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**研究報告の概要**

○血小板濃厚液の病原体を不活化するUVC照射の効果と限界

背景：輸血による疾患伝播を引き起こす血液製剤の病原体汚染は現在も懸念されている。本試験において、血小板濃厚液のUVC照射の病原体不活化能を検討した。複数のウイルス・細菌不活化の用量依存性を血小板の質への影響と比較した。

試験デザインおよび方法：さまざまな脂質エンベロープ(LE)、非脂質エンベロープ(NLE)ウイルスおよび細菌を用いてUVC照射の効力を調べた。

LEウイルスは、ウシ・ウイルス性下痢ウイルス(BVDV)、ヒト免疫不全ウイルス(HIV)、仮性狂犬病ウイルス(PRV)、伝播性胃腸炎ウイルス(TGEV)と水疱性口内炎ウイルス(VSV)とし、NLEウイルスは、イヌ・パルボウイルス(CPV)とシミアンウイルス40(SV40)、細菌は、表皮ブドウ球菌、黄色ブドウ球菌、大腸菌とセレウス菌とした。スパイクおよび照射後の検体について、残存する感染性と減少率(RF)を調べた。さらに、in vitroでUVC照射が血小板の品質に及ぼす影響を調べた。

結果：UVC量500J/m²では、血小板の品質の変化は許容できるものであった(pH、乳酸産生、CD62P発現、ホスファチジルセリン曝露にて測定)。CPV、TGEV、VSV、表皮ブドウ球菌、黄色ブドウ球菌、大腸菌のRFは高く(>4log)、BVDV、PRV、セレウス菌のRFは中等度(約3log)であり、HIVおよびSV40のRFは低かった(約1log)。cell-freeウイルス、cell-associatedウイルス間で、ウイルスの軽減に差は認められなかった。

結論：UVC照射は、血小板の品質に影響を及ぼさずに、細菌および広範なウイルス(HIVを除く)を不活化することのできる、血小板濃厚液の有望な病原体低減技術である。しかし、HIVのような血液感染性ウイルスに対応するには、UVC法をさらに最適化することが必要である。

**使用上の注意記載状況・その他参考事項等**

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血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

| 報告企業の意見 | 今後の対応 |
|---|---|
| UVC照射は、血小板の品質に影響を及ぼさずに、細菌および広範なウイルス(HIVを除く)を不活化することができるが、HIVのような血液感染性ウイルスに対応するには、UVC法をさらに最適化することが必要であるとの報告である。 | 日本赤十字社では8項目の安全対策の一つとして、不活化技術の導入について、各不活化技術の効果、血液成分への影響、製造作業への影響などについて評価検討を行っている。外国での不活化実施状況や効果、新たな技術、副作用等の情報収集も含め総合的に評価し、導入について関係機関と協議しているところである。 |

(19)

# Potential and limitation of UVC irradiation for the inactivation of pathogens in platelet concentrates

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**BACKGROUND:** Pathogen contamination, causing transfusion-transmitted diseases, is an ongoing concern in transfusion of cellular blood products. In this explorative study, the pathogen-inactivating capacity of UVC irradiation in platelet (PLT) concentrates was investigated. The dose dependencies of inactivation of several viruses and bacteria were compared with the effect on PLT quality.

**STUDY DESIGN AND METHODS:** The potential of UVC irradiation was studied with a range of lipid-enveloped (LE) and non–lipid-enveloped viruses (NLE) and bacteria. LE viruses were bovine viral diarrhea virus (BVDV), human immunodeficiency virus (HIV), pseudorabies virus (PRV), transmissible gastroenteritis virus (TGEV), and vesicular stomatitis virus (VSV). NLE viruses were canine parvovirus (CPV) and simian virus 40 (SV40). Bacteria were *Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli,* and *Bacillus cereus.* After spiking and irradiation, samples were tested for residual infectivity and reduction factors (RFs) were calculated. Furthermore, the effect of UVC irradiation on PLT quality was determined by measuring in vitro quality variables.

