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Increased Responsiveness of Stored Platelets after Short-Term Refrigeration

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Platelets · Storage · Refrigeration · Vasodilator-stimulated phosphoprotein · Aggregation

Abstract
Introduction: Refrigeration of platelets is considered to provide advantages in therapy of acute hemorrhage due to increased platelet responsiveness. The alleviation of inhibitory signaling caused by cold temperature (CT) has been identified as an important mechanism contributing to enhanced platelet reactivity, detectable in freshly prepared platelets within 1 h of cold storage. The aim of this study was to confirm the effects of short-term refrigeration in platelets from apheresis-derived platelet concentrates (APC). ***Methods:*** APC were stored under standardized conditions for 1 day or for 2 days at room temperature and then refrigerated for 1 h, followed by sampling of platelets for analysis. Platelet reactivity was measured by aggregation studies using threshold concentrations of different agonists and by detection of fibrinogen binding using flow cytometry. The exploration of inhibitory signaling comprised the detection of VASP phosphorylation using flow cytometry or Western blot and the measurement of cyclic nucleotide levels. ***Results:*** Aggregation responses induced with ADP, collagen, or thrombin receptor-activating peptide-6 (TRAP-6) were increased in APC after cold storage for 1 h, associated with elevated TRAP-6-induced fibrinogen binding. VASP phosphorylation levels were decreased after cold exposition, detectable in 1-day- and 2-day-stored APC with flow cytometry, and in 2-day-stored APC with Western blot technique. Induced cGMP levels were lower after storage at CT in APC on day 1 and on day 2, whereas cAMP levels were reduced on 2-day-stored APC. ***Conclusion:*** Short-term refrigeration for 1 h is sufficient to induce an attenuation of inhibitory signaling, accompanied with increased aggregation responses in APC stored for up to 2 days. The "on demand" refrigeration of PC may be a reasonable approach for the preparation of platelets with enhanced responsiveness to treat patients with hemorrhage more effectively, which should be further addressed in consecutive studies.

Introduction

Platelet concentrates (PC) are important blood components used for the treatment or prophylaxis of hemorrhage caused by thrombocytopenia or platelet function disorders [1]. After manufacturing, PC are commonly stored in gas-permeable containers, under continuous agitation on flat-bed shakers, and at room temperature (RT, 20–24°C) [2]. Ex vivo, platelets experience a loss of function, a process called storage lesion, influenced, e.g., by sampling, manipulation, or storage conditions [3]. Therefore, different approaches have addressed the maintenance of platelet integrity under storage, among them the optimization of storage temperature [4].

Until the 1980s, storage of PC was additionally performed at cold temperature (CT, 2–6°C) with the advantage of lower risk of bacterial growth [4] and the opportunity for prolonged storage [5]. Furthermore, cold-stored platelets are considered to be beneficial in the

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treatment of acute hemorrhage, since they show a higher hemostatic reactivity [6–8].

However, storage at CT is associated with short circulation and rapid clearance of transfused platelets in vivo, which are caused by clustering of glycoprotein Ib and desialylation on the platelet surface [9, 10]. In consequence, RT has become the standard in platelet storage, since most patients requiring transfusions of PC suffer from chronic thrombocytopenia and benefit from long circulation periods of platelets [11]. In addition, the provision of two different product types – stored at RT or at CT – for different indications would be an organizational challenge.

In previous studies, we could show that higher platelet reactivity upon cold storage is mediated by attenuation of inhibitory signaling, contributing to emphasized aggregation responses and increased platelet adhesion under flow conditions [12]. Interestingly, the cold-induced effects are initiated within 1–2 h of storage CT, characterized by decreasing levels of induced vasodilator-stimulated phosphoprotein (VASP) phosphorylation levels, as an essential marker of platelet inhibition [10]. In this way, short-term refrigeration for 1–2 h has the capacity to generate platelets with increased responsiveness, which is potentially beneficial in patients with acute hemorrhage [12].

These previous experiments were performed with freshly prepared platelet-rich plasma from citrated whole blood [12]. Therefore, the rationale of this study was to expose RT-stored apheresis-derived platelet concentrates (APC), representing the blood product used under clinical conditions, to CT for a short period of time (short-term refrigeration for 1 h), as a procedure to obtain platelets with higher hemostatic capacity "on demand" before transfusion.

Since "fresh" APC, being less affected by storage lesion, would be used preferably for clinical situations with acute bleeding, APC stored at RT for 1 day or for 2 days were used for this study. APC continuously stored at RT served as controls for comparison. The analytical procedures comprised aggregation studies, the detection of fibrinogen binding (activating capacity), and the measurement of VASP phosphorylation and cyclic nucleotide concentrations (inhibitory capacity).

Materials and Methods

Materials

ADP and thrombin receptor-activating peptide-6 (TRAP-6) were obtained from Haemochrom Diagnostica GmbH (Essen, Germany), and collagen reagent HORM (a suspension of native equine tendon collagen type I) was from Takeda (Linz, Austria). Ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetra-acetic acid (EGTA), prostaglandin E1 (PGE1), Ponceau S, and Tyrode's salt solution were from Sigma-Aldrich Chemie/Merck (Munich, Germany). Nitric oxide (NO) donor DEA/NONOate (DEA/NO) was from Enzo Life Sciences GmbH (Lörrach, Germany). FITC-conjugated mouse anti-fibrinogen antibody and an appropriate FITC-conjugated isotype control were from BioCytex SARL (Marseille, France). Mouse monoclonal FITC-conjugated phospho-VASP-Ser^{239}, unconjugated phospho-VASP-Ser^{239}, and unconjugated phospho-VASP-Ser^{157} antibodies were from Nanotools (Teningen, Germany). StarBright Blue 520-conjugated goat anti-mouse antibody was from Bio-Rad Laboratories, Inc. (Munich, Germany).

