Does Offline Beat Inline Treatment: Investigation into Extracorporeal Photopheresis

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Keywords

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Abstract

Background: Extracorporeal photopheresis is a therapy based on the induction of apoptosis to cells harvested from peripheral blood, followed by direct retransfusion. Currently, there are two approaches: inline procedures, where cell harvesting, 8-methoxypsoralen (8-MOP) incubation, and UV irradiation is performed with a single device, and offline procedures, with collection in one device, followed by 8-MOP incubation/UV irradiation using a second device. **Study Design and Methods:** In a prospective crossover study, we compared an inline (Cellex, Therakos) with an established offline procedure (Optia, Terumo, and MacoGenic G2, Macopharma) in 6 patients, focusing on cell composition and apoptosis induction after 24 h. In total, 32 photopheresis treatments per device were performed. **Results:** We observed an overall 2-fold higher number of apoptotic “target” cells for each patient with offline treatment. All yields were stratified per patient. Yields were compared as ratio offline/inline for CD3+ (2.5-fold), CD4+ (2-fold), CD8+ (2.8-fold), CD56+ (2.8-fold), CD19+ (1.8-fold), CD15+ (0.5-fold), and CD14+ (2.2-fold) cells. Apoptosis induction was measured after 24 h with Annexin V/7-AAD for early and late apoptosis rates of CD3+ (CD4+, CD8+) and CD56+ cells. CD3+ cells of the inline treatment had an average of 88% (26% early, 62%

late) of apoptotic cells compared to 75% (34% early, 41% late) in the offline treatment. Procedure duration ranged from 80 to 100 min inline, with a maximum of 1,500 mL processed blood, and 125–140 min offline, with at least 3,000 mL processed blood, depending on blood flow. Average hematocrit levels of the products were 2.7% inline versus 1.7% offline. **Conclusions:** The offline procedure, as established in our department, provides more apoptotic cells for treatment. The increased number of mononuclear cells collected outweighs a slightly reduced apoptosis rate after 24 h in comparison to the inline procedure. Besides this, the final decision for one or the other procedure has to take into account additional aspects, such as peripheral white blood cell count, hematocrit, and weight of the patient, required before apheresis, extracorporeal volume, and, last but not least, overall costs. The final criterion, however, has to be the reported clinical efficacy of the system applied.

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Introduction

Extracorporeal photopheresis (ECP) is an immune-modulating therapy based on the induction of apoptosis of cells harvested from peripheral blood (PB) via apheresis. This therapy has been established for several diseases, such as cutaneous T-cell lymphoma, as a second-line therapy of graft-versus-host disease (GvHD), organ transplant rejection, and autoimmune-like diseases [1–

6]. Especially ECP treatment of acute and chronic GvHD has been a subject of intense investigation [7–12]. Guidelines recommend ECP as second-line therapy of both acute and chronic steroid-refractory GvHD [13].

ECP requires an apheresis procedure where collected cells are incubated with 8-methoxypsoralen (8-MOP), subsequently irradiated with UV light, and then retransfused. The 8-MOP treatment is postulated to cause an intercalation of 8-MOP with DNA strands, with a subsequent induction of apoptosis of all treated cells [14–16].

For this therapy, either an inline or an offline procedure can be chosen [17]. The inline procedure is performed with a device that includes both the apheresis and irradiation unit, while the offline procedure collects cells in one device and irradiates them in a second device. This study focused on cellular yield and apoptotic cell dose obtained from extended quality control of both ECP procedures. To the best of our knowledge, this is the first study to compare directly cell composition and number of apoptotic mononuclear cells (MNCs) between an inline and offline system.

Material and Methods

Photopheresis Procedures

For the inline procedure, Cellex (Therakos, Mallinckrodt, Dublin, Ireland) was used, for the offline procedure Optia (Terumo BCT, Tokyo, Japan) with the UV irradiation device MacoGenic G2 (Macopharma, Tourcoing, France), with a protocol already established in our department. Six patients were treated in a crossover design, both inline and offline, with an expected number of 6 photopheresis procedures on each system. Cell analysis was part of the extended quality control of photopheresis products, utilizing the routinely drawn quality control samples.

