Platelet Storage Lesion Accelerates Desialylation of Platelets and Increases Hepatic Thrombopoietin Production

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Directed by Professor Hyun Ok Kim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Jooyoung Cho

June 2017

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< ACKNOWLEDGEMENTS >

I believe that happiness is the desired result created from enormous effort and passion. Although there were some moments when I had feeling of hardness and giving up, now I know that all of the process toward the doctoral degree made me much firmer and what I am.

First of all, I would like to give special thanks to my supervisor, professor Hyun Ok Kim, who has encouraged and led me to this position with her sincere guidance and persistent help. I am grateful for the lab member, Hyunkyung Kim, for technical assistance. Also, I am grateful to professors of thesis committee, Jaewoo Song, Jeong Won Shin, June-Won Cheong, and Woo Ick Yang, for their valuable advice. I attribute a great deal of my motivation and desire to succeed to my mother, Hee Sook Lee. Her love and devotion allowed me to successful graduation. And I would like to express gratitude for my family members, my wife, Insun Kim, and my son, Eun Chan Cho. I also extend my thanks to all of those who supported me during my course for the thesis.

Now I am about to put a period on my long journey toward the doctoral degree. All of the moments passed in the blink as a midsummer night's dream. And I am convinced that from this moment on, my new dream is just beginning. I know that I am at the starting point now. I will keep on trying my best to be a highly qualified and dedicated professional. Thank you.

Jooyoung Cho
June 2017

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ABSTRACT

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Background: Stored platelets undergo deleterious changes, referred to as platelet storage lesions (PSLs), which accelerate the desialylation of platelets and result in their phagocytosis and clearance by hepatic macrophages. Recent studies have reported that Ashwell-Morell receptor binds to desialylated platelets, thereby inducing hepatic thrombopoietin (TPO) production in a mouse model. Therefore, this study aimed to demonstrate these relationships between PSL and hepatic TPO production in human study.

Methods: Platelet concentrates (PCs) were obtained from 5 healthy volunteers and remaining samples in blood bank. PCs were divided into two halves, and stored either at 22℃ or 4℃. Experiments were conducted using serial samples. Desialylation was assessed using flow cytometry, and structural changes were

visualized using electron microscopy. Following co-culture of HepG2 cells (HB-8065, ATCC) with isolated platelets, hepatic TPO production was determined using real-time quantitative polymerase chain reaction (qPCR) and the supernatant TPO level was measured using a Luminex kit.

Results: For 5 days of storage duration, platelet counts themselves were not influenced by the storage conditions. The degree of desialylation was proportional to the storage duration and dependent on hypothermal stress. Changes on platelet surface and structure in electron microscope were significant according to storage conditions. And HepG2 cells that reacted with aged or refrigerated platelets expressed more *TPO* mRNA than those that reacted with fresh platelets. But, the changes of supernatant TPO level were not significant.

Conclusions: This study demonstrated that, in vitro, aging and refrigeration affect the integrity of human platelets, resulting in induction of hepatic *TPO* mRNA expression, as proven in a mouse model.

Key words: Platelet storage lesion (PSL), Desialylation, Thrombopoietin (TPO), Ashwell-Morell receptor (AMR)

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I. INTRODUCTION

Transfusion of platelet components is widely used as a life-saving therapy to repair bleeding and maintain stable hemostatic status.[1,2] Platelet components are routinely provided in the form of platelet concentrates (PCs) derived from donated whole blood or plateletpheresis, and can be stored for up to 5 days at room temperature (22±2℃) with continuous gentle agitation.[3,4] Longer storage of platelets has not been recommended until now because of the possible risk of bacterial contamination,[5,6] as a result, blood transfusion services are faced with a chronic shortage of platelets.[3] Although new methods of pathogen inactivation are being developed for application in clinical settings,[7] questions have been raised about whether a storage duration of longer than 5 days is helpful for blood banks, considering the many existing risks.[2,6,8] Refrigeration may lower the risk of bacterial

contamination; however, investigators have noted that hypothermic conditions (temperatures lower than 15℃) can have deleterious structural and functional effects on platelets.[3,5,9,10] For these reasons, the storage duration of PCs is currently limited to 5 days.

In addition to the possibility of bacterial contamination, platelet aging can affect their structural and functional integrity.[2,4,6,11,12] Platelets in PCs, which are collected in gas-permeable plastic bags and used in routine blood banks, undergo many physiologic changes during collection, processing, and storage; the process can alter the structure and function of platelets and is referred to as platelet storage lesion (PSL).[2,6,12] Platelets secrete sialidase under many circumstances, such as the cold-rewarming process, aging, and other pathologic statuses.[1,8] Sialidase induces hydrolysis of sialic acid from glycoprotein (GP) 1bα or GPV on the platelet surface, forming irreversible clustering of the von Willebrand factor (vWF) receptor.[6,8,13] Moreover, Hoffmeister *et al*. demonstrated that the recognition of β-N-acetylglucosamine (β-GlcNac) residues on clustered GPIbα by the αMβ2 integrin receptor on hepatic macrophages (Kupffer cells) produced *in vivo* platelet phagocytosis in mice.[5] Therefore, PCs stored at hypothermic condition can be more easily altered than those stored at room temperature, and as a result, desialylation and rapid clearance can occur.[3,8,11,12]

Thrombopoietin (TPO) is the main regulator of megakaryopoiesis and thrombopoiesis.[14-17] It stimulates platelet production by promoting differentiation of hematopoietic stem cells (HSCs) into the megakaryocytic lineage.[15,16] This cytokine is predominantly produced in the liver at a constant rate, but its level is regulated by circulating platelets. Platelets and megakaryocytic cells express the TPO receptor,

known as the myeloproliferative leukemia oncogene (Mpl) receptor, which binds and internalizes TPO in thrombocytosis.[14,15] In thrombocytopenia, low levels of Mpl receptor expression and TPO clearance facilitate an increase of thrombopoiesis. Folman *et al*. demonstrated that the plasma TPO level is inversely proportionate to circulating platelet counts, and that the Mpl receptor plays a major role in TPO clearance and platelet homeostasis.[14]