Manufacturing and Storage of APC

APC ($2.0–2.5 \times 10^{11}$ platelets in 250 mL of plasma) were collected using Trima Accel devices with software Version 7.0 and the Trima Accel LRS Platelet, Plasma Set (Terumo BCT, Lakewood, CO, USA) according to current guidelines and the approval of regulatory authorities. The ratio of inlet blood volume to anticoagulant (ACD-A) was 10:1.

Corresponding pairs of APC from one donor (double-dose donation) were stored under standardized blood bank conditions at RT (20–24°C). One day (day 1) and 2 days (day 2) after manufacturing, one of the two APC was divided into two parts under sterile conditions by transferring each half of the material into a storage container with half volume. One part of APC was then stored for 1 h in the refrigerator at 4°C (2–6°C) without shaking (CT), whereas the second part was left at RT under agitation. After that, both parts were left quiescent at RT for 30 min followed by sampling for analysis.

Measurement of Temperature in the APC Storage Container at CT

The course of temperature in the liquid content of (split and total) APC during storage at CT was measured with the temperature sensor Saveris 2-T2 (Testo SE & Co. KGaA, Neustadt-Titisee, Germany). The values were recorded every 30 s over a period of 1 h after placing the APC in the refrigerator at 4°C (2–6°C).

Measurement of Metabolic Parameters and Platelet Count in APC

In APC, the parameters potassium, pH, glucose, and lactate were detected with the blood gas system cobas b 123 POC, software version 4.14 (Roche Diagnostics GmbH, Mannheim, Germany), and platelet count with the hematology analyzer KX21N (Sysmex GmbH, Norderstedt, Germany).

Platelet Aggregation

Light transmission aggregometry was determined with material from APC diluted with autologous plasma to 3×10^8 platelets/mL under continuous stirring at 1,000 rpm and 37°C using an APACT 4004 aggregometer (LabiTec, Ahrensburg, Germany). Individual threshold concentrations of agonists were used to induce submaximal, reversible aggregation (5–15 μM ADP, 2.5–7.5 μg/mL collagen, and 10–20 μM TRAP-6 on day 1; 7.5–20 μM ADP, 5–20 μg/mL collagen, and 10–25 μM TRAP-6 on day 2). Threshold concentrations were first assessed for RT-stored PC (as the lowest agonist concentrations induced weak or reversible aggregation responses). Consecutively, the same concentration was used to induce aggregation in material from short-term refrigerated APC for comparison.

Flow Cytometric Detection of Platelet VASP Phosphorylation

For detection of VASP phosphorylation, according to [13], 30 μL APC was stimulated with PBS buffer, 3 μM DEA/NO, or 1 μM PGE1 (final concentration) for 5 min at 37°C, followed by 10 min

fixation with 2.5% formaldehyde at RT. Consecutively, the samples were centrifuged for 1 min at 20,000 *g*, and the pellets were permeabilized in 50 µL of PBS/BSA/Glc buffer (Dulbecco's PBS [Ca^{2+}, Mg^{2+} free], 5.5 mM D-glucose, 0.5% BSA) containing 0.2% Triton X-100 for 10 min at RT. Permeabilized platelets were stained for 30 min at RT with 0.5 µg of FITC-conjugated phospho-VASP-Ser^{239} antibody in the dark. Then, samples were diluted with 500 µL of PBS/BSA/Glc buffer and analyzed by flow cytometry using a FACS Calibur flow cytometer from Becton Dickinson (Franklin Lakes, NJ, USA) and the CELL-Quest software, version 6.0. The platelet population was identified by its forward and side scatter distribution. 20,000 events were analyzed for the calculation of mean fluorescence.

Flow Cytometric Detection of Fibrinogen Binding

For determination of basal and TRAP-6-stimulated fibrinogen binding, 10 µL of APC were initially stained with 10 µL of FITC-conjugated anti-fibrinogen antibody for 10 min at 37°C. After that, platelets were incubated with PBS (basal fibrinogen binding) or with 5 µM TRAP-6 for 5 min at 37°C and fixed with 1% formaldehyde (final concentration) for 10 min at RT. Samples stained with 10 µL FITC-conjugated isotype control and stimulated with PBS or with TRAP-6 served as isotype controls. Finally, the samples were diluted with 300 µL of PBS/BSA/Glc and analyzed by flow cytometry as described above.

Preparation of Washed Platelets for Western Blot Analysis

Washed platelets were prepared as described [14]. After addition of 3 mM EGTA, 10 mL APC were centrifuged at 430 *g* for 10 min. Then, pelleted platelets were washed once in CGS buffer (120 mM sodium chloride, 12.9 mM trisodium citrate, 30 mM D-glucose, pH 6.5) and resuspended in Tyrode's buffer to the final concentration of 3×10^8 platelets/mL. After resting for 15 min in a water bath at 37°C and addition of 1 mM $CaCl_2$, washed platelets were used for stimulation.