Six patients were included: 1 with Sezary syndrome and 5 with acute GvHD after hematopoietic stem cell transplantation for acute myeloid leukemia ($n = 2$), myelodysplastic syndrome ($n = 2$), and primary myelofibrosis ($n = 1$). Four were males, 2 were females, body weight ranged from 50 to 82 kg, and hematocrit (HCT) from 25 to 38.2%.

On Cellex, preset 1,500 mL of blood was processed to retrieve the buffy coat with a Latham bowl. The manufacturer's instructions require the patient's HCT to be at least 27% to start the procedure. The volume of the buffy coat is variable based on the WBC count and was diluted within the process with physiological saline solution. 8-MOP was added according to the manufacturer's protocol (product volume $\times$ 0.017), usually totaling around 60 μ g 8-MOP per treatment. Irradiation time was automatically set by the device depending on HCT (20–40 min) at 1.5 J/cm². Product volumes were recorded based on the device's display.

On Optia, the MNC continuous collection program was used to collect 120 mL product with a collection rate of 1 mL/min. The processed blood volume (>3,000 mL) depended on the blood flow. The collected product was transferred into an irradiation bag and diluted to 200 mL with a physiological saline solution. One mL of 8-MOP (Uvadex, Therakos)/100 mL product was applied according to the manufacturer's protocol of the irradiation device, totaling 40 μ g 8-MOP per treatment. Irradiation time was set to 10 min with a dosage of 2 J/cm², based on an HCT <2%.

PB cell counting was obtained before each apheresis procedure, a quality control sample (2 mL) of the product was drawn before and after irradiation. Of each 2-mL sample, 300 μ L were sterilely removed for cell counting. Initial cell composition was measured on FACS Navios (Beckman Coulter, Krefeld, Germany) based on a life gate for CD45-KrO in two tubes, one with CD56-PE, CD3-PB, CD8-AF 700, and CD4-APC, the other with CD14-APC, CD15-PB, and CD19-PE (all Beckman Coulter). Approximately 5×10^5 cells were incubated with a cocktail of 10 μ L of each antibody, except for 5 μ L CD4-APC, for 15 min and subsequently measured. To culture the treated and untreated samples, a sterile filtered culture medium was prepared with 9 mL Iscove basal medium (Biochrom GmbH, Berlin, Germany), 1 mL AB Octaplas (Octapharma), 100 μ L penicillin/streptomycin, and 4 μ L heparin.

Measurement of Apoptosis

Apoptosis was measured for CD3 with the subsets CD4 and CD8 as well as CD56-positive cells. The samples were centrifuged with 433 g/5 min/4 °C. The supernatant was removed, cells were resuspended in 0.5 mL added to a flask with 4.5 mL culture medium and kept in the incubator at 37 °C. No Ficoll was used for separation in order to reduce any additional stress for the cells. Apoptosis was measured at start of culture and after 24 h for both treated and untreated cells. No red cell lysis was used. 5 to 10×10^6 cells were taken from culture into a tube prefilled with 4 °C PBS and centrifuged (433 g/5 min, 4 °C), supernatant was removed, and set to a concentration of 5×10^3 / μ L with ice-cold binding buffer of the Annexin A5 FITC/7-AAD kit (IMMUNOTECH S.A.S., Beckman Coulter). 100 μ L of cell suspension was then incubated with an apoptosis antibody cocktail. The antibody cocktail contained 10 μ L CD45-KrO, 10 μ L CD56-PE, 10 μ L CD3-PacificBlue, 10 μ L CD8-AF 700, 5 μ L CD4-APC, 10 μ L Annexin V-FITC, and 20 μ L 7-AAD, was gently mixed and incubated for 15 min on ice in the dark. After addition of 400 μ L ice-cold binding buffer, the cells were measured within 30 min. Cells were life-gated on CD45 and then gated for CD3, CD4, CD8, CD56, and the gated populations plotted in quadrants for Annexin V-FITC and 7-AAD. Baselines of the quadrants were set based on the day 0 of the untreated cells and kept constant.

Statistical Analysis

A repeated-measurements linear mixed-effects model was performed as a restricted maximum likelihood (REML) approach. Two analyses with the percentage of CD3+ apoptosis and absolute CD3+ apoptotic number as the dependent variable in the model were performed. Separator (Cellex and Optia) and type (untreated or treated) were included as within-group effects. "Patient" was considered as a person-specific random effect. A Wilcoxon signed-rank test was used to compare CD4+ and CD8+ apoptosis. A two-tailed p value of <0.05 was considered as statistically significant. All statistical tests were performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA).