As mentioned above, aged, refrigerated, or damaged platelets undergo more physiologic changes, such as PSL.[2,6,12] These altered platelets are more susceptible to phagocytosis by hepatic macrophages than normal platelets.[3] In addition to platelet clearance, recent studies noted that aged platelets stimulate hepatic TPO production.[1,18,19] Regardless of the plasma platelet count, the rate of hepatic TPO production is constant.[15] However, Grozovsky *et al*. demonstrated that desialylated platelets are cleared by the hepatic endocytic Ashwell-Morell receptor (AMR) and thereby stimulate hepatic TPO production by the JAK2-STAT3 pathway.[1]

This study aimed to investigate the relationship between desialylation of platelets and hepatic TPO production. And it was hypothesized that aged or refrigerated PCs are cleared more than control PCs by hepatic endocytic AMR, and thereby hepatic TPO clearance would be increased. Therefore, this study plans to investigate the many possible changes in platelets during storage, and to propose guidelines about appropriate storage conditions for platelet components.

II. MATERIALS AND METHODS

1. Samples and subjects

Five healthy volunteers, who agreed to participate in this study and signed the informed consent, were enrolled. Three men and two women participated, and their ages ranged from 26 to 58 years. All participants did not have hemato-oncologic diseases and were not taking medications that could affect platelet function. They visited the blood bank in Severance Hospital (Seoul, Korea) once for the purpose of blood donation from March to August 2016.

The median vein near the antecubital fossa was punctured with an 18-gauge needle attached to blood collecting set; the average time for collecting 400 mL of whole blood (WB) sample was less than 10 minutes. Each sample of WB was drawn into a triple blood bag (Triple BSDC-NP-SB3; Green Cross Medical Science Corp., Yongin, Korea) containing 56 mL of citrate phosphate dextrose adenine (CPDA)-1. Component processing was performed using centrifugation with a Kubota 8730 (Kubota corp., Osaka, Japan) at 2000 rpm for 5 minutes in order to separate red blood cells (RBCs). This was followed by centrifugation at 4000 rpm for 6 minutes in order to separate the plasma component. Approximately 50 mL of PC was obtained from each WB sample during the blood processing procedure, and each PC was transferred to a gas-permeable transfer plastic bag (Green Cross Medical Science Corp.) for analysis. Besides the donated PCs, some of the remaining PCs for disposal in blood bank were also used under the authorization of the institutional review board (IRB).

To study the effect of refrigeration on platelets, each PC was subdivided into 2 groups and stored either at 4℃ or at 22℃. Samples from serially stored platelets were examined at 0 (fresh), 1, 3, and 5 days after donation. This study was approved by the IRB of Severance Hospital (no. 4-2015-0105).

2. Platelet preparation

Platelet counts of each group were determined using an ADVIA 2120i (Siemens Diagnostics, Tarrytown, NY, USA) automated complete blood cell (CBC) analyzer. In order to prepare for flow cytometry, a washing procedure was performed as described by Jansen el al.[8] Platelets were washed with a buffer composed of 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, and 1 μg/mL prostaglandin E_1 with a pH of 6.0 (buffer A; washing buffer). They were then resuspended in 10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 10 mM glucose, and 0.5 mM $NaHCO_3$ with a pH of 7.4 (buffer B; suspension buffer).[1,8] Platelets treated with α2-3,6,8 neuraminidase from *Clostridium perfringens* (sialidase) (Sigma-Aldrich, Saint Louis, MO, USA) to remove surface sialic acid were used as a positive control. Isolated platelets (1×10^8) from each group were mixed with 1 mL of buffer A, followed by centrifugation using an Eppendorf Centrifuge 5424 (Eppendorf Inc., Hamburg, Germany). After removal of the supernatant, platelet pellets were resuspended in 1 mL of buffer B and were subdivided into 2 microtubes for performing assays on different kinds of lectins. All of these procedures were conducted at room temperature (22℃).

3. Flow cytometry

Desialylation of platelets was confirmed by lectin binding, using flow cytometric analysis. Fluorescein isothiocyanate (FITC)-conjugated *Ricinus Communis* agglutinin I (RCA-I; Vector Laboratories, Burlingame, CA, USA) was used for lectin binding for platelet surface β-GlcNac exposure.[8,13] RCA-I at 5.0 μg/mL was added to each of isolated platelets after resuspension in buffer B. In the positive control group, 0.3 U/mL of sialidase was also added. The groups of platelets stored at 4℃ were then incubated at 37℃ for 20 minutes, using a WiseTherm® HB-R (Daihan Sci., Seoul, Korea) water bath, and the groups of platelets stored at 22℃ were incubated with continuous gentle agitation. The lectin binding of platelets was analyzed using a Beckman Navios flow cytometer (Beckman Coulter Inc., Brea, CA, USA). Platelets were gated according to their forward scatter (FSC) and side scatter (SSC) characteristics.

4. Electron microscopy

Structural changes in platelets were examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM, platelets were fixed with 2% glutaraldehyde-paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 for 6 hours and washed twice for 30 minutes in 0.1 M PB. They were post-fixed with 1% OsO_4 dissolved in 0.1 M PB for 2 hours and dehydrated in an ascending gradual series (50–100%) of ethanol. Afterwards, they were infiltrated using isoamyl acetate and subjected to a Critical Point Dryer (HCP-2; Hitachi, Tokyo, Japan). They were coated with gold using ion sputter (IB-3, Eiko,

Fukuoka, Japan) at 6 mA for 6 minutes. Secondary electron images of the surfaces were obtained using scanning electron microscopy (SU-8220 FE-SEM; Hitachi) at an acceleration voltage of 20 kV in the KBSI Seoul Western Center.