Western Blot Analysis

VASP phosphorylation in washed platelets was additionally determined by Western blot analysis, as previously described [15]. For this purpose, 100 µL of washed platelet suspension was supplemented with 1 mM $CaCl_2$ followed by stimulation with buffer, 1 µM DEA/NO, or 1 µM PGE1 for 5 min at 37°C. Samples were lysed with 50 µL SDS loading buffer (200 mmol/L Tris-HCl, pH 6.7, 10% 2-mercaptoethanol, 6% SDS, 15% glycerol, 0.03% bromophenol blue) and boiled for 5 min at 95°C. The cell lysates were loaded onto the gel, separated by SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were incubated with mouse monoclonal phospho-VASP Ser^{239} (clone 16C2) and phospho-VASP Ser^{157} (clone 5C6) antibodies overnight at 4°C. For visualization of the signal, goat anti-mouse IgG conjugated with StarBright Blue 520 was used as secondary antibody, followed by detection with ChemiDoc MP imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analysis with the corresponding Image Lab Software Version 6.0. Band intensities were given in arbitrary units (AU), which were normalized to loading control (visualized by Ponceau S staining).

cAMP and cGMP Measurement

For measurement of the basal and the stimulated platelet cAMP or cGMP levels, 200 µL of washed platelets (3×10^8 platelets/mL) were supplemented with 1 mM $CaCl_2$ followed by 5 min stimulation with buffer (basal concentration), 1 µM DEA/NO (stimulated cGMP), or 1 µM PGE1 (stimulated cAMP) at 37°C. After that, platelets were lysed by addition of 20 µL of cold 50% trichloroacetic acid. The precipitates were removed by centrifugation at 10,000 *g* for 10 min at 4°C. After trichloroacetic acid extraction with water-saturated ether, the levels of cAMP and cGMP were determined by cGMP and cAMP (enzyme-linked immunoassay) EIA kits, following the manufacturer's instructions (Cayman Chemical, Hamburg, Germany).

Statistical Analysis

Descriptive data were calculated with GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Data distribution analysis was performed using the Shapiro-Wilk test. Differences of variances between groups were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey-Kramer test for normally distributed data, or by paired Student's *t* test as appropriate. $p < 0.05$ was considered statistically significant.

Results

Metabolic Parameters and Platelet Count in APC Are Maintained after Short-Term Refrigeration

Platelet count, potassium levels, glucose, and lactate concentration were comparable between RT-stored and CT-stored APC. Only pH values were marginally higher after storage at CT in APC on day 1. Details are shown in Table 1.

The Temperature in Split and Total APC Comparably Decrease to Less than 6°C within 1 h of Cold Storage

The temperature in cold-stored total APC and in split APC continuously decreased under cold storage (at 2–6°C) comparably. In average, the values reached 10°C after 26.8 ± 2.8 min (total APC) or after 24.3 ± 1.0 min (split APC), and the "target" level of 6°C after 53.7 ± 4.3 min (total APC) or 49.8 ± 2.3 min (split APC), respectively. The course of temperature during 1 h of refrigeration is shown in online supplementary Figure S1 (for all online suppl. material, see https://doi.org/10.1159/000533274).

Aggregation Responses Are Enhanced upon Cold Storage for 1 h

The effect of cold exposition for 1 h on platelet responsiveness was measured by light transmission aggregometry (Fig. 1). ADP-induced threshold aggregation increased after CT storage from 21.6 ± 6.6% to 37.6 ± 8.8% in 1-day-stored APC, and from 32.7 ± 6.8% to 44.8 ± 9.6% in 2-day-stored APC (Fig. 1a). Using collagen as platelet agonist, aggregation was also enhanced in cold-stored platelets, from 22.3 ± 10.1% to 64.4 ± 2.0% in 1-day-stored APC, and from 26.9 ± 10.4% to 66.8 ± 6.8% in 2-day-stored APC (Fig. 1b). Threshold aggregation induced by TRAP-6 was supported by cold exposition, rising from 34.7 ± 8.3% to 58.4 ± 6.9% in 1-day-stored APC, and from 41.1 ± 7.7% to 56.6 ± 5.8% in 2-day-stored APC (Fig. 1c).

Table 1. Metabolic parameters and platelet count of APC

Parameter	Unit	APC day 1, RT	APC day 1, CT	APC day 2, RT	APC day 2, CT
Platelets	$\times 10^3/\mu L$	1,087±269	1,148±281	1,093±268	1,079±265
Potassium	mmol/L	3.14±0.08	3,14±0.71	3.15±0.09	3.17±0.09
pH (22°C)	--	7.52±0.02	7.55±0.02*	7.56±0.02	7.57±0.03
Glucose	mg/dL	343±7	342±6	329±6	329±7
Lactate	mmol/L	2.3±0,1	2.3±0.2	3.8±0.3	3.8±0.2

Data are shown as mean ± SEM; $n = 6$. APC, apheresis-derived platelet concentrate; RT, room temperature; CT, cold temperature for 1 h. *$p < 0.05$, compared with APC on day 1 at RT.

Fibrinogen Binding Is Promoted by Refrigeration for 1 h

In APC on day 1, cold storage did not influence basal fibrinogen binding (Fig. 2). In APC stored for 2 days, basal values were slightly elevated from 21.6 ± 5.2 MFI to 26.4 ± 6.2 MFI after cold exposition. Storage at CT provoked an increase of TRAP-6-stimulated fibrinogen from 241.4 ± 57.6 MFI to 309.3 ± 73.4 MFI in 1-day-stored APC and from 210.8 ± 52.6 MFI to 307.9 ± 73.8 MFI in 2-day-stored APC. For illustration, an exemplary histogram of flow cytometry is shown in online supplementary Figure S2.