**RESULTS:** A UVC dose of 500 J per $m^2$ resulted in acceptable PLT quality (as measured by pH, lactate production, CD62P expression, and exposure of phosphatidylserine) and high RFs (>4 log) for CPV, TGEV, VSV, *S. epidermidis, S. aureus,* and *E. coli.* Intermediate RFs (approx. 3 log) were observed for BVDV, PRV, and *B. cereus.* Low RFs (approx. 1 log) were found for HIV and SV40. No differences in virus reduction were observed between cell-free and cell-associated virus.

**CONCLUSION:** UVC irradiation is a promising pathogen-reducing technique in PLT concentrates, inactivating bacteria, and a broad range of viruses (with the exception of HIV) under conditions that have limited effects on PLT quality. Further optimization of the UVC procedure, however, is necessary to deal with blood-borne viruses like HIV.

Next to careful selection of donors, safety of cellular blood products is primarily based on screening systems to detect markers for viral contamination (e.g., specific antibody testing and nucleic acid testing [NAT]) and bacterial contamination (e.g., BacT/ALERT culturing). In donors experiencing primary virus infections, however, antibodies are not yet detectable in the early phase of infection and NAT might also score negative (the so-called window phase). Moreover, in case of emerging infections, both safety measures will fail. Also, despite screening for bacteria, cases of bacterial transmission have been reported, due to the limited sensitivity of the system.[1] Hence, there is a strong need for in-process steps with broad pathogen-inactivating capacity. An additional requirement for such a step is that the quality of the appropriate blood products is not compromised.

For pathogen inactivation in platelet concentrates (PCs), a number of different techniques involving ultraviolet (UV) light have recently been described. One method utilizes the psoralen compound S-59 (amotosalen hydrochloride) in combination with UVA light. In this photochemical process S-59 intercalates into and binds to

**ABBREVIATIONS:** BVDV = bovine viral diarrhea virus; CA virus = cell-associated virus; CPV = canine parvovirus; LE virus = lipid-enveloped virus; NLE virus = non–lipid-enveloped virus; PC(s) = platelet concentrate(s); PRV = pseudorabies virus; PS = phosphatidylserine; RF(s) = reduction factor(s); SV40 = simian virus 40; TGEV = transmissible gastroenteritis virus; VSV = vesicular stomatitis virus.

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nucleic strands. Upon irradiation with UVA light (320-400 nm), this binding becomes irreversible and the strands are cross-linked, resulting in inactivation of the pathogen.[2]

Another method takes advantage of the properties of a naturally occurring vitamin supplement, riboflavin. Riboflavin interacts between the bases of DNA or RNA and upon irradiation with broadband UV light (265-370 nm), riboflavin oxidizes guanine in nucleic acids, resulting in irreversible damage to the pathogen.[3]

A third method applies a two-step procedure of a photodynamic treatment with thionine and/or light to inactivate free viruses, followed by low-dose UVB treatment to inactivate white blood cells (WBCs) and bacteria.[4] Thionine has a high binding affinity to nucleic acids and specifically binds strongly in G-C–rich regions, but also formation of singlet oxygen is important.[5]

A disadvantage of most of the photochemical and photodynamic treatments developed to date is the need to add and to remove the sensitizer and/or its breakdown products. An alternative in this field is the use of UV light alone. The theoretically optimal wavelength for DNA damage without the need for a photosensitizer is 254 nm, that is, in the UVC range. A UVC technique applying light of 254 nm has, among others, been described by Caillet-Fauquet and colleagues[6] and shown to be effective in treating purified plasma-derived products. UVC mainly causes dimerization of adjacent pyrimidines[7] and the resultant intranucleotide cross-link abrogates subsequent pathogen replication. In addition, UVC also generates free radicals such as singlet oxygen.[8]

In this study, we explored the potential of UVC irradiation for pathogen inactivation in PCs. For a dose of 500 J per $m^2$, we found good pathogen reduction for bacteria and a broad range of viruses (with the exception of human immunodeficiency virus [HIV]) in a mixture of 10 percent plasma and additive solution (Composol-PS, Fresenius HemoCare, Emmer-Compascuum, the Netherlands), with a limited effect on several in vitro variables of platelet (PLT) quality.

## MATERIALS AND METHODS

### Preparation of PCs

PCs were prepared from whole blood–derived buffy coats by a modification of the standard protocol.[9,10] In short, 500 ± 50 mL of blood from nonremunerated, informed donors was collected in 70 mL of citrate-dextrose-phosphate in bottom-and-top blood collection systems (Fresenius HemoCare, Emmer-Compascuum, the Netherlands). After collection, the blood was stored for 12 to 16 hours at 20 ± 2°C.[11] After centrifugation (2780 × g, 8 min), the blood was separated in a plasma, buffy coat, and red cell (RBC) fraction with the aid of a semiautomated component preparation device (Compomat G4, Fresenius HemoCare).[12] Three buffy coats were mixed together and 240 mL of Composol-PS (consisting of 90 mmol/L NaCl, 5 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 27 mmol/L acetate, 23 mmol/L gluconate, 3.2 mmol/L citrate, pH 7.0; Fresenius HemoCare)[13] was added by means of sterile docking. Instead of a low-speed centrifugation step to prepare the PC, the pooled buffy coats were subjected to a high-speed centrifugation (2780 × g, 8 min), the supernatant was removed, and an additional 180 mL of Composol-PS was added to lower the plasma content of the final product. The buffy coats were then subjected to a low-speed centrifugation (5 min, 260 × g), and the PLT-rich supernatant was transferred across a leukoreduction filter (Compostop CS, Fresenius HemoCare) to an empty 1.3-L polyvinylchloride (PVC)-citrate storage bag (Compoflex, Fresenius HemoCare) with the use of a Compomat G4. This procedure had a yield of 60 to 65 percent and resulted in PCs with a residual plasma content of less than 15 percent, as determined by the protein content of the cell-free supernatant and a PLT count of $0.9 \times 10^9$ to $1.2 \times 10^9$ per mL. Residual WBCs and RBCs were less than $0.1 \times 10^6$ and $0.01 \times 10^9$ per mL, respectively, the detection limits of the hematologic counter used (Ac·T 10, Beckman Coulter, Mijdrecht, the Netherlands). After overnight storage at 22 ± 2°C (horizontally shaking with 1 cycle/second; PLT incubator, Model PF96, Helmer, Noblesville, IN), the PC was split into two equal volumes and the plasma concentration was adjusted to 10 percent (by addition of Composol-PS) or 30 percent (by addition of autologous plasma) with equal PLT counts. The final PLT counts were $0.6 \times 10^9$ to $0.8 \times 10^9$ per mL. The PLT was then subjected to UVC irradiation in portions of 5 mL, with or without pathogens added, as described below.

### Bench-scale UVC irradiation

The UVC irradiation was performed as described previously.[6] Briefly, unless indicated otherwise, 5 mL of spiked material was irradiated from above in an open petri dish (84 mm in diameter) placed on an orbital shaker (50 r.p.m.). This resulted in a fluid layer with a thickness of approximately 1 mm. The irradiation device consisted of a UVC lamp with a low-pressure mercury arc (emission line at 254 nm, Germicidal 15T/8, General Electric, Fairfield, CT), a ventilator, filter, photoradiometer with UV sensor, and toothed rack.

### In vitro measurements of PLT quality

After UVC irradiation, 5 mL of PC was transferred to 50-mL culture flasks with the addition of 10 mmol per L glucose, 12 mmol per L $HCO_3^-$, and 5 percent $CO_2$ in the gas phase and stored for 5 days at 22 ± 2°C (horizontal

shaking with 1 cycle/second). Control experiments had indicated that this down-scaled version of PLT storage results in similar loss of PLT quality as observed in whole PCs stored in PVC bags (data not shown). After the storage period, the PLTs were analyzed for pH (at 37°C), CD62P expression, exposure of phosphatidylserine (PS), and lactate production.

The expression of the activation antigen CD62P was measured essentially as described previously[14] with minor revisions. Briefly, PLTs were diluted to a concentration of $3 \times 10^8$ per mL with an electrolyte solution (Isoton II, Beckman Coulter, Mijdrecht, the Netherlands), and 5 µL of PLT suspension was then incubated with 45 µL of Isoton containing CD62P antibody (clone CLB-Thromb/6 conjugated with fluorescein isothiocyanate [FITC], Immunotech, Marseille, France) for 30 minutes at 22°C in the dark. Afterward, the PLTs were fixed by adding 0.5 mL of 0.5 percent (vol/vol) methanol-free formaldehyde (Polyscience, Inc., Warrington, PA; diluted in PBS) and analyzed via flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ). A parallel incubation with FITC-labeled murine immunoglobulin G (Sanquin Reagents, Amsterdam, the Netherlands) was used as negative control.

PS exposure after treatment and storage was determined by means of annexin V binding as follows. PLTs were diluted to a concentration of $1 \times 10^8$ per mL in HEPES medium (136 mmol/L NaCl, 3.2 mmol/L KCl, 2 mmol/L $MgSO_4$, 1.2 mmol/L $K_2HPO_4$, 10 mmol/L HEPES, pH 7.4), and 20 µL of PLT suspension was incubated with 180 µL of HEPES medium in the presence of annexin V-FITC (0.6 µg/mL, added from a stock solution of 250 µg/mL, VPS Diagnostics, Mijdrecht, the Netherlands) and 2.5 mmol per L $CaCl_2$. As negative control, all samples were also stained in the presence of 2.5 mmol per L ethylene glycol-bis(beta-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

Lactate was measured in cell-free supernatants (obtained by centrifugation of PLT samples for 5 min at 14,000 × g) by enzymatic conversion with lactoperoxidase (Trinity Biotech plc, Bray, Ireland). Rates of lactate production were calculated by comparison with cell-free supernatants obtained just before UVC irradiation.

### Selection of viruses and cells

Viruses were selected for being blood-borne and pathogenic and/or to represent various genome types (RNA or DNA, single- or double-stranded) and sizes and with or without lipid envelopes[15,16] (Table 1). Five lipid-enveloped (LE) viruses were used: bovine viral diarrhea virus (BVDV; model for hepatitis C virus [HCV]), HIV (relevant blood-borne virus), pseudorabies virus (PRV; model virus for LE DNA viruses like hepatitis B virus [HBV]), transmissible gastroenteritis virus (TGEV; model for the corona virus causing severe acute respiratory syndrome), and vesicular stomatitis virus (VSV; model for LE RNA viruses). Two non–lipid-enveloped (NLE) viruses were used: canine parvovirus (CPV) and simian virus 40 (SV40), a specific model for parvovirus B19 and a general model for NLE DNA viruses, respectively.

BVDV, strain NADL (VR-534; ATCC, Rockville, MD), was cultured on MDBK cells (CCL-22; ATCC) and titrated on EBTr cells (ID-Lelystad, the Netherlands). HIV, strain HTLV-IIIB (National Cancer Institute, Bethesda, MD), was cultured on H9 cells (National Cancer Institute) and titrated on MT2 cells (Wellcome, Beckenham, UK). PRV, strain Aujeszki Bartha K61 (Duphar, Weesp, the Netherlands), was cultured and titrated on VERO cells (CCL-81; ATCC). TGEV, strain Purdue (VR-763; ATCC), was cultured and titrated on ST cells (CRL-1746; ATCC). VSV, strain Indiana (Sanquin Pharmaceutical Services, Amsterdam, the Netherlands), was cultured and titrated on BHK21 cells (CCL-10; ATCC). CPV, strain 780916 (Erasmus University Rotterdam, Rotterdam, the Netherlands), was cultured and titrated on A72 cells (Erasmus University Rotterdam). SV40, strain PML-2 (VR-821; ATCC), was cultured and titrated on BSC-1 cells (Organon, Oss, the Netherlands).

### Test for cytotoxicity and interference

Before the actual spiking experiments, assays were performed to determine cytotoxic effects of the plasma-Composol mixture on the cell lines used for virus titrations and interference of the plasma-Composol mixture with the titration assays, as described previously.[17] Briefly, threefold serial dilutions of the plasma-Composol mixture

**TABLE 1. Properties of the viruses used in UVC irradiation studies**

| Virus group | Virus | Size (nm) | Virus family | Genome | Size (kb) | Model virus for |
|---|---|---|---|---|---|---|
| LE | BVDV | 37-50 | Flavi | ss RNA | 10-12 | HCV |
| | HIV | 100 | Retro | 2 ss RNA | 2 × 10 | Relevant virus |
| | PRV | 100-200 | Herpes | ds DNA | 140 | Large LE ds DNA viruses |
| | TGEV | 100-120 | Corona | ss RNA | 27-32 | SARS |
| | VSV | 75 × 180 | Rhabdo | ss RNA | 11-15 | Large LE ss RNA viruses |
| NLE | CPV | 18-26 | Parvo | ss DNA | 5 | Human parvovirus B19 |
| | SV40 | 45 | Polyoma | ds DNA | 30 | Human parvovirus B19 |

ss = single-stranded; ds = double-stranded; SARS = severe acute respiratory syndrome.

were prepared and tested on cells to determine cytotoxicity. Then, with the first dilution of the plasma-Composol mixture without cytotoxic effects, a known amount of virus was spiked and incubated. Subsequently, the infectivity of the virus inoculum and the spiked plasma-Composol mixture was measured to determine possible interference with the detection system. In none of the virus assays, interfering effects were observed for the plasma-Composol mixtures used.

### Virus assays

Infectivity was measured in $TCID_{50}$ assays and bulk culture tests. For $TCID_{50}$ assays, 1-in-3 serial dilutions of samples were prepared in culture media and 50-µL (or 0.5 mL for HIV) volumes were tested in eight replicates. To detect small amounts of virus, up to 60 mL of prediluted sample was tested in duplicate bulk culture tests, with 25- and 175-$cm^2$ flasks. BVDV, CPV, PRV, SV40, TGEV, and VSV cultures were inspected microscopically for cytopathic effects at 6, 7, 5, 21, 3, and 4 days postinfection, respectively. HIV cultures were inspected microscopically twice a week for the formation of syncytia until 21 days postinfection. In all experiments, virus titers were calculated by the Spearman-Kärber method[18] with the exception of the factorial design experiment, in which the most probable number[19] method was used, and titers were expressed as $TCID_{50}$ per mL. If all cultures were negative, the titer ($TCID_{50}$/mL) was considered to be less than 1 ÷ total test volume (mL).

Reduction factors (RFs) were calculated by the formula

RF = log (total amount of virus spiked as derived from the reference sample ÷ total amount of virus recovered from the treated sample).

### Bacteria assays

For these studies four different bacteria, representing potential contaminants in PCs as derived from the screening results in the Netherlands,[20] were selected: *Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli,* and *Bacillus cereus* (for each species, two different clinical isolates were used). Bacterial stocks were produced by overnight culture on regular blood agar plates, and colonies were picked and diluted in PBS to a concentration of approximately $3 \times 10^7$ colony-forming units (CFUs) per mL. After treatment, samples were collected and titrated in CFU assays: samples were serially diluted 1 in 10 with ice-cold saline. For *B. cereus*, after UVC irradiation an extra sample was taken and given an additional 10 minutes' incubation at 80°C to check for spores. Subsequently, 10-µL samples were tested on blood agar plates in duplicate and incubated at 35°C during 18 to 24 hours. All samples were tested in serial dilutions and for selected samples also a 200-µL sample was plated, in duplicate. Subsequently the number of bacteria was counted and CFU values were calculated.

RFs were calculated by the formula

RF = log (total amount of bacteria spiked as derived from the reference sample ÷ total amount of bacteria recovered from the treated sample).

## RESULTS

In a first explorative study, efficacy of UVC irradiation for virus inactivation was tested by spiking BVDV in Composol containing varying amounts of residual plasma (5, 10, and 30%) in the absence of PLTs. Given the poor penetration of UVC light in plasma, an experimental setting with a thin plasma layer of approximately 1 mm was chosen, an orbital mixing speed of 50 r.p.m., UVC irradiation at a light intensity of 0.25 mW per $cm^2$, and doses ranging from 250 up to 1000 J per $m^2$ (1000 J/$m^2$ equals exposure for 400 sec). For both the 5 and the 10 percent plasma suspensions, reductions of approximately 6 log were observed at 1000 J per $m^2$, although the kinetics for the 10 percent plasma suspension was clearly slower than the 5 percent plasma suspension. In case of 30 percent plasma, the reduction was approximately 2.6 log at 1000 J per $m^2$ (Fig. 1). Although these data clearly indicated that the plasma concentration should be kept as low as possible, we used 10 and 30 percent plasma in subsequent experiments to avoid compromising PLT quality beyond acceptable limits.

The effect of UVC irradiation on PLT quality was evaluated in a similar experimental setup as above. Before analysis, the PLTs were stored for 5 days to allow detection of long-term effects of the treatment. In Fig. 2, pH, lactate production, CD62P expression, and PS exposure (as

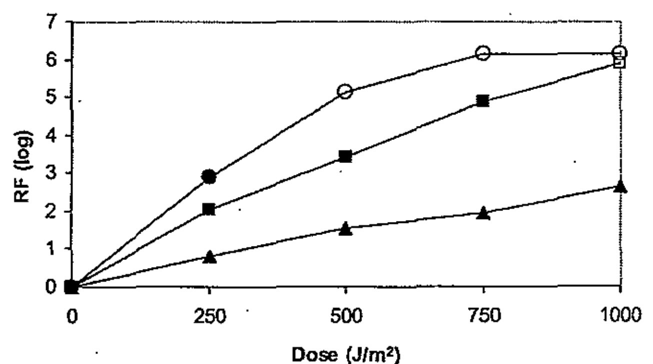


**Fig. 1. Effect of UVC irradiation on BVDV inactivation in 5 (●), 10 (■), and 30 percent (▲) plasma. BVDV inactivation was tested with UVC irradiation in Composol containing 5, 10, or 30 percent plasma; RF values (log) are shown. Light intensity was 0.25 mW per $cm^2$, suspension depth 1 mm, and orbital mixing 50 r.p.m. Open symbols indicate maximal reduction.**

measured by annexin V binding) are shown, as indicators of PLT quality. Increasing doses of UVC resulted in a clear deterioration in all of these variables, especially in the PCs suspended in 10 percent plasma. The pH had decreased from 7.23 to 6.52 with the highest UVC dose tested (Fig. 2A), concomitant with an increase in lactate production (Fig. 2B). CD62P expression increased up to 40 percent after a dose of 500 J per $m^2$ and leveled off at higher doses (Fig. 2C), probably due to shedding of the antigen. PS exposure also increased with increasing doses of UVC, but in 10 percent plasma and with 500 J per $m^2$ of UVC, the PS exposure remained below 30 percent (Fig. 2D). In 30 percent plasma, all PLT quality indicators remained within an acceptable range at all UVC doses tested, but, as noted above, under these circumstances virus inactivation may be limited.

To further optimize the UVC irradiation, three variables, light intensity, plasma percentage, and PLT concentration, were tested in a full factorial design (in duplicate) with inactivation of BVDV as readout, as described previously.[17] In this factorial design, light intensity was tested at 0.25 and 1.0 mW per $cm^2$, percentage plasma at 10 and 30 percent, and PLT concentration at 0 and $1.0 \times 10^9$ cells per mL. The irradiation dose was fixed at 500 J per $m^2$ based on the results obtained in the PLT quality experiments, and the depth was fixed at approximately 1 mm. Results for this series of experiments are shown in Table 2. Linear regression analysis of the complete data set showed that the percentage plasma was the most important determinant of UVC efficacy, with the highest virus reduction in the presence of 10 percent plasma. In addition, light intensity was also a significant parameter with highest virus reduction at 0.25 mW per $cm^2$. Finally, the presence or absence of PLTs did have a significant effect on the virus reduction obtained, but the contributing effect of the PLT concentration was slightly smaller than the other two variables. Given the relative low contribution of the PLT concentration, all subsequent virus studies were performed with the optimal setting as determined by the factorial design (i.e., 10% plasma, 0.25 mW/$cm^2$), but in the absence of PLTs.