For TEM, after dehydration in ethanol, specimens were embedded using a Poly/Bed 812 kit (Polysciences Inc., Warrington, PA, USA). After pure fresh resin embedding, polymerization was conducted at 65℃ in an electron microscope oven (TD-700; Dosaka EM, Kyoto, Japan) for 24 hours. Sections of approximately 200–250 nm thick were initially cut and stained with toluidine blue (T3260; Sigma-Aldrich) for light microscopy. Thin sections of 70 nm were double stained with 6% uranyl acetate for 20 minutes (Electron Microscopy Sciences, Catalog No. 22400; Hatfield, PA, USA) and lead citrate for 10 minutes (Thermo Fisher, Waltham, MA, USA) for contrast staining. The sections were cut using a Leica EM UC-7 (Leica Microsystems, Wetzlar, Germany) with a diamond knife (Diatome Ltd., Biel, Switzerland), and transferred on copper and nickel grids. All thin sections were examined using transmission electron microscopy (JEM-1011; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV in the Severance Biomedical Science Institute.

5. Co-culture of HepG2 cells with isolated platelets

HepG2 cells (HB-8065, ATCC) were cultured in 75 cm^2 cell culture flasks (SPL Life Sciences, Gyeonggi, Korea); cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/low glucose (Gibco, Green Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/

streptomycin (P/S; Gibco) at 37℃ and 5% CO_2. For subculture, HepG2 cells were washed first with Dulbecco's phosphate buffered saline (PBS; Biowest, Nuaillé, France), and cell detachment was performed using 0.5% trypsin-EDTA (Invitrogen, Waltham, MA, USA), followed by resuspension in cell culture medium.

For the assays, 1×10^6 HepG2 cells were seeded into 6-well dishes (Nunclon Delta Surface; Thermo Scientific, Waltham, MA, USA). HepG2 cells were then counted using a Neubauer-improved (0.0025 mm^2, depth 0.1 mm; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) hemocytometer. After overnight incubation at 37℃ and 5% CO_2, cells were washed using PBS and resuspended in calcium-free DMEM supplemented with 2% FBS and 1% P/S, followed by incubation with 1×10^7 isolated platelets for 6 hours. Supernatants from each well were then collected in a 2 mL microtube, and the cells were detached using cell scrapers. All of the cells and supernatants were stored at -20℃ until analysis.

6. Measurement of TPO in supernatant of HepG2 cell culture

TPO concentration in the cell culture supernatant was measured using a Magnetic Luminex® Performance Assay Human TPO kit (catalog No. LUHM288) (R&D Systems Inc., Minneapolis, MN, USA). This assay has a working range from 81 pg/mL to 11,379 pg/mL. The intraassay coefficient of variation was <5.6% and the interassay coefficient of variation was <8.3%, according to the manufacturer.

7. *TPO* mRNA expression

For the polymerase chain reaction (PCR) study, total RNA was extracted using an RNeasy® Plus Mini kit (Qiagen, Hilden, Germany). The RNA concentration was measured using a Nanodrop Lite (Thermo Scientific) at an absorbance of 260/280 nm, and about 1 μg of extracted RNA was used for cDNA synthesis. First-strand cDNA was performed using oligo(dT)-primed reverse transcription with genetically engineered Moloney murine leukemia virus (MMLV) reverse transcriptase (SuperScript® III First-Strand; Invitrogen). Synthesized cDNA was stored at -20℃ for real-time quantitative PCR (qPCR). For real-time qPCR, commercially available *TPO* (Hs01061346_m1; Thermo Scientific) and *GAPDH* (Hs02758991_g1; Thermo Scientific) probes were used. Required cycles for PCR amplification were 27 cycles (*GAPDH*) and 35 cycles (*TPO*), and each cycle consisted of 30 seconds of denaturation at 94℃, 30 seconds of annealing at 55℃, and 1 minute of extension at 72℃. PCR was performed using a StepOnePlus Real Time PCR system (Applied Biosystems) and analyzed using StepOne Software version 2.3 (Applied Biosystems). Following real-time PCR, threshold cycle values (C_T values) were obtained on the amplification curves. All samples were measured in triplicate and the mean C_T value for each was used for quantitation. *TPO* mRNA quantitation was calculated using the E-ΔC_T formula, and *GAPDH* expression was used as a control.

8. Statistical analysis

Numerical values were expressed as the mean ratio compared with that of the fresh controls (day 0). Data were analyzed using a Student's *t*-test (for 2 groups) or a one-way analysis of variance (ANOVA) (for more than 3 groups) followed by post-hoc analysis. SPSS software (Version 18.0; IBM corp., Armonk, NY, USA) and Microsoft® Excel software (Version 14.0; Microsoft corp., Redmond, WA, USA) were used for statistical analysis. A *P* value of less than 0.05 was considered statistically significant.

III. RESULTS

1. Platelet counts according to different storage conditions

Table 1 and Fig. 1 show the mean platelet counts and mean platelet volumes at various storage durations and temperatures. Platelet counts determined using an automatic CBC analyzer were approximately 1,300–1,500$\times 10^3$/μL for each of the groups. A Student's unpaired *t*-test revealed there were no statistically significant differences between the 22℃ and 4℃ groups. Moreover, a one-way ANOVA followed by post-hoc analysis showed there were no significant changes in platelet counts according to the time course (P>0.05). No correlation was observed between storage conditions and platelet counts *in vitro*.

Table 1. Platelet counts in different storage durations and temperatures (n=5). Data are expressed as means ± standard deviation in each group.

