group were treated for a second time and sacrified 24h later. Following parameters were examined: haematology, clinical biochemistry and ECG. A complete histopathology was carried out.

Genotoxicity studies. Methylene blue, Azure B, Azure A/C have been tested in a variety of genotoxicity assays, namely: Bacterial reverse mutation test (Ames test); in vitro mammalian cell gene mutation test (HPRT test); in vitro mammalian chromosome aberration test with human lymphocytes); in vivo micronucleus test with rat bone marrow and peripheral blood cells (dose 20 mg/kg b.w., 24h infusion); in vivo UDS test in rats (dose 20 mg/kg b.w., bolus infusion).

All studies were conducted according to GLP and international guidelines

Results

Pharmacokinetics

Excretion and Recovery of Radioactivity Following Oral and 24h Intravenous Administration of Methylene Blue

Fig. 1 Recovery of ^{14}C-labelled Methylene blue (MB). The recovery was examined in Sprague-Dawley rats following oral administration (p.o.) and 24h i.v. infusion at a dose level of 20 mg MB/kg body weight. Urine, faeces, organs, expired air, rinse water and infusion site were analysed. The radioactivity recovery rate in organs and at the infusion site was determined 96h

Distribution of Radioactivity 96h After Administration of ^{14}C-Methylene Blue

Fig. 2 Mean recovery of radioactivity after oral (gavage) application and 24h i.v. infusion of 20 mg MB/kg body weight in rats

Organ Distribution of ^{14}C-Labelled Methylene Blue Following Oral and 24h i.v. Administration

Fig. 3 Distribution of radioactivity in different organs of rats 96h after administration of 20 mg MB/kg body weight. Less than 1% of the dose was found in the different organs.

Pharmacokinetics

Pharmacokinetics of Radio-Labelled Methylene Blue

Sex/Route of application	C_{max} (µg-eq/ml)	T_{max} (min.)	$T_{1/2}\ \alpha$ (min.)	$T_{1/2}\ \beta$ (h)	$AUC_{0-\infty}$ (µg eq·h/ml)	Systemic bioavailability
Males p.o.	1.58	10.9	2.8	13.7	13.1	44%
Females p.o.	1.55	8.5	2.8	18.4	12.2	56%
Males 24h i.v.	0.93	12	3	12.6	30.0	
Females 24h i.v.	0.77	12	2.8	16.0	22.0	-

Tab. 1 Pharmacokinetic parameters of plasma radioactivity in rats. Pharmacokinetics were determined in male and female rats after oral (gavage) application and 24h i.v. infusion of 20 mg MB/kg body weight

AUC: Area under the curve

Pharmacokinetic Parameters in Rats Following 24h i.v. Infusion of ^{14}C-Labelled MB and Azure B (AzB)

Compartment	C_{max} (µg-eq/g tissue) T_{max} 30 min.		$AUC_{0-\infty}$ (µg-eq·h/g)		Terminal $T_{1/2}$ (h)	
	MB	AzB	MB	AzB	MB	AzB
Plasma	0.72 *SD: 0.25*	1.27 *SD: 0.61*	18.7	33.3	17.7	16.1
Bone marrow	0.48 *SD: 0.21*	0.81 *SD: 0.45*	17.04	31.44	19.1	23.3
Heart	1.39 *SD: 0.90*	0.97 *SD: 0.22*	57.85	50.48	29.8	29.1
Kidneys	2.22 *SD: 0.80*	2.74 *SD: 0.99*	88.04	129.83	15.2	37.1
Liver	2.30 *SD: 0.64*	2.94 *SD: 1.28*	75.43	106.35	15.5	20.2
Lungs	1.05 *SD: 0.28*	1.30 *SD: 0.48*	31.90	47.73	15.0	18.2

Tab. 2 Pharmacokinetic parameters following 24h i.v. infusion of 20 mg/kg b.w. ^{14}C-labelled MB or Azure B (AzB). MB and Azure B have an initial half-life of only several minutes and a longer terminal elimination half-life at a very low MB concentration level (< 1% of the dose)

Summary of Pharmacokinetics

- **Pharmacokinetics of ^{14}C-labelled MB after 24h infusion and oral application were comparable in T_{max}, $T_{1/2}\ \alpha$ and $T_{1/2}\ \beta$. It indicated:**
  - **a biphasic elimination of MB with an initial half-life of only several minutes and a longer terminal half-life of several hours but at a very low MB concentration level**
  - **less than 1% radioactivity in plasma and the examined organs**
  - **that radioactivity was almost completely excreted after 96h**
  - **that the oral dose of Methylene blue was well absorbed. The systemic bioavailability of MB was approx. 50%**
  - **no accumulation or storage of Methylene blue**
- **Administration of degradation product Azure B revealed a similar pharmacokinetic profile as MB**