Cold Exposition for 1 h Induces the Suppression of Induced VASP Phosphorylation

Initially, VASP phosphorylation levels at Ser^{239} were measured by flow cytometry (Fig. 3). In APC stored for 1 day or for 2 days, basal values remained unchanged after cold exposition (Fig. 3a, b). Storage at CT for 1 h reduced DEA/NO- and PGE1-induced VASP phosphorylation in both, 1-day- and 2-day-stored APC. DEA/NO-induced levels were decreased from 94.7 ± 28.2 MFI to 72.6 ± 24.0 MFI in 1-day-stored APC and from 122.1 ± 27.4 MFI to 88.9 ± 22.0 MFI in 2-day-stored APC (Fig. 3a, b). PGE1-induced VASP phosphorylation was diminished from 161.4 ± 28.3 MFI to 141.0 ± 25.5 MFI on day 1 and from 157.6 ± 25.2 MFI to 115.2 ± 19.0 MFI on day 2, respectively (Fig. 3a, b).

VASP phosphorylation was additionally investigated by Western blot analysis (Fig. 4). In APC stored for 1 or 2 days, basal VASP phosphorylation at Ser^{239} and at Ser^{157} was not significantly reduced after cold storage for 1 h (Fig. 4a, b).

In APC stored for 1 day, VASP phosphorylation at Ser^{239} reached 2.5 ± 0.7 AU after stimulation with DEA/NO and 1.9 ± 0.5 AU after stimulation with PGE1 (Fig. 4a). After storage at CT for 1 h, the levels were similar. However, in APC stored for 2 days, induced VASP phosphorylation at Ser^{239} showed slightly but constantly lower levels after cold exposition for 1 h, with 2.1 ± 0.5 AU (DEA/NO) and 1.7 ± 0.4 (PGE1), compared to 2.5 ± 0.7 AU or 2.1 ± 0.6 AU at continued RT storage.

Analyzing Ser^{157} in APC stored at RT for 1 h, phosphorylation levels were 1.3 ± 0.4 AU upon stimulation with DEA/NO and 1.2 ± 0.3 AU upon stimulation with PGE1. After exposition at CT for 1 h, the levels remained unchanged (Fig. 4 b). In APC stored for 2 days, VASP phosphorylation was slightly reduced after cold exposition for 1 h, from 1.1 ± 0.3 AU to 1.0 ± 0.3 AU (DEA/NO) and from 1.4 ± 0.4 AU to 1.1 ± 0.3 AU (PGE1), respectively (Fig. 4b).

Induced Cyclic Nucleotide Concentrations Are Reduced after Storage at CT

Inhibitory signaling was further investigated by the detection of basal and DEA/NO- or PGE1-induced cyclic nucleotide concentrations, measured by ELISA technique (Fig. 5). Basal values were not affected by refrigeration for 1 h and comparable on day 1 and on day 2 of APC storage (Fig. 5a, b).

PGE1-induced increase of cAMP levels of 4.9 ± 0.5 fold in 1-day-stored APC, remaining stable with 4.7 ± 0.6 fold elevation after CT storage for 1 h. In contrary, in 2-day-stored APC, the PGE1-induced elevation dropped from 7.6 ± 1.1 fold to 3.9 ± 0.8 fold (Fig. 5a). The induction of cGMP levels was reduced after cold storage for 1 h, from 15.8 ± 3.7 fold to 4.4 ± 0.7 fold in 1-day-stored platelets, and from 19.2 ± 3.5 fold to 11.9 ± 2.9 fold in 2-day-stored APC (Fig. 5b).

Discussion

In this first study addressing short-term refrigeration of stored APC, it was able to demonstrate a supporting effect on platelet responsiveness. Similar to freshly prepared platelets [12], cold exposition of stored APC for 1 h led to enhanced aggregation responses induced by different activators like ADP, collagen, or TRAP-6 used in threshold concentrations. The observation of facilitated aggregation upon refrigeration is also illustrated by emphasized fibrinogen binding since aggregation is based on the conformational change of