		D+0	D+1	D+3	D+5	D+7
22℃	Platelet count ($\times 10^3/\mu L$)	1383.3±149.9	1375.7±73.4	1334.7±221.3	1312.7±75.1	1401.3±246.2
	MPV (fL)	9.1±0.2	9.3±0.2	9.0±0.4	9.4±0.7	8.8±0.9
4℃	Platelet count ($\times 10^3/\mu L$)		1363.3±76.0	1302.7±40.8	1246.3±151.2	1428.0±175.9
	MPV (fL)		7.1±0.8	7.0±0.8	7.5±0.5	7.2±0.6

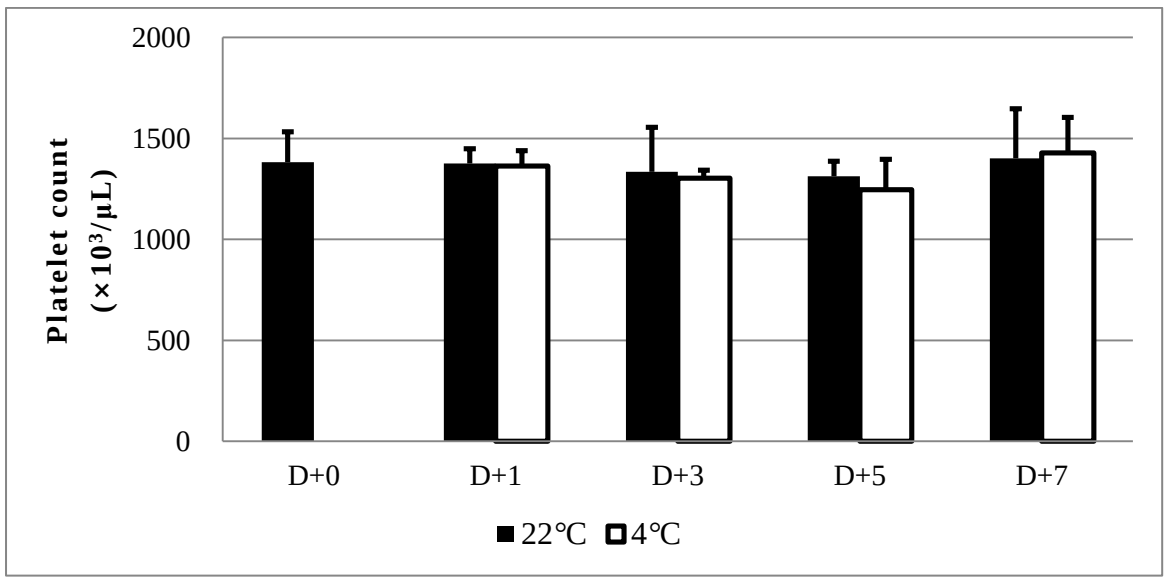


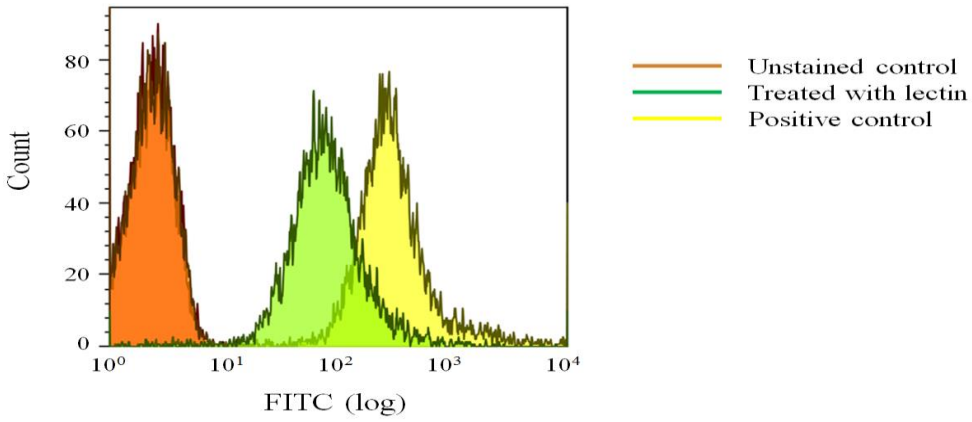
Fig. 1. Platelet counts in different storage durations and temperatures (n=5). Closed bars represent the platelets stored at 22℃ and open bars represent the platelets stored at 4℃.

2. Flow cytometric analysis of desialylation of platelets

Human platelets treated with RCA-I lectin showed more positivity than platelets not treated with RCA-I lectin (unstained control). Human platelets treated with both RCA-I and sialidase (positive control) showed more desialylation than platelets treated with RCA-I lectin only (Fig. 2A). Both storage duration and temperature affected desialylation of the platelets. Compared with fresh (day 0) platelets, those stored for 1 day showed increased lectin binding, and platelets stored at 4℃ showed more positivity than those stored at 22℃ (Fig. 2B).

According to storage duration, flow cytometric analysis of β-GlcNac exposure on the surface of platelets showed a positive correlation with storage duration. The level of desialylation was expressed as the mean relative ratio of samples compared with the mean of fresh controls. As expected, β-GlcNac exposure on the surface of platelets increased depending on the storage duration, as determined in a RCA-I lectin binding assay using flow cytometry; however, this was not statistically significant except on day 5. Refrigeration also influenced desialylation of platelets, but was not statistically significant ($P>0.05$). Positive controls (groups treated with both RCA-I lectin and sialidase) showed more positivity to the ratio of RCA-I lectin binding with statistical significance ($P<0.05$) (Fig. 3).

