Wound-healing properties of trehalose-stabilized freeze-dried outdated platelets

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BACKGROUND: The wound-healing applications of platelet (PLT)-derived cytokines, proteins, and membranes is accepted but continues to be investigated. In this study, it is demonstrated that stabilized freeze-dried PLTs prepared from outdated PLTs (FDPOs) accelerate wound healing and form tube structures as well as stabilized indated freeze-dried PLTs (FDPIs) and room-temperature fresh PLTs (RT-PLTs).

STUDY DESIGN AND METHODS: Experiments were designed to compare in vitro and in vivo wound-healing properties of FDPI, FDPO, and RT-PLT preparations. The concentration of PLT-derived growth factor (PDGF)-ββ and transforming growth factor (TGF)-β1 was determined, and the abilities of FDPIs, FDPOs and RT-PLTs to induce endothelial cell proliferation and promote endothelial cell tube formation (cells formed solid spouts connecting neighboring cells to form tube structures) were observed. Wound-healing characteristics were measured by surgically inducing 1-cm^2, full-thickness wounds on db/db mice (n = 10 per group). The wounds were treated with single or multiple doses of FDPIs and FDPOs. Wound closure rate was determined, and histology samples were evaluated for cellular makeup.

RESULTS: FDPOs retained the same levels of PDGF-ββ and TGF-β1 and were able to promote endothelial cell proliferation and tube formation in vitro as well as FDPIs or RT-PLTs. Multiple applications of FDPO accelerated wound closure and enhanced reepithelialization when compared to untreated wounds in db/db mice.

CONCLUSION: FDPOs enhanced wound healing in db/db mice as well as FDPIs and RT-PLTs. Wound closure was obtained 6 days earlier than untreated wounds and histologic examination revealed reduced granulation and increased cellular angiogenesis.

Platelets (PLTs) have been shown to actively promote and participate in angiogenesis and blood vessel maturation and accelerate wound healing.[1-4] PLTs also play a critical role in wound healing and matrix remodeling,[5,6] tissue regeneration,[7] and recruitment of marrow progenitor cells to the site of injury.[8] These effects are largely due to the content of PLTs' granules, among which there are a number of important growth factors that contribute to the wound-healing process.[9] Studies have found that PLTs' growth factors such as PLT-derived wound-healing factors, PLT-derived growth factor (PDGF), transforming growth factor (TGF), and insulin growth factors, among others, are important in different stages of the wound-healing cascade and greatly influence mitogenic and cellular differentiation

ABBREVIATIONS: FDP(s) = freeze-dried platelet(s); FDPI(s) = stabilized indated freeze-dried platelets; FDPO(s) = freeze-dried platelets prepared from outdated platelets; HUVEC(s) = human umbilical vein endothelial cell(s); MTT = tetrazolium salt; PDGF = platelet-derived growth factor; RT-PLT(s) = room-temperature fresh platelets.

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activities.[10-12] These findings have led to the development of strategies based on recombinant single growth factors for enhancing wound healing. For example, a recombinant human PDGF in a carrier gel is used to treat diabetic wounds (Regranex, Ethicon, Inc., Irvine, CA),[13,14] while others such as TGF[12,15,16] are currently being tested for FDA approval.

The approach of the use of single growth factors has met with limited success and mixed clinical results probably because wound healing is a complex integration of cascades, which requires multiple growth factors for different stimulatory and inhibitory functions at different phases within the process. Owing to the intricate interactions between the growth factors[17] over time, it is probable that the application of multiple PLT derived factors is more likely to recreate and propagate the normal sequence of events. Thus, concentrated PLT preparations containing all molecular components in PLTs allows for the delivery of multiple growth factors at a higher concentration than the physiological plasma levels that are released from the α-granules upon contact with area of injury.