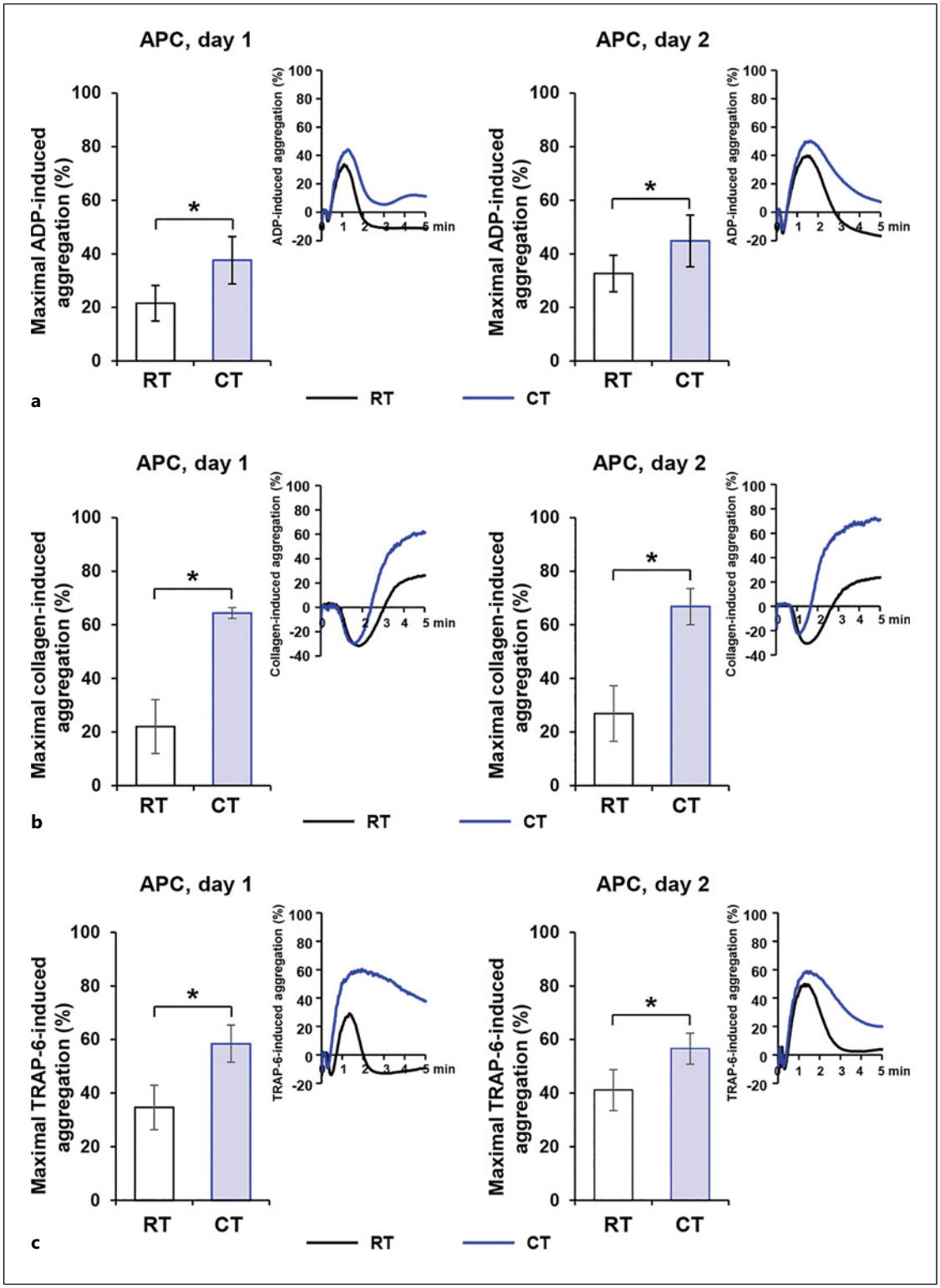

Fig. 1. Short-time refrigeration enhances platelet aggregation in stored APC. Samples from APC, continuously stored at RT or additionally at CT for 1 h, were stimulated with individual threshold concentrations of ADP (**a**), collagen (**b**), or TRAP-6 (**c**), followed by measurement of light transmission aggregometry for 5 min. Results in the histograms are presented as mean of maximal aggregation ± SEM, accompanied by a representative aggregation curve; $n = 6$; $^*p < 0.05$.

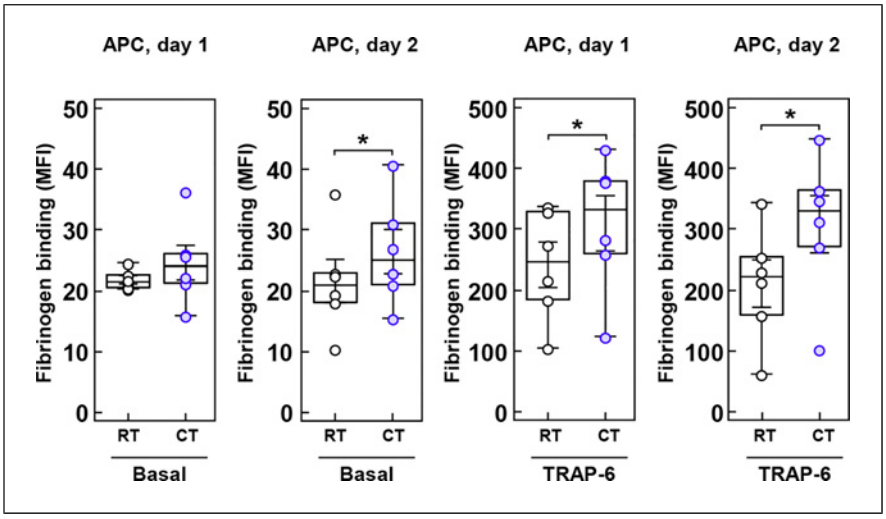

Fig. 2. Basal and TRAP-6-stimulated fibrinogen binding is increased in short-time refrigerated APC. The box-and-whisker plots show the distribution of basal and TRAP-6-induced fibrinogen binding in RT-stored and short-term refrigerated APC, measured by flow cytometry and given as MFI (mean fluorescence intensity). Results are presented as mean ± SEM; $n = 6$; $^*p < 0.05$.