To determine the generality of the results obtained with BVDV, we tested BVDV and six additional viruses with different genome type, size, and envelope status in a kinetic setting with irradiation values up to 1000 J per $m^2$. For CPV, TGEV, and VSV, high virus kill was observed, resulting in RF values of greater than 4 log already at 500 J per $m^2$ (sensitive viruses; Fig. 3A). For BVDV and PRV, the

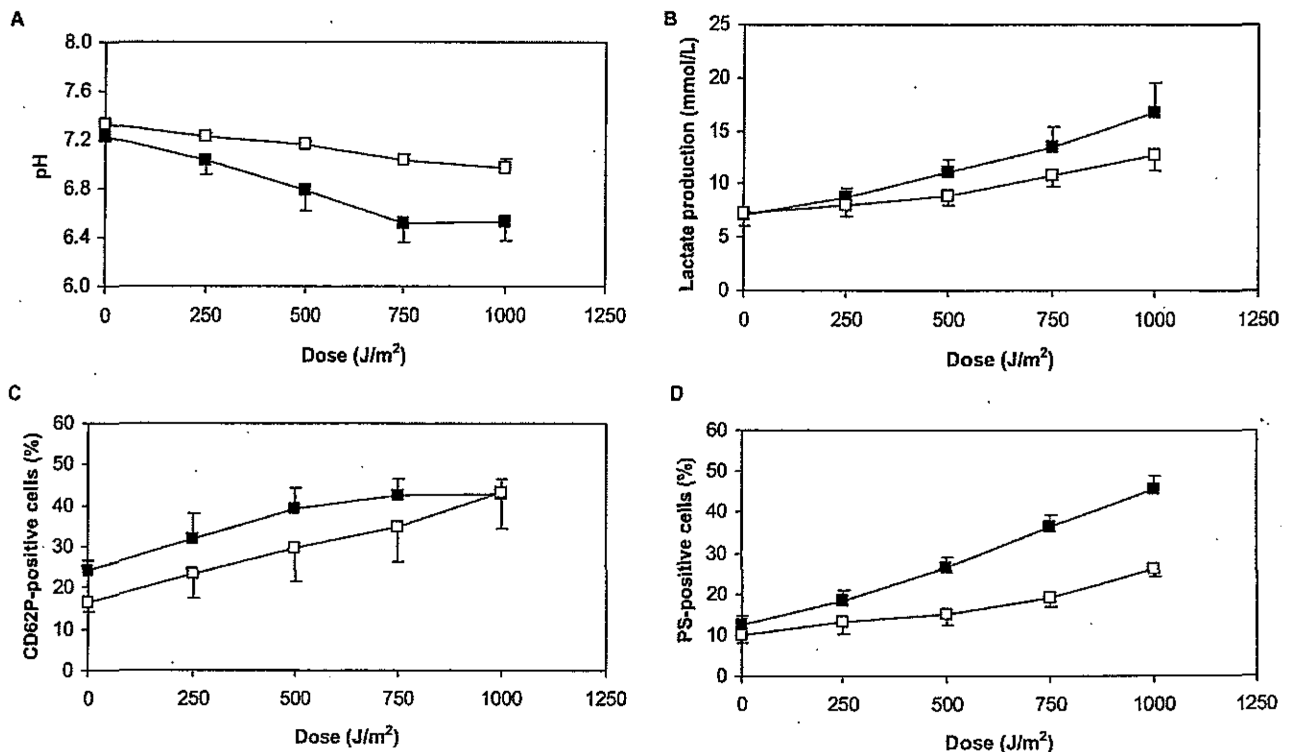


**Fig. 2. Effect of UVC irradiation on PLT quality. PCs were subjected to UVC irradiation as described in the legend to Fig. 1. PLT quality variables after subsequent storage for 5 days were determined as described under Materials and Methods. (A) Medium pH; (B) lactate production; (C) CD62P expression; (D) PS-positive cells. (□) PCs in 30 percent plasma; (■) PCs in 10 percent plasma. Results shown are the mean ± SEM of 6 PCs.**