(A)



(B)

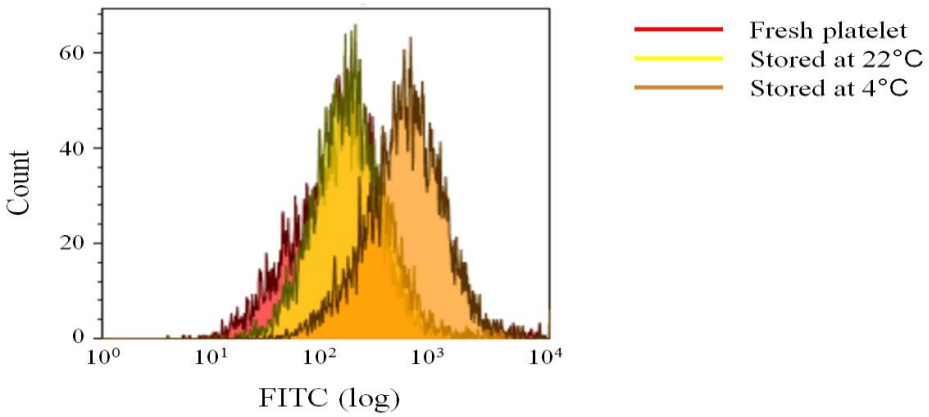


Fig. 2. Desialylation of platelet surface *in vitro*. (A) Human platelets treated with RCA-I lectin, not treated with lectin (unstained control), or treated with both RCA-I lectin and α2-3,6,8 neuraminidase from *Clostridium perfringens* (positive control). (B) Comparison between platelets with different storage conditions: day 0 (fresh), day 1 (stored at 22℃), and day 1 (stored at 4℃).

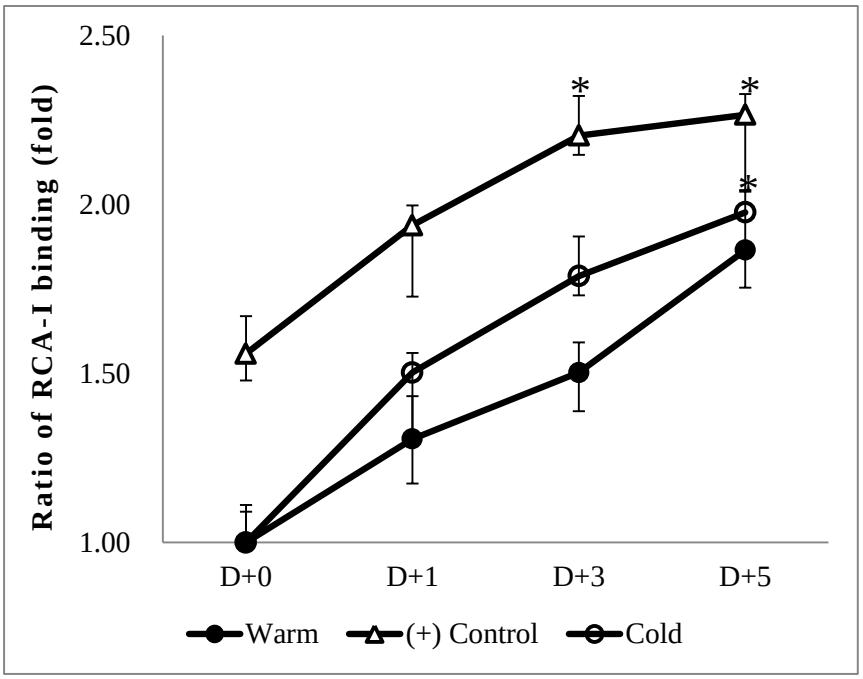


Fig. 3. Flow cytometric analysis of β-GlcNac exposure on platelets. Arithmetic mean values of FITC(log) were used for evaluation. Values in these graphs represent the relative ratio to fresh platelets (n=5). RCA-I lectin was used for detection using flow cytometry. Closed circles represent the platelets stored at 22℃, open circles represent platelets stored at 4℃, and open triangles represent positive controls with neuraminidase. A one-way ANOVA was conducted followed by post hoc analysis to compare all groups with the fresh controls. *P < 0.05 represents statistical significance.

3. Structural changes of aged or refrigerated platelets *in vitro*

Electron microscopic visualization was conducted using both SEM and TEM. At 20,000 × magnification, SEM analysis showed that surface damage and multiple pseudopodia of platelets were increased according to the time course and hypothermal stress (Fig. 4). In TEM analysis at 12,000 × magnification, fresh platelets showed many electron-dense chains and clusters. However, aging and refrigeration stimulated damage to the platelets, including to their cell membrane, dense bodies, alpha granules, and other cell organelles (Fig. 5).[20,21] Both a longer storage duration and refrigeration irreversibly influenced the shape and structure of the platelet surface.

4. Supernatant TPO levels of HepG2 cell cultures

As mentioned above, it was hypothesized that as more aged platelets react with HepG2 cells, supernatant TPO levels increase. And in supernatant of HepG2 cell cultures that reacted with platelets stored at 4℃, TPO level was higher than in that of HepG2 cell cultures that reacted with platelets stored at 22℃, but the difference was not significant (Fig. 6) (P>0.05).