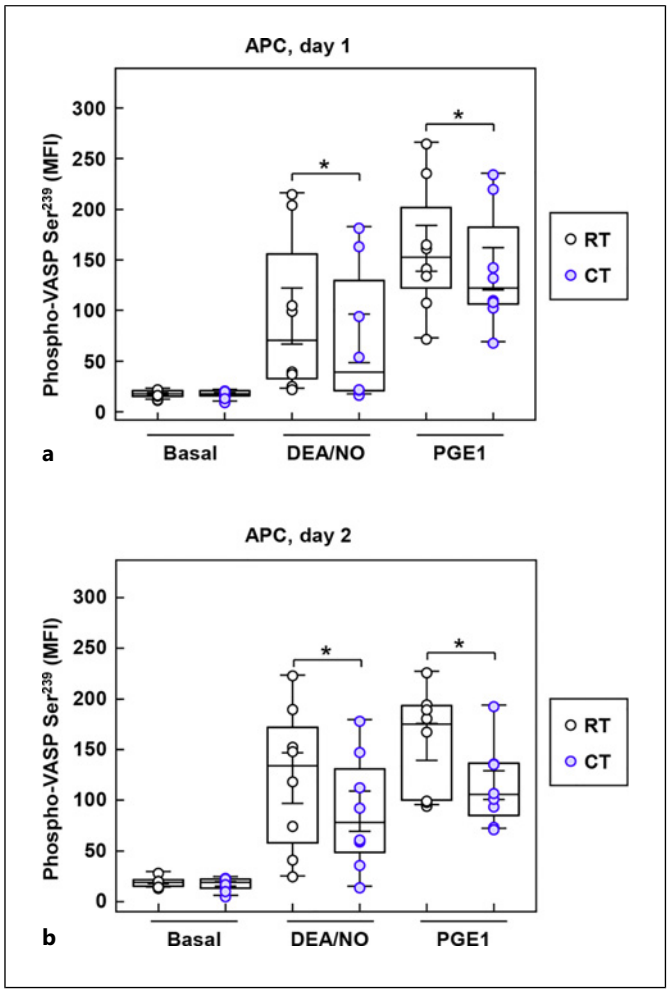

Fig. 3. Short-time refrigeration dampens inhibitory signaling in platelets from stored APC. The box-and-whisker plots show the distribution of basal, 3 μM DEA/NO-, and 1 μM PGE1-induced VASP phosphorylation, measured by flow cytometry in APC stored for 1 day (**a**) or for 2 days (**b**) and given in mean fluorescence intensity (MFI). Results are presented as mean ± SEM; $n = 8$; $^*p < 0.05$.

glycoprotein IIb/IIIa on the platelet surface, acting as fibrinogen receptor and linking platelets via bound fibrinogen [16].

Recently, the attenuation of inhibitory signaling was identified as an important mechanism contributing to enhanced platelet responsiveness under cold storage [12, 15]. In this context, VASP phosphorylation, serving as a major substrate for protein kinase A and protein kinase G, is a common marker for platelet inhibition [14]. In APC on day 1 and on day 2, the increments of induced VASP phosphorylation were reduced after refrigeration by approximately 20–30%, as measured by flow cytometry. In Western blot analysis, relevant effects on induced VASP phosphorylation were not detectable on day 1, presumably caused by differences in methodological sensitivities compared to flow cytometry.

PGE1 stimulates platelet inhibition via the IP (prostacyclin) receptor, triggering the activation of adenylyl cyclase and the production of cAMP, which results in an increase of cAMP-dependent VASP phosphorylation. The second inhibitory system in platelets, the NO-dependent pathway, can be stimulated by NO-releasing compounds, activating soluble guanylyl cyclase, and inducing the production of cGMP [17]. In general, the cAMP-dependent pathway represents the more robust system, whereas cGMP-dependent mechanisms are prone to be affected by storage, with an accumulation over the storage period at RT [18]. In line with these results, short-term refrigeration does not affect cAMP levels on day 1 and only partially on day 2. In contrary, induced cGMP levels are exceedingly suppressed on day 1 and day 2.

As a limitation, it should be considered that only APC stored for 1 day and for 2 days were used in this study, but not APC at the end of their shelf-life, e.g., stored for 4–5 days. However, the use of APC on storage days 1 and 2 was a rational first approach to confirm the previous results performed with freshly prepared platelet-rich plasma from whole blood [12]. In this way,