The current clinical use of PLTs for wound-healing applications is restricted to autologous PLTs donated at the bedside with subsequent formation and application of PLT gels.[18,19] The procedure to generate autologous PLT gel is impractical for expanded use and difficult to standardize. Different patients have different PLT profiles and different methods produce dramatic differences in PLT yield and effect.[9,18,19] Although some mixed results were reported, several studies about the use of PLT concentrates or gel showed accelerated bone regeneration, reduced inflammation, decreased blood loss, reduced postoperative narcotic requirements, and improved hard and soft tissue wound healing.[9,18,19]

PLTs have a very short life at room temperature (RT; 7 days) and incur storage damage, albeit by different mechanisms, when stored at RT, in the cold[20] or frozen states.[21] Moreover, federally regulated storage limitations apply to PLTs incorporated in wound-healing applications, although PLTs retain the ability to induce proliferation for up to 3 weeks.[22] In addition, there is considerable waste of 5- or 7-day RT-PLTs because of inability to accurately predict demand, the time needed for preparation, and infectious disease testing.[23] As a result a significant number of outdated units are discarded every year making PLTs expensive to process and maintain. Even though overproduction occurs the short shelf life and unpredictable need has resulted in annual shortages. Short- and long-term approaches have been taken to attempt to lengthen the shelf life to 10 to 14 days, freeze the cells to allow 2-year storage at –80°C, or freeze-dry the cells to permit storage for as long as 1 year at RT.

The use of protective carbohydrate for preserving PLTs during lyophilization has been previously described in the literature dating back to the mid 1980s.[24,25] Protective sugars are overproduced by both plants and animals in response to environmental stressors including chilling, freezing, and drying.[26-29] The mechanism of action of protective carbohydrate preservation likely involves many stabilizing events that result in preservation of cellular structure and function[30] during freezing and drying. Studies focused on the interaction of trehalose and sucrose with biologic membranes have defined one mechanism of action in preventing harmful phase transitions in membranes as they pass through temperature- or hydration-induced phase boundaries. This action is mediated by a direct bonding of the hydroxyl groups on these carbohydrates to bimolecular surfaces (and aggregate surfaces such as membranes) at which water normally resides, creating a water replacement "structure" that holds native conformation in place during the environmental stress. In addition, stabilizing sugars create a stable matrix that retards molecular diffusion, particularly of harmful oxygen and nitrogen radical species. In effect, in the presence of trehalose, PLTs can undergo the freeze-drying process and preserve particular PLT functions.[24]

A method to preserve outdated RT-PLTs and incorporate them into treatment methods for chronic wounds would represent significant advancement for wound healing. Salvaging outdated RT-PLTs and stabilizing them in the dry state would reduce costs and improve safety by minimizing the potential of bacterial and viral contamination through the addition of reduction steps during processing, thereby making use of the outdated units of this critically short blood component and significantly increasing the availability of PLT-derived growth factors for wound care beyond the operating room venue. The use of expired and discarded PLT units for wound-healing applications would represent a dramatic and cost-efficient advance over the current methods with recombinant proteins.

In this study, we investigate the wound-healing properties of freeze-dried PLTs prepared from indated RT-stored PLTs (FDPIs) and compare them with freeze-dried PLTs prepared from outdated RT stored PLTs (FDPOs) in single and multiple applications.

MATERIALS AND METHODS

Freeze-dried PLT preparation

All chemical reagents were obtained through Sigma (St. Louis, MO), except where otherwise noted. Lyophilized PLTs were prepared from single Type O positive random-donor units, obtained from Lifeblood Mid-South Regional Blood Center in Tennessee. Upon arrival, the random-donor units were immediately placed on an RT orbital shaker until use. PLT counts were monitored by a hematology analyzer (ACT-10, Beckman Coulter, Miami, FL).