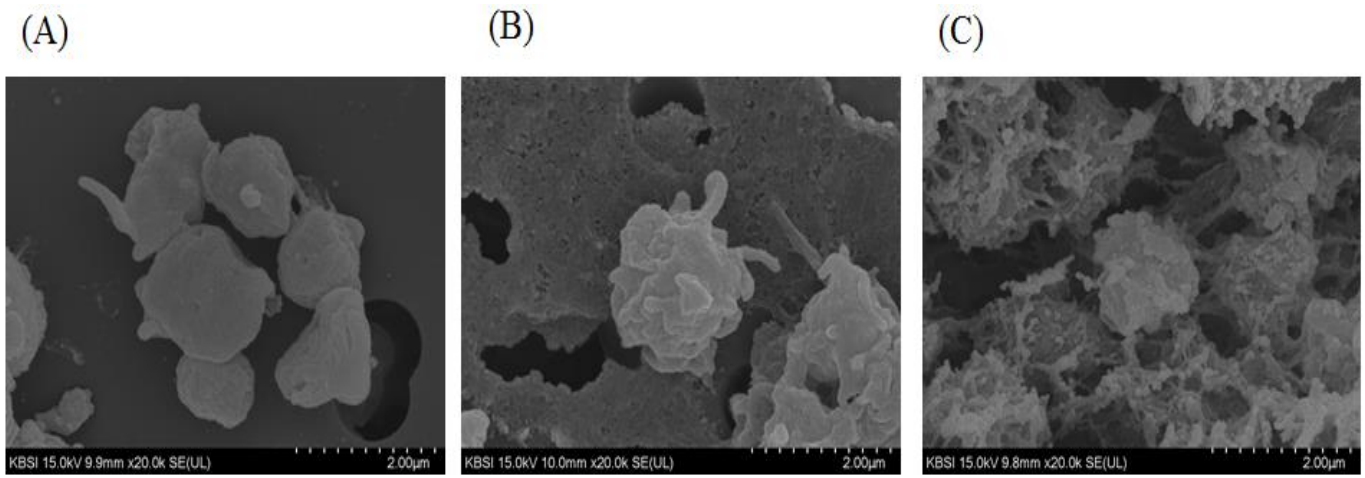


Fig. 4. Scanning electron microscopy (SEM) of the platelets at different storage durations and temperatures. Magnification, ×20,000: fresh platelets (A), platelets stored at 22℃ for 2 days (B), and platelets stored at 4℃ for 2 days (C).

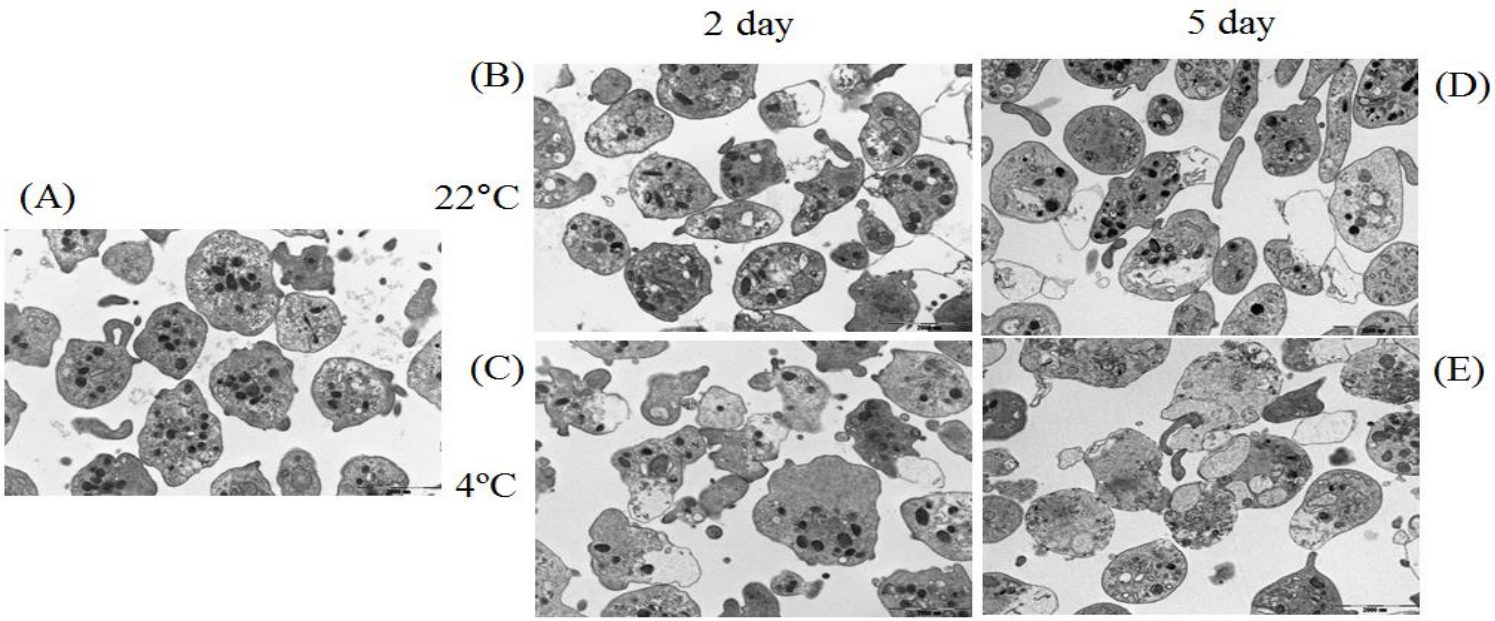


Fig. 5. Transmission electron microscopy (TEM) of the platelets at different storage durations and temperatures. Magnification, ×12,000: fresh platelets (A), platelets stored at 22℃ for 2 days (B), platelets stored at 4℃ for 2 days (C), platelets stored at 22℃ for 5 days (D), and platelets stored at 4℃ for 5 days (E).