➡ **Results of the National Toxicological Program (NTP) can be used to asses the toxicological profile of Methylene blue following intravenous application**

Toxicological Profile

Study Type	BSD	NTP
Route of application	24h or bolus infusion	Oral (by gavage)
Toxicokinetics	MB, Azure B (rat)	MB (rat, mouse)
ADE	MB (rat)	
Acute toxicity	MB, Azure B, Azure A/C (rat)	
4 and 12 weeks toxicity		MB (rat, mouse)
Teratology	MB (rat, rabbit)	MB (rat, rabbit)
Tolerance test	MB light-treated plasma (beagle)	
Genotoxicity in vitro	MB, Azure B, Azure A/C	MB
Genotoxicity in vivo	MB, Azure B (rat)	MB
Carcinogenicity		[1]MB (mouse, rat)

Tab. 3 Toxicology program for Methylene blue and its photoproducts. Test items are mainly administered clinically as 24h infusion. ADE: Absorption, Distribution, Excretion; NTP: American National Toxicology Program

[1] Laboratory reports completed but not yet published by NTP

Mutagenicity of Methylene Blue and Methylene Blue-Treated Plasma *in Vitro*

	MB	AzB	AzA/C	[1]MB-plasma (10µM)
In vitro				
Ames-test (TA98)	Pos. (> 1µg/plate)	Pos. (> 0.3 µg/ml)	Neg.	Neg.
Chromosome aberration test (human lymphocytes)	Pos. (> 1.25µg/ml)	Pos. (> 2.5 µg/ml)	Neg.	Neg.
HPRT test V79 cells	Neg.	Neg.	Neg.	Neg.
In vivo				
[1]Micronucleus test (rat bone marrow and peripheral blood cells)	Neg.	Neg.		
[1]UDS test	Neg.	Neg.		

Tab. 4 Treatment conditions *in vivo*: 24h i.v. infusion of 20 mg/kg body weight Methylene blue (MB) or Azure B (AzB). [1]Light treated MB plasma before removal of MB and its photoproducts.

Study Type	Clinical signs	Thresholds
4 and 12 weeks toxicity rat, mouse 0, 25, 50, 100, 200 mg/kg b.w. (oral)	Dose related increase in haematopoises, methaemoglobinemia, Heinz bodies.	Threshold for haematological effects (slight methaemoglobinemia) < 25 mg/kg b.w.
Teratology rat 4, 12, 36 mg/kg b.w. (i.v.)	Increase in resorption rate. No teratogenic properties	NOEL (foetal organism) 4 mg/kg b.w.
Rabbit i.v. 2, 6, 18 mg/kg b.w.	Highest dose leads to death of the foetus and increase resorption rate. No significant teratogenic properties	NOEL (foetal organism) 6 mg/kg b.w.
Tolerance test Beagle light-treated MB plasma (1.6 and 16 µg MB/kg b.w.)	No signs of intolerance	

Tab. 5 Toxicological findings detected during 12 week toxicity (NTP), developmental toxicity (teratogenicity) and tolerance test after application of methylene blue and light-treated MB plasma (tolerance test only)

NOEL: No observed effect level

Safety Margins for Toxicity from *in vivo* Studies with Methylene blue and Methylene Blue Treated Plasma

Fig. 4 Safety margins for toxicity from in vivo studies with methylene blue. Calculations are based on the thresholds NOEL (no observed effect level) (teratology) and LOAEL (lowest observed effect level) (slight methaemoglobinemia) (12 weeks toxicity) and a normal clinical exposure of 0.1µg MB/kg body weight (≤ 6 units MB plasma). No toxicity occurred, therefore the safety margins are based on the highest dose tested.

Summary of toxicological findings:

- **The threshold for a haematological effect was below 25 mg MB/kg body weight. Subchronic administration (13 weeks) of Methylene blue in rats and mice resulted in gross and microscopic findings which are consistent with the development of haemolytic anaemia.**
- **The no observed effect level (NOEL) for the foetal organism was 4 mg and 6 mg/kg b.w./day in rats and rabbits, respectively. High dose of Methylene blue (≥12 mg/kg b.w.) intravenously administered to pregnant rats and rabbits resulted mainly in loss of implants and increased number of early resorptions. No teratogenic properties were detected.**
- **Genotoxicological (i.e. clastogenic) effects of MB and Azure B were only found *in vitro*.**
- **No indication of genotoxic effects on bone marrow, peripheral blood cells and hepatocytes after application of 20 mg/kg b.w. MB and Azure B.**
- **No signs of intolerance (haematology, clinical biochemistry and ECG) or sensitization after infusion of 1µM or 10µM MB light-treated plasma before removal of MB and photoproducts were observed.**

➡ **Thresholds for no or low toxic properties which occurred after administration of MB in preclinical studies are > 160 to 200000**

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