Indated (1-5 days after collection) or outdated random-donor unit PLT concentrates (6-15 days after collection) were centrifuged at 500 × *g* for 5 minutes to remove red blood cells. The PLT-rich plasma supernatant was then removed and acid citrate dextrose (85 mmol/L sodium citrate, 65 mmol/L citric acid, and 111 mmol/L glucose) was added, as needed, to bring pH down to between 6.6 and 6.8. PLT-rich plasma was then centrifuged at 1500 × *g* for 15 minutes to pellet PLTs. The PLT-poor supernatant was removed and discarded. Afterward, the pellet was resuspended in 5 mL of cation-free Tyrode's buffer (9.5 mmol/L HEPES, 100 mmol/L NaCl, 4.8 mmol/L KCl, and 12 mmol/L $NaHCO_3$) containing 50 mmol per L trehalose, pH 6.8, and adjusted to a count of 1.3×10^9 PLTs per mL with the same buffer. The mixture was then incubated for 2 hours at 37°C, mixing once by gentle inversion every half-hour. Finally, human serum albumin was added to a final concentration of 5 percent for lyophilization. PLT suspensions were cooled from 22 to –50°C with freezing rates between –2° and –1°C per minute. The PLT suspension was kept frozen for approximately 1 hour until vacuum had reached a maximum set point (approx. 100 mTorr) to prevent any meltback while the chamber was evacuated. During primary drying, the temperature of the samples was slowly ramped up over time (approx. 10°C/hr) until RT was reached. Secondary drying held the samples at 25°C for 12 hours under vacuum. Samples were sealed under vacuum and directly used for experiments or stored at RT for later use. The water content after freeze-drying was typically between 3 and 5 percent.

In vitro assays

Human umbilical vein endothelial cells maintenance and propagation. Cryopreserved human umbilical vein endothelial cells (HUVECs), at the density of 10,000 cells per mL, were purchased from Cambrex (Walkersville, MD) at Passage 2. Cells were thawed and plated on a sterile flask (T75, Fisher Scientific, Newark, DE) and maintained with cells media system (Clonetics, Cambrex, Walkersville, MD) in a standard incubator (5% CO_2, 37°C, and 100% humidity with standard O_2). When the confluency of the cells reached approximately 80 to 90 percent, cells were passaged to Passage 3. To do this, the cells were washed one time with 1× Dulbecco's phosphate-buffered saline (PBS; Sigma) and aspirated. Then, 5 mL of 1× Trypsin-ethylenediaminetetraacetate (EDTA; Fisher, Suwanee, GA) was added to the flask until cells detached. Cells were collected in a 15-mL tube (Falcon, BD Biosciences, San Jose, CA) and centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the cells were resuspended and plated in three T75 flasks (1:3 split ratio) in the Clonetics cells media system. With the same method, cells were split and passaged to Passage 7 to achieve sufficient number of cells to be used in bioassays.

Proliferation and tube formation bioassays for endothelial cells. For the bioassays with endothelial cells, at Passage 7 when the confluency of the cells reached approximately 80 percent, the cells were starved by adding starvation medium (Clonetics cells medium without fetal bovine serum [FBS]). At 24 hours after addition of the starvation medium, the cells were washed one time with 1× Dulbecco's PBS (Sigma) and aspirated. Then, 5 mL of 1× Trypsin-EDTA (Fisher) were added to the flask until cells detached. Cells were collected in a 15-mL Falcon tube and centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the cells were resuspended in starvation medium. Cells were stained with trypan blue to distinguish viable from dead or dying cells (the dead and dying cells will take up the blue stain).

The starved cells prepared from above were used for the tube formation assay. Viable HUVECs (10,000 cells), supplemented with 2 percent FBS and antibiotics, were added to uncoated wells in a 96-well plate (Fisher). After 2 to 3 hours, 100 µL of FBS was added to the cells as a positive control while cells without FBS were considered a negative control. Various PLT samples were freeze-dried, ranging from indated (3 days old) up to 15 days after collection. These freeze-dried PLT (FDP) samples were reconstituted with water to a concentration of approximately 1×10^9 FDP per mL, and 100 µL samples were added to appropriate wells. Cells were allowed to grow for an additional 48 hours before any quantitative analysis. At the end of the 48-hour incubation period, the cells were observed under the microscope for confluency, growth, and tube formation (angiogenic growth patterns). When cultured in the presence of angiogenic agents, HUVEC cells become stimulated and form secondary tube-like structures instead of growing in a random manner. The formation of tubes was rated by imaging and scored by visual inspection of cells within a defined field, 10 being the highest level of angiogenesis and 0 indicating no angiogenesis. Scoring of angiogenesis was performed by two observers in blinded fashion. The criteria used for scoring the angiogenesis were consistent with other published reports for scoring angiogenesis.[31]