For descriptive analysis, cell yields of apheresis products were averaged for each patient in a first step in order to account for the patient-specific variation, resulting in a relative benchmark of the inline system as 1. Then, the offline/inline ratio of the averaged patient yields was calculated and given as mean and a standard deviation. Apoptosis percentages were averaged over all samples of each patient. Relative number of apoptotic cells was determined by first calculating the total number of apoptotic cells based on the 24-h measurements and then averaging these amounts in a two-step approach, i.e., comparing the averages of each patient, corresponding to the previously described cell yield comparison, providing mean and standard deviation.

Fig. 1. Relative yield of cells per patient ($n = 6$). The yield of each cell type has been stratified per patient; thus, the yield of the inline system (Cellex) is displayed with the value “1.” The variation represents the variation of the averaged values of each patient.

Table 1. Summary of collection data

	Inline ($n = 32$)	Offline ($n = 32$)
Blood volume processed, mL	1,500±0	5,911±711
Collection time, min	102±22	145±19 + 25 irradi./transf.
Product volume, mL	189±23	212±10
Hematocrit, %	2.7±0.7	1.7±0.5
Hemoglobin, g/dL	0.6±0.1	0.3±0.1
Range of MNCs collected/patient, ×10 ⁹	0.36±0.12 to 2.25±0.71	1.02±0.39 to 7.65±2.19

Results

Although the inline ECP system was consistently able to induce higher percentages of apoptotic MNCs, the overall apoptotic cell number was significantly (2-fold) higher in the offline system. Four patients were included with 6 ECP procedures using both the inline and offline ECP systems, one with 5 and one with 3 ECP treatments, totaling 32 ECP procedures for each system. The peripheral WBC count had a range from 1.3 to $11.5 \times 10^3/\mu\text{L}$, with the MNC in a range from 0.15 to $4.07 \times 10^3/\mu\text{L}$. Apheresis data are summarized in Table 1. Apheresis duration ranged from 80 to 100 min using the inline system and from 125 to 140 min using the offline system. Average HCT of the product after addition of physiological saline solution was 2.7% inline versus 1.7% offline. The ratio of offline/inline yield resulted in values as shown in Figure 1: CD3+ (2.5-fold), CD4+ (2.0-fold), CD8+ (2.8-fold), CD56+ (2.8-fold), CD19+ (1.8-fold), CD15+ (0.5-fold), and CD14+ (2.2-fold) cells. Apoptosis measurement on FACS differentiated between early and late apoptosis, early with Annexin V-positive/7-AAD-nega-

tive events, late apoptosis represented by both Annexin V- and 7-AAD-positive events. Measurement required inclusion of a distinct smaller and more fragmented cell cluster, representing mainly late apoptotic cells (Fig. 2). Apoptosis after 24 h was determined for CD3+, CD4+, CD8+, and CD56+ cells (Table 2). Untreated CD3+ cells of the inline procedure had an average of 55% (38% early, 16% late) apoptotic cells and 48% (37% early, 11% late) with offline treatment as shown in Table 2. Treated CD3+ cells had an average of 88% (26% early, 62% late) apoptotic cells in inline treatment and 75% (34% early, 41% late) in offline treatment. REML analysis was applied on apoptotic CD3+ cells with inline outperforming offline with a $p < 0.001$. CD4+ cells were significantly less susceptible to apoptosis (inline $84 \pm 11\%$, offline $59 \pm 16\%$) than CD8+ cells (inline $91 \pm 8\%$, offline $82 \pm 10\%$; Wilcoxon signed-rank test, $p < 0.001$).

The predicted number of apoptotic cells is shown in Figure 3 and Table 2, based on the apoptosis measurement of treated cells, without adjustments based on the values of untreated cells. The calculation resulted in an averaged 2-fold increased number of apoptotic MNCs for

Fig. 2. After 24 h, two distinct populations of cells can be identified in the forward scatter/side scatter plot. The smaller, less granulated events represent the late apoptotic cells and have to be included in the analysis.

the offline in comparison with the inline treatment. The difference in these numbers was significant (REML, $p < 0.001$). An adjustment based on the observed apoptosis of untreated cells increased the dosage in favor of the offline treatment. The offline system required an additional time of 35 min for handling, incubation with 8-MOP, irradiation, and retransfusion, the treatment time totaling to almost 3 h in contrast to around 100 min for the inline procedure.