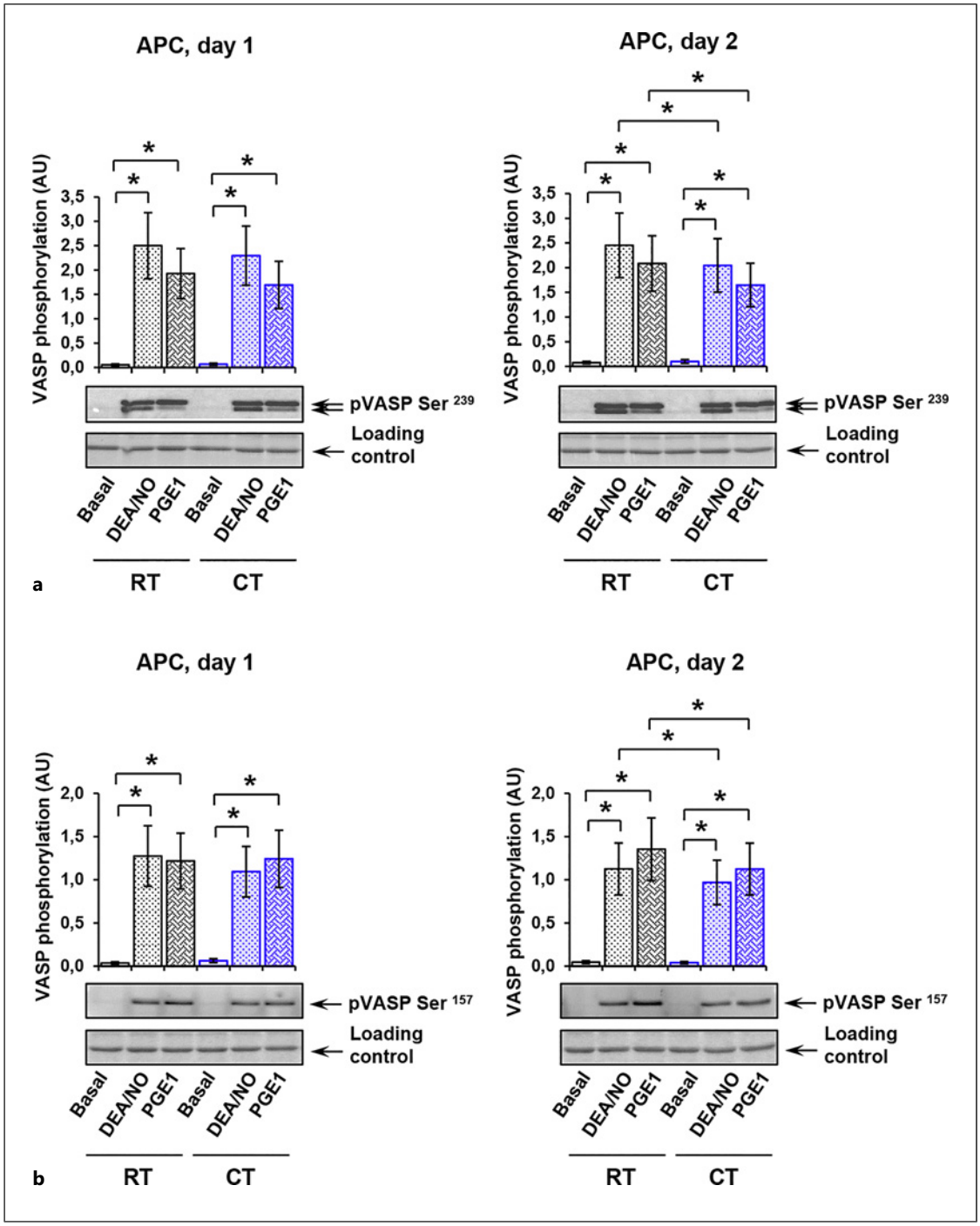

Fig. 4. Western blot analysis shows cold-dependent attenuation of induced VASP phosphorylation at both Ser^{239} and Ser^{157} in 2-day-stored APC. Quiescent washed platelets from 1-day- and 2-day-stored APC, continuously stored at RT or additionally at CT for 1 h, were stimulated with 1 μM DEA/NO or 1 μM PGE1 as indicated and visualized by Western blot analysis using phospho-VASP Ser^{239} (**a**) and phospho-VASP Ser^{157} (**b**) antibodies. After scanning, bands were quantified by the Image Lab program and normalized to loading controls. Results are presented in arbitrary units (AU) as mean ± SEM; $n = 5$; $^*p < 0.05$.

the number of APC required for this study could be reduced. In addition, "fresh" APC in the first half of their shelf-life, characterized by a higher level of hemostatic capacity compared to APC at the end of shelf-life, are commonly preferred for the treatment of acute hemorrhage.

In consecutive studies, it is also mandatory to analyze APC at the end of shelf-life since "fresh" APC are not always available for therapy. After extended storage at RT, however, platelets may show a more complex functional deterioration requiring more laborious