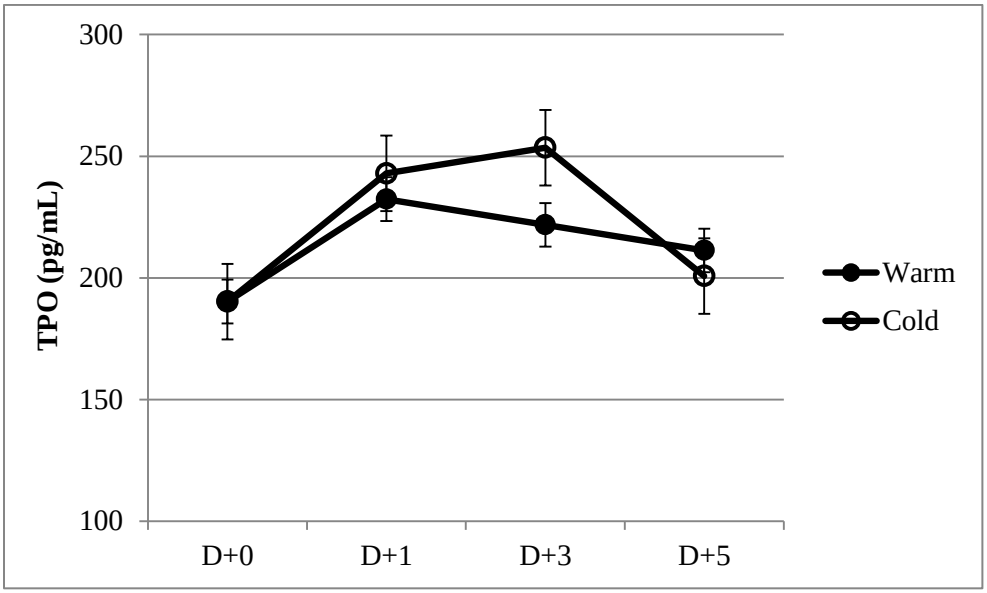


Fig. 6. TPO levels in supernatant of HepG2 cell cultures after incubation with human platelets (n=3). After incubation with human platelets for 6 hours under various conditions, supernatant TPO levels were measured using a Magnetic Luminex® Performance Assay Human TPO kit. Closed circles represent the platelets stored at 22℃ and open circles represent the platelets stored at 4℃. There were no significant changes in supernatant TPO levels according to time course and storage temperature (P>0.05).

5. Hepatic *TPO* mRNA expression

HepG2 cells treated with isolated human platelets expressed *TPO* mRNA. Measurement of hepatic *TPO* mRNA was performed using real-time qPCR. Fig. 7 shows the relative ratio of *TPO/GAPDH* compared with the mean ratio of fresh controls. Groups of platelets measured after 6 hours of incubation showed more significant changes than those measured after 24 hours of incubation (data not shown). The relative ratio of *TPO/GAPDH* mRNA expression increased according to storage duration and refrigeration ($P<0.05$ for days 3 and 5).

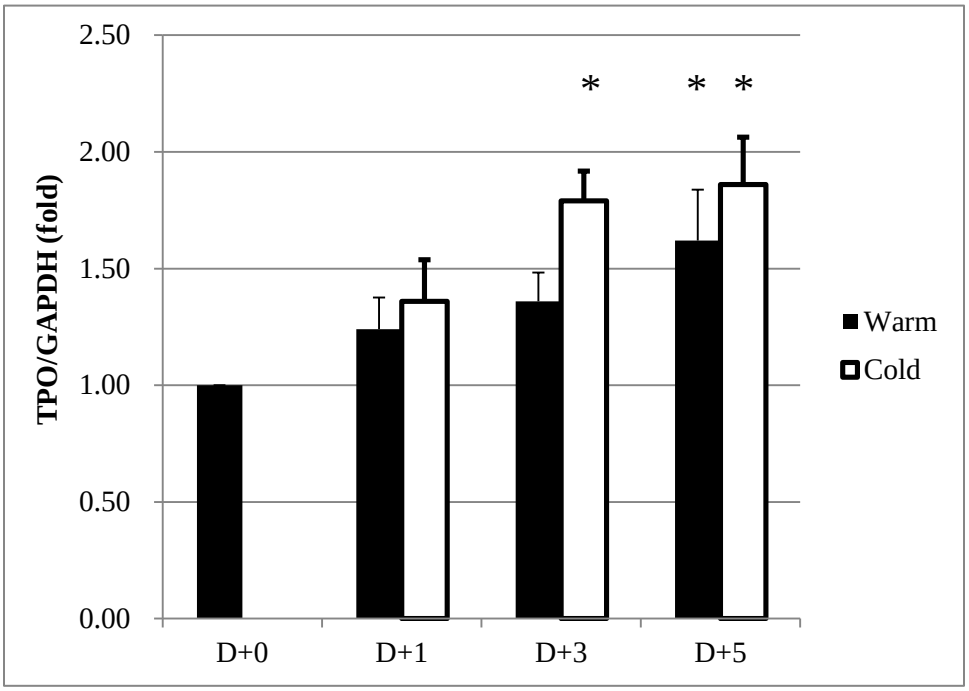


Fig. 7. Quantitation of hepatic *TPO* mRNA expression using real-time qPCR. Values in these graphs represent the relative ratio to fresh (D+0) platelets (n=5). 1×10^6 HepG2 cells were incubated with 1×10^7 isolated platelets for 6 hours. Closed bars represent the platelets stored at 22℃ and open bars represent the platelets stored at 4℃. *P<0.05 represent statistical significance.

IV. DISCUSSION

Timely and adequate supply of PCs is essential for many blood banks and healthcare facilities. However, a chronic shortage of PCs remains a problem.[3] Since the 1960s, PCs have been stored at room temperature with gentle agitation, but their storage duration has been limited to 5 days until now.[4,6,8] Technical developments for longer storage of PCs have been established continuously; however, extension of the storage duration is restricted in routine blood banks because of the possible risk of bacterial contamination.[5,6] And it is known that hypothermal conditions alter the structural and functional characteristics of platelets, which are referred to as PSL.[2,6,12] And it is also known that damaged platelets are easily cleared from the circulation by phagocytosis of hepatic Kupffer cells.[3,5,8]

As mentioned above, Grozovsky *et al*. identified that aged platelets are recognized by hepatic endocytic AMR, and this is followed by stimulation of hepatic *TPO* mRNA expression via the JAK2-STAT3 pathway.[1] Aging and refrigeration also accelerate desialylation of platelet surface. Therefore, it was hypothesized that uptake of aged or refrigerated platelets triggers hepatic TPO production in a human model *in vitro*. This study investigated human platelet desialylation and hepatic *TPO* mRNA expression to establish the relationship between storage conditions (such as storage duration and temperature) and changes in platelets in routine blood banks. And, the data supported the hypothesis about these relationships presented in this study.

First, stored platelet counts were not influenced by storage conditions. Actual platelet levels were not increased or decreased significantly *in vitro*. So, it might be

assumed that phagocytosis by hepatic or splenic macrophages affect on platelet counts in the circulation.

Second, platelet surface β-GlcNac exposure was proportional to the storage duration and temperature. Positive controls that were treated with α2-3,6,8 neuraminidase from *Clostridium perfringens* showed more desialylation, as expected. Platelets stored at 4℃ showed more desialylation than those stored at 22℃, which was determined as the lectin binding ratio (RCA-I) using flow cytometric analysis. Furthermore, by the storage duration of 5 days, the lectin binding ratio had increased proportionally in a time-dependent manner. At 5 days of storage, statistical significance in PSL was observed.

Third, structural changes in aged or refrigerated human platelets were observed using electron microscopy (SEM and TEM). Through SEM analysis, surface changes were observed on the platelet surface, which might be due to the result of vWF receptor aggregation, and thereby exposure of β-GlcNac, and multiple pseudopodia. Through TEM analysis, structural changes in the platelets were observed, including changes in the cell membrane and the cytoplasmic organelles.

Finally, the relationship between desialylation of platelets and TPO production was determined using HepG2 cells. HepG2 cells were co-cultured with isolated PCs, and hepatic *TPO* mRNA expression was measured at various time points and storage temperatures. And it was demonstrated that hepatocytes that reacted with aged or refrigerated platelets expressed higher levels of *TPO* mRNA than those that reacted with fresh platelets. However, changes in supernatant TPO levels were not significant.

In fact, in this study, PCs treated with sialidase (positive control) were also co-cultured with HepG2 cells. It was proved that sialidase induces desialylation of platelets in this study. But the level of hepatic *TPO* mRNA expression were not significantly increased (data not shown). And this study conducted the analysis serially up to the 7th day of storage. However, the mean value of *TPO* mRNA expression decreased compared with that from the 3rd or 5th day of platelet storage (data not shown). It might be thought that sialidase and longer duration of platelets result in more deleterious changes, such as reduced or loss of viability and functional efficacy. Therefore, extension of the storage duration to 7 days is difficult with the current technology for blood processing. Some studies defined old platelets as those with storage duration of ≥4 days, and demonstrated that the transfusion of these old platelets increases the risk of adverse reactions or worse outcomes.[22,23]

On the one hand, although desialylated platelets cause more hepatic *TPO* mRNA expression, the differences in supernatant TPO levels were not significant. This might be because of differences in the *in vitro* and *in vivo* conditions or limitation due to small sample size. And, this study did not investigate the effect of storage duration and temperature on the JAK2-STAT3. This is a limitation of this study because, as mentioned above, the mechanism of these relationships is known as the result due to JAK2-STAT3 pathway.[1,19] Therefore, further studies are required, and the risk of bacterial contamination and PSL should be assessed in human model *in vivo*.

V. CONCLUSION

In conclusion, this study demonstrated that, *in vitro*, aging and refrigeration affect the integrity of human platelets in terms of their structural and functional aspects; this induces in the stimulation of hepatic *TPO* mRNA expression, as proved in mouse model.[1] Refrigeration alters the integrity of platelets, thereby causing β-GlcNac exposure. Desialylated platelets are taken up by AMR on hepatocytes, and hepatic *TPO* mRNA expression is triggered. But, the effect of induced TPO production is not studied *in vivo*. To the best of our knowledge, this is the first human study which demonstrates about the relationship between desialylation of platelets and TPO production in hepatocytes according to storage temperature and duration *in vitro*. Furthermore, this study might aid researchers and blood bank staffs to study and establish the strategies about appropriate storage conditions for stored platelet components.

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ABSTRACT (IN KOREAN)

혈소판 저장 병변에 의한 혈소판의 탈사이알릴화와 간세포의 트롬보포이에틴 합성 증가에 대한 규명

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배경: 저장된 혈소판은 혈소판 저장 병변이라고 하는 변화를 겪게 되며, 이는 혈소판 표면 당단백의 탈사이알릴화를 유발하여 간세포에 의해 포획 및 제거된다. 최근 연구에서는 간에 존재하는 Ashwell-Morell 수용체가 탈사이알릴화된 혈소판을 흡수하여 간세포에서의 트롬보포이에틴 합성을 유발한다는 내용이 마우스 모델에서 보고된 바 있어, 본 연구에서는 이러한 인과관계에 대해 인간 모델에서 규명하고자 하였다.

방법: 5명의 건강인 성인으로부터 자발적 동의에 의해 전혈 헌혈을 받은 후 농축혈소판 제제를 제조하였으며 또한 본원 혈액은행의 잔여 혈소판

제제도 실험에 사용하였다. 혈소판은 두 군으로 나누어 4℃ 혹은 22℃에서 보관하면서 연속적으로 실험을 수행하였다. 혈소판의 탈사이알릴화 정도는 유세포 분석법으로 측정하였고, 구조적 변화는 전자현미경으로 관찰하였다. HepG2 cell (HB-8065, ATCC) 을 사용하여 보관된 혈소판과의 공배양을 통해 간세포내의 트롬보포이에틴 합성은 실시간 정량적 중합효소연쇄반응으로, 상층액에서의 사이토카인 양은 luminex kit 를 이용하여 측정하였다.

결과: 5일간의 저장기간 동안 혈소판의 수에는 변화가 없었으나, 탈사이알릴화 정도는 저장 기간과 온도와 양의 상관관계를 보였으며, 구조적 변화 역시 전자현미경으로 관찰할 수 있었다. 그리고 오래되거나 냉장 보관한 혈소판과 반응한 간세포에서 신선한 혈소판과 반응한 간세포보다 트롬보포이에틴의 합성이 증가하였다. 하지만 상층액에서의 트롬보포이에틴의 양적 변화는 뚜렷하지 않았다.

결론: 본 연구는 보관 기간의 증가와 냉장보관이 인간의 혈소판의 상태에도 병적 영향을 미치며, 이로 인해 간에서의 트롬보포이에틴 발현이 증가한다는 것을 최초로 규명한 체외 연구이다.

핵심되는 말: 혈소판 저장 병변, 탈사이알릴화, 트롬보포이에틴, Ashwell-Morell 수용체