Discussion

A clinical department offering photopheresis treatment has to decide which system to use under various points of view: efficacy of the in vitro induced treatment

and its clinical outcome, admission criteria for patients to undergo treatment, surveillance of the patient during treatment, user friendliness of the procedures, and, last but not least, the costs involved per treatment.

Although extracorporeal photopheresis treatment is widely used as a second-line treatment of GvHD, the exact “pharmaceutical” mechanism of this therapy remains unclear. There are no hard facts or data points that can be used to predict the clinical efficacy of an ECP apheresis product [18, 19], although there is some indication that distinct cell populations within the recipient might be a prerequisite for successful treatment [20]. A factor that can be measured as quality control of ECP therapy is the dose of apoptotic MNCs [21] or the number of lymphocytes and MNCs/kg body weight given per single procedure [22]. The main factor contributing to the number of

Table 2. Apoptosis ($n = 32$) of cells immediately after culture and after 24 h and relative number of apoptotic cells

	CD3+ apoptosis, %			CD4+ apoptosis, %			CD8+ apoptosis, %			CD56+ apoptosis, %		
	total	early	late	total	early	late	total	early	late	total	early	late
<i>Apoptosis at 1 h after cultivation ($n = 32$)</i>												
Untreated												
Inline	27±24	26±23	1±1	19±19	17±18	1±2	33±28	32±28	1±1	15±13	14±13	0±0
Offline	16±9	16±9	0±1	10±5	9±5	1±1	19±11	19±11	0±0	7±3	6±3	0±0
8 MOP/UV												
Inline	32±24	31±23	1±1	22±18	21±17	1±1	38±28	37±28	1±1	17±13	16±13	0±0
Offline	18±12	18±12	0±0	11±7	11±7	0±1	22±14	21±14	0±0	9±6	9±6	0±0
<i>Apoptosis at 24 h after cultivation ($n = 32$)</i>												
Untreated												
Inline	55±20	38±13	16±11	41±15	30±11	11±7	63±21	44±13	19±13	39±21	31±19	8±7
Offline	48±17	37±13	11±8	34±15	26±12	8±6	57±16	43±12	13±9	34±16	29±16	5±6
8 MOP/UV												
Inline	88±11	26±9	62±11	84±11	23±10	61±12	91±8	29±8	62±11	81±15	26±10	55±15
Offline	75±12	34±10	41±13	59±16	29±10	30±14	82±10	38±10	44±14	73±17	29±11	44±17
	CD3+ dosage			CD4+ dosage			CD8+ dosage			CD56+ dosage		
	total	early	late	total	early	late	total	early	late	total	early	late
Offline/inline	2.1±1.21	2.9±0.76	1.8±1.42	1.5±0.93	2.5±1.15	1.2±0.97	2.4±1.1	3.3±0.83	2.1±1.29	2.6±1.46	3.2±1.63	2.3±1.33

Apoptosis values of untreated and MOP/UV treated cells after start of culture and after 24 h are displayed in the upper section, the resulting relative dosages, stratified per patient, are listed in the lower section. Early apoptosis is represented by Annexin V-positive, 7-AAD-negative cells, late apoptosis by both Annexin V- and 7-AAD-positive cells. The ratio of 2.1 indicates a 2.1-fold number of apoptotic cells in the offline in comparison to the inline system.

Fig. 3. Average relative apoptotic dose per patient after psoralen/UVA and 24 h. The dosage of each cell type has been stratified per patient; thus, the yield of the inline system (Cellex) is displayed with the value “1.” The variation represents the variation of the averaged values of each patient. Inline: blue; offline: red. The lighter colors represent the early, the darker colors the late apoptotic cells.

apoptotic MNCs is the concentration of the respective “target” cells in the PB and, thus, the yield of cells during apheresis (data not shown). We were able to collect constantly and significantly more MNCs with the offline system Optia/MacoGenic in comparison to the inline Cellex system ($p < 0.001$). This difference has its origin, on the one hand, in the already established offline procedure in our department with an endpoint of 120 mL of apheresis product, which resulted in a higher processed blood volume (up to 4-fold) in comparison to the inline procedure. On the other hand, the inline procedure followed recommendations released by the manufacturer with a fixed processed blood volume of 1,500 mL. The upper limit of the processed volume is restricted to 2,000 mL. The inline system collected more granulocytes, as also shown by other authors [23, 24]. Recent findings indicate a promoting role of neutrophils at GvHD onset [25]; however, there is a lack of data on whether apoptotic neutrophils might have any effect in this regard. Although apoptosis is an ongoing process, the time point 24 h after irradiation treatment provides the largest increase in apoptosis [26] and was therefore used as a representative marker of apoptosis. In general, apoptosis levels were consistent with findings published by other groups [27]. Apoptosis induction was significantly higher in the inline system ($p < 0.001$), which also had a higher “background” apoptosis of untreated cells after 24 h compared to the offline

procedure. The observed apoptosis level of untreated cells was higher than published elsewhere [26, 28]. The underlying effect for this slight difference in apoptosis is unclear and has not been further investigated; one explanation could be the higher number of granulocytes, which were co-cultured during the 24-h period.

The increased amount of MNCs collected with the offline method by far outweighed the lower apoptosis ($p < 0.001$), resulting in a more than 2-fold higher dose of apoptotic cells for the recipient. In that regard, the offline system clearly outperforms the inline system. However, no significant correlation between apoptotic cell numbers reinfused and clinical response has been reported so far.

An unexpected observation is the difference in CD4+ and CD8+ apoptotic cell levels that can be seen both in inline and offline treatment and which is highly significant ($p < 0.001$), contradicting a report by another group [26].

The collection volume of the offline system was kept at 120 mL because of the already established procedure. With a continuous collection system, this volume can be adjusted if indicated. The inline system, however, cannot regulate the apheresis procedure based on the product volume and features a fixed volume of processed blood by the manufacturer.

The apheresis procedure of the offline system requires a constant extracorporeal volume (290 mL), while in the inline system, the extracorporeal volume correlates indirectly with the HCT. Below an HCT of 39%, the inline system results in an extracorporeal volume >290 mL. In consequence, some patients with a low HCT and body weight, who can undergo apheresis in the offline system, require a transfusion of at least 1 red blood cell unit before starting the inline apheresis procedure according to the manufacturer's instructions. Patients monitored in this paper did not require transfusion before treatment, but in other cases, treatment had to be delayed because of low HCT. However, patient-specific information (weight, HCT) itself is utilized only by the Optia system, which prevents an excessive infusion rate of anticoagulant. This is not controlled in the Cellex system. Handling of 8-MOP incubation and irradiation was more comfortable with the inline system. In the inline system, only one step, the addition of 8-MOP, is required. The offline system requires labelling of the irradiation bag, addition of a saline solution and 8-MOP, incubation, data entry at the irradiation device, irradiation, and retransfusion.

Time required per patient from start of apheresis to end of retransfusion with the inline system was 80–100 min, while the offline system required about 3 h in our setup. Additionally, the inline device is limited to ECP, while the offline device can be utilized in a whole spectrum of apheresis procedures.

Conclusion

Based on our findings, there is no clear-cut recommendation for one system or the other. In any case, the documented and published clinical efficacy of each system is a prerequisite for using it for treatment. If patients have low MNC PB counts, the offline Optia/MacoGenic combination outperforms the inline Cellex system. Still, no significant correlation between apoptotic cell numbers reinfused and clinical response has been reported so far. The offline procedure is preferable if ECP requires a high number of lymphocytes/MNCs/kg body weight treated [22] or if additional apheresis procedures are part of the routine services of an institution. With close to normal peripheral MNC counts, the inline system has its benefits. If sufficient cells can be collected and time is relevant, the inline system will be preferred.

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Statement of Ethics

The study protocol was part of the established extended quality control of photopheresis products and did not require an ethics approval.

Disclosure Statement

The authors declare no competing financial interests.

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Author Contributions

W.H., S.S., A.G.-S., H.G., and P.S. provided substantial contributions to study conception and design; provided substantial contributions to acquisitions of data; provided substantial contributions to analysis and interpretation of data; drafted the article or critically revised it for important intellectual content; and provided final approval of the article to be published.

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