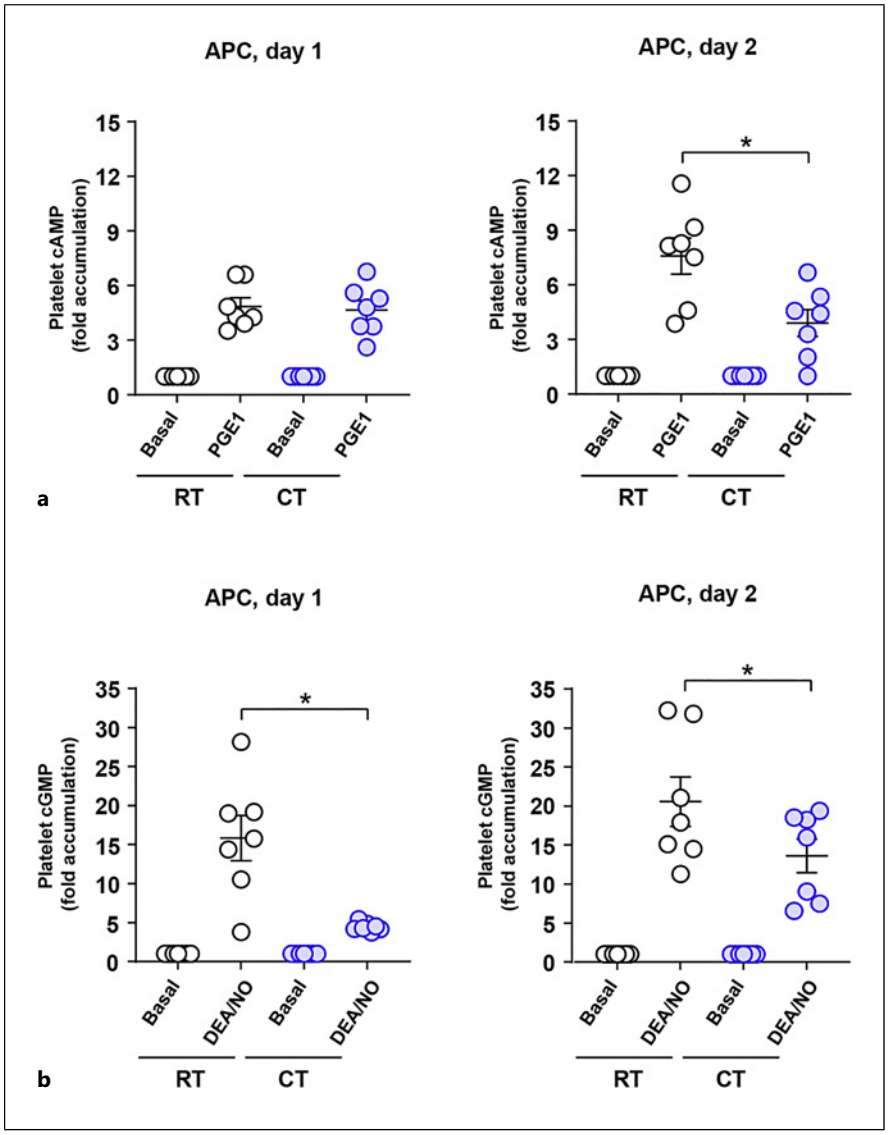

Fig. 5. Short-time refrigeration diminishes DEA/NO- and PGE1-induced cyclic nucleotide levels in stored APC. Washed platelets from RT- and CT-stored APC were incubated for 5 min with 1 μM DEA/NO or 1 μM PGE1 as indicated. The intracellular cAMP (**a**) and cGMP (**b**) concentrations were determined with immunoassay kits. Results are given as fold accumulation compared to basal values and presented as mean ± SEM; $n = 7$; $^*p < 0.05$.

experiments and a wider spectrum of methods to detect potential effects of short-term refrigeration. For example, adenosine diphosphate-induced aggregation is severely abrogated after 4 days of storage at RT storage [12, 15], making it more difficult to work out threshold aggregation responses. The progressive development of storage lesion also tampers inhibitory signaling, especially cGMP-dependent integrity, resulting in an elevation of VASP phosphorylation and enhanced susceptibility to NO donors [18, 19], possibly masking effects of refrigeration. Therefore, experimentation with APC stored for 4–5 days should also include the analysis of platelet receptor function or platelet-activating signaling cascades. Since the presented results refer to static in vitro experiments, it is required to confirm the promoting effect in adhesion studies and flow chamber experiments, as an important prerequisite for the design of clinical studies addressing the hemostatic effectiveness of short-term refrigerated APC.

Furthermore, it must be kept in mind that short-term refrigeration may bear potential risks. The advantage of higher platelet reactivity for the treatment of acute hemorrhage may be counteracted by lower increments or by rapid clearance of cold-exposed platelets [4], possibly resulting in the need of additional transfusions, especially in patients with chronic thrombocytopenia. Facilitated adhesion or aggregation after short-term refrigeration may also be associated with a higher risk of thrombotic complications, although an elevated frequency of adverse events has not been reported for cold-stored PC in previous studies [4, 8, 20, 21].

It is also important to explore additional time periods of refrigeration to define an optimal strategy for rapid but effective generation of "reactive" platelets. Storage at CT for more than 1 h may have induced more substantial effects on platelet responsiveness reaching a higher hemostatic potential. However, longer periods of cold storage are less acceptable in urgent clinical situations requiring the rapid availability of PC for the stabilization

of hemorrhage. In this context, experiments with temperatures below 4°C or temporary freezing are crucial to minimize the duration for generation of reactive platelets. Another question is raised for short-term refrigerated APC not used for patients and restored at RT. It would be of interest, if cold exposition for a short time interferes with glycoprotein Ib integrity on the platelet surface and desialylation [9], a relevant issue for the treatment of patients with thrombocytopenia.

Our experimental design with splitting of APC pairs from one donor allowed the comparison of two timepoints. For future studies, it is important to use standardized APC, although we could show that the course of temperature was comparable in total and split APC, reaching the range of 2–6°C in both cases.

In summary, short-term refrigeration for 1 h is sufficient to induce an alleviation of inhibitory signaling and to improve aggregation responses in APC stored for up to 2 days. The "on demand" refrigeration of PC may be a reasonable approach for the preparation of platelets with enhanced responsiveness to treat patients with hemorrhage more effectively. The clinical benefit and potential side effects have to be investigated more closely in further studies.

Statement of Ethics

Our studies with human platelets and the consent procedure were approved by our Local Ethics Committee of the University of Würzburg (approval number 101/15). The participants provided their written informed consent to participate in this study. The study was performed according to our institutional guidelines and to the Declaration of Helsinki.

Conflict of Interest Statement

The authors have no other conflicts of interest to declare.

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Author Contributions

A.Kob. and J.K. conceived the experiments; M.B., J.Z.-H., and K.W. conducted the experiments; A.Kob., M.B., and J.Z. analyzed the results; and A.Kob., A.Koe., M.B., J.Z.-H., and J.K. drafted the article. All authors reviewed the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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