For the proliferation assay, HUVEC metabolic activities were measured by the uptake and reduction of tetrazolium salt (MTT) to a formazan dye by cellular microsomal enzymes (MTT proliferation assay MTT kit, ATCC, Manassas, VA). The same plates that were used to assess angiogenic growth patterns were used for the MTT assay, 10 µL of MTT dye was added to each well and incubated for 2 to 3 hours. Next, 100 µL of detergent reagent included within the kit was added to each well to lyse the cells and solubilize the formazan dye. The absorbance from cell lysate was read at 590 to 650 nm with a plate reader. The optical density was plotted against the final PLT concentration.

Growth factor content (PDGF-ββ and TGF-β1) by enzyme-linked immunosorbent assay. Growth factor levels were determined with the enzyme-linked immunosorbent assay (ELISA) and were tested according to the manufacturer's instructions (Allevex Life Sciences, San Jose, CA). To release the growth factors from the α-granules, both RT-PLTs and FDPs (1×10^9/mL) were sonicated, centrifuged ($1000 \times g$, 15 min) to remove cellular debris, and the supernatants were kept on ice for later use. PDFG-ββ standards, 100 µL of RT-PLTs, and reconstituted FDP supernatant samples was added to a microplate coated with an antibody against either PDGF-ββ or TGP-β1. Any growth factor present was bound by the immobilized receptor. After any unbound substances were rinsed away, an enzyme-linked polyclonal antibody specific for each growth factor was added to the appropriate wells. After a second wash, a substrate solution was added and color developed in proportion to the amount of bound growth factor in the first step. The color development was stopped, and the intensity of the color was measured at 450 nm with a microplate reader (Safire 2, Tecan, Research Triangle Park, NC). The amount of growth factors was calculated per individual PLT by taking the growth factor concentration divided by 1×10^8 RT PLTs or FDP.

In vivo studies

Experimental animals. Homozygous genetically diabetic 8- to 12-week-old, Lep/r– db/db male mice (The Jackson Laboratories, Bar Harbor, ME) were housed in an AAALAC facility under an approved animal protocol following guidelines from the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education and Human Services, Publication no. 86-23 [National Institute of Health], revised 1985). They were acclimatized for 24 hours before use, maintained on a 12-hour light-dark cycle, and allowed ad libitum access to food and water.

Study design and surgical protocol. Animals were anesthetized with pentobarbital (Nembutal), at the dosage of 60 mg per kg. The back of the animals, shaved 1 day before surgery, was first prepared with iodine solution and subsequently with 70 percent alcohol. A sterile 1×1-cm^2 template was used to mark the area that was excised through the level of the panniculus carnosus muscle; the wounds were photographed, measured, and kept for wound size analysis. The study therapeutics were applied onto the wound bed in the dry state and then covered with dressing (Tegaderm, Nexcare, 3M, St. Paul, MN) to seal the treatment in place and to prevent physical contact with the wound bed.

The study contained four treatment groups of db/db mice wounded in the dorsal region. The first group (n = 10) was not treated. The second group (n = 10) received 5×10^8 FDPIs applied to the wound site on Day 0. The third group (n = 10) received 5×10^8 FDPOs applied to the wound site on Day 0. The fourth group (n = 10) was treated with the same FDPO samples with the exception that applications of 5×10^8 FDPs were given on the day of surgery and on Days 2, 5, 9, and 12 after surgery. All FDP were applied in the dry form directly on to the wound bed.

Wound size measurements. Open wound areas, defined as areas that were not epithelialized, were measured manually. The length and width were used to calculate the wound area ($A = L \times W$). Wounds were measured by two observers, who were blinded to the treatments, twice a week from Day 0 to Day 24. Each wound was measured at least three times per observer. Because there were 10 mice per group, each individual data points represented six independent measurements. All measurements were tabulated and expressