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

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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

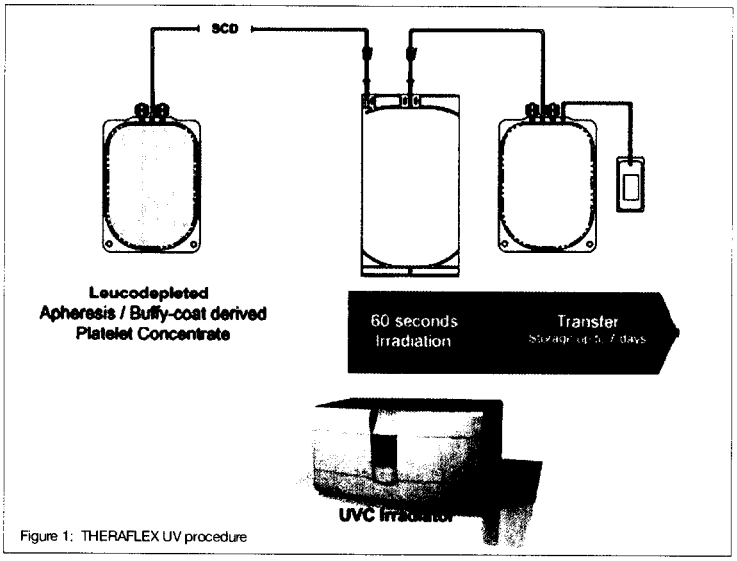

Figure 1: THERAFLEX UV procedure

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+ (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 and the plasma concentration was approx. 35%. PCs were transferred into irradiation bags (Fig.2) for UVC treatment. After insertion into the irradiation device (Fig.3) PCs were treated with UVC light at a dose of 0.4 J/cm² (approx. 60 sec). They were strongly agitated during irradiation. The system was microprocessor-controlled. The control unit enables monitoring and recording of relevant treatment parameters like UV dose, UV intensity, temperature and irradiation time.

Figure 2: Twin-bag kit

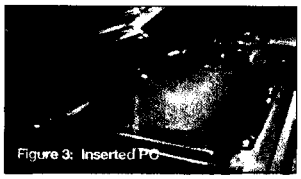

Figure 3: Inserted PC

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 [x10⁹/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/cm² UVC light has only a minor influence on in vitro parameters of PCs and on their storage stability until day 8 after blood donation.

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

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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

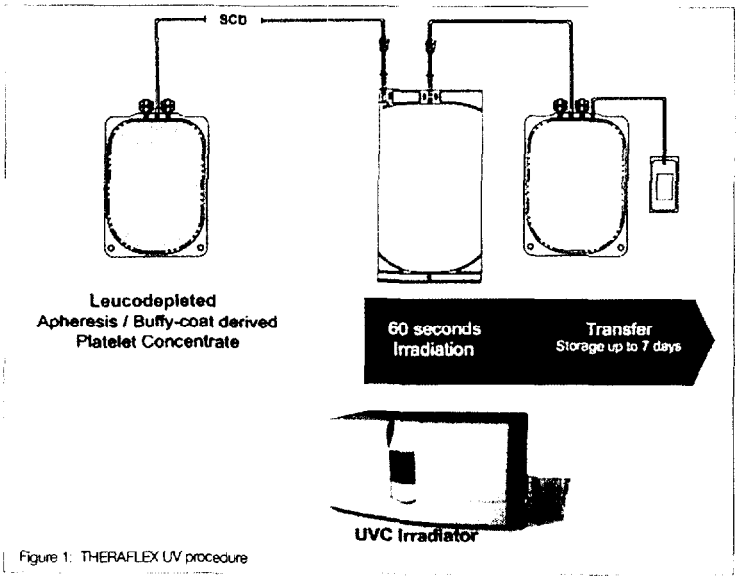

Figure 1: THERAFLEX UV procedure

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) for UVC treatment. After insertion into the irradiation device (Fig. 3) PCs were treated with UVC light at a dose of 0.4 J/cm² (approx. 60 sec). They were strongly agitated at a frequency of 1.8 Hz during irradiation. The system was microprocessor-controlled. The control unit enables monitoring and recording of relevant treatment parameters like dose, intensity, temperature and irradiation time.

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)

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

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

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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/cm^2. 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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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/cm^2 of UVC, i.e. from approx. 10xE7/ml genome copies to 10xE5/ml.

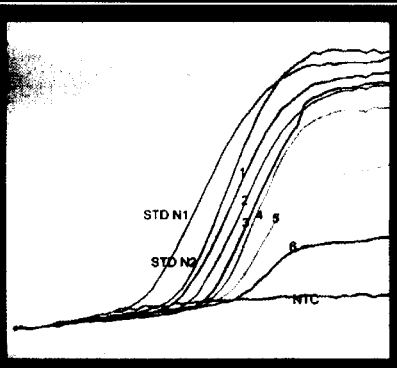

Figure 1: Amplification plot of LR-PCR before and after pathogen inactivation procedure with UVC of HBV in platelet concentrates
1) untreated HBV sample; ***2-6)*** *UVC- treated HBV sample;0.1, 0.3, 0.4, 0.5 and 0.6 J/cm^2*
STD N1 and N2) *calibration standards;* ***NTC)*** *no template control*

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

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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
Plts [x10⁹/mL]	10.8 ± 0.6	10.2 ± 0.6	9.6 ± 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	15.7 ± 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

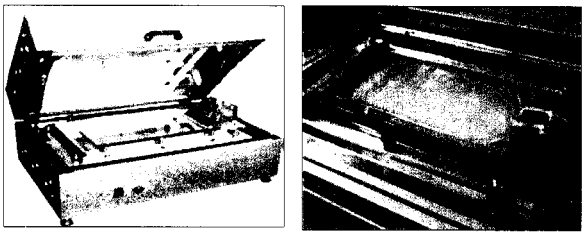

Fig. 1: **Irradiation device for UVC treatment of PCs**

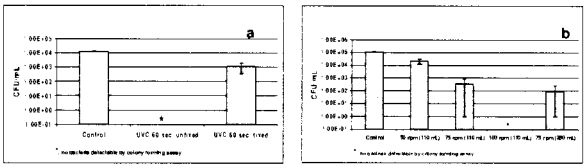

Fig. 2: **Inactivation of *St. epidermidis* in PC aliquots (110 or 280 mL) by irradiation with UV light: fixed vs. loosely placed irradiation bags (a); dependence of bacteria inactivation in loosely placed irradiation bags on the agitation speed (b). n=3, mean ± SD**

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)

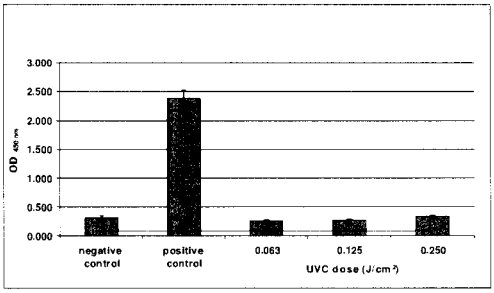

Fig. 3: Inactivation of T-lymphocytes in platelet concentrates by irradiation with UVC. Viability was assayed by mixed lymphocyte culture.

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

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

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 ± 0.5	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	68 ± 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).

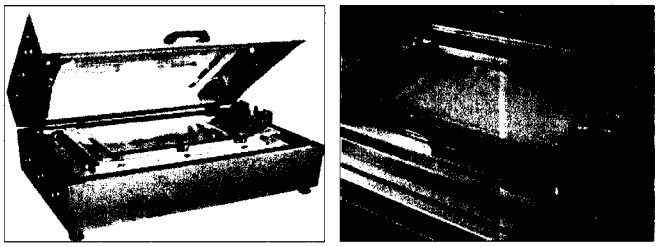

Fig. 1 and 2: Irradiation device for UVC treatment of PCs

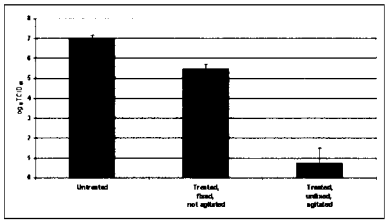

Fig. 2: Inactivation of vesicular stomatitis virus by irradiation with UVC (n=.3). Comparison of two treatment modes: Fixed bags (champed between 2 quartz plates) vs. unfixed bags (loosely put on a quartz plate), both with agitation (100 rpm)

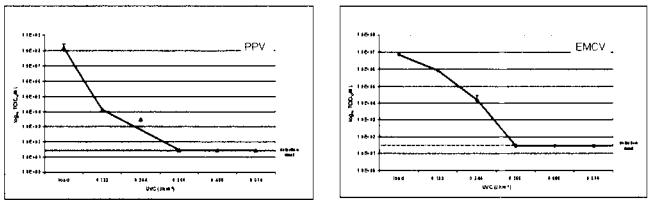

Fig. 3: Inactivation kinetics of PPV and EMCV in plasma-reduced PCs irradiated with UVC. (n= 2).

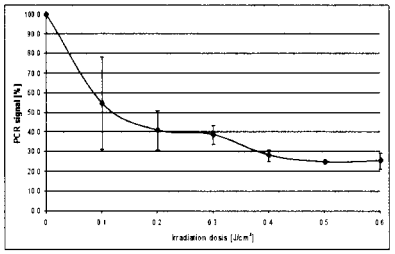

Fig. 4: Degradation of the DNA of parvovirus B19 spiked into plasma-reduced PCs by irradiation with UVC. (n= 3, mean ± SD).

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.

References

1. Welch J, Bienek C, Gomperts E, Simmonds P. Resistance of porcine circovirus and chicken anemia virus to virus inactivation procedures used for blood products. Transfusion 2006;46(11):1951-8.
2. Lin L, Hanson CV, Alter HJ, et al. Inactivation of viruses in platelet concentrates by photochemical treatment with amotosalen and long-wavelength ultraviolet light. Transfusion 2005;45(4):580-90.
3. Mohr H, Lambrecht B, Selz A. Photodynamic virus inactivation of blood components. Immunol Invest 1995;24(1-2):73-85.

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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 sacrificed 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

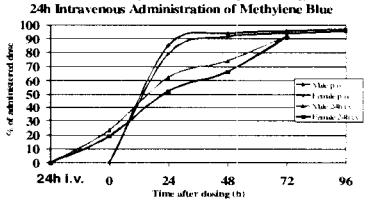

Fig. 1 **Recovery of ^{14}C-labelled Methylene blue (MB).** The recovery was examined in Sprague-Dawley rats following oral administration (p.o.) and 24h i.v. infusion at a dose level of 20 mg MB/kg body weight. Urine, faeces, organs, expired air, rinse water and infusion site were analysed. The radioactivity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

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

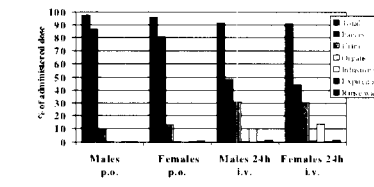

Fig. 2 Mean recovery of radioactivity after oral (gavage) application and 24h i.v. infusion of 20 mg MB/kg body weight in rats.

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

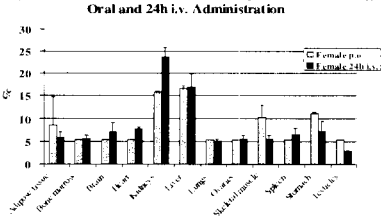

Fig. 3 Distribution of radioactivity in different organs of rats 96h after administration of 20 mg MB/kg body weight. Less than 1% of the dose was found in the different organs.

References:

Hejtmancik MR, Ryan MJ, Toft JD, Persin RL, Kurtz PJ, Chabra RS. Hematological effects in F344 rats and B6C3F1 mice during the 13-weeks gavage toxicity study of methylene blue trihydrate. Toxicol Sciences 2002; 65: 126-134

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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		^{3}MB (mouse, rat)

BSD = Toxicology program for Methylene blue and its photoproducts. Treatments are mainly administered clinically as 24h infusion. ADE = Absorption, Distribution, Excretion. NTP: American National Toxicology Program.

^{3} 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 (TA 98)	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				
^{2}Micronucleus test (rat bone marrow and peripheral blood cells)	Neg.	Neg.		
^{2}UDS test	Neg.	Neg.		

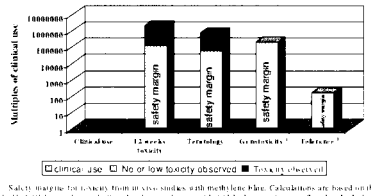
Tab. 4 Treatment conditions *in vivo*: 24h i.v. infusion of 20 mg/kg body weight. Methylene blue (MB) or Azure B (AzB). ^{1}Light-treated MB plasma before removal of MB and its photoproducts.

Study Type	Clinical signs	Thresholds
4 and 12 weeks toxicity rat, mouse (0, 25, 50, 100, 200 mg/kg b.w./rat)	Dose related increase in haematoporines, methaemoglobinemia, Heinz bodies	Threshold for haematological effects (slight methaemoglobinemia) < 25 mg/kg b.w.
Teratology rat (4, 12, 36 mg/kg b.w./day)	Increase in resorption rate. No teratogenic properties	NOEL (foetal organism) 4 mg/kg b.w.
Rabbit (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 and 10 µg MB/kg b.w.	No signs of intolerance	

Tab. 3 Toxicological findings detected during 12 week toxicity, (NTP), developmental toxicity (teratogenicity) and tolerance test after application of methylene blue and light-treated MB plasma (clinical trial).

NOEL: No observed effect level.

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



1 Safety margins for toxicity from in vivo studies with methylene blue. Calculations are based on the thresholds NOEL = no observed effect level (teratogen) and LOAEL = lowest observed effect level (slight methaemoglobinemia <12 weeks toxicity) and a normal clinical exposure of 1 (1 app MB/kg body weight ≙ 6 units MB plasma). ^{2} No toxicity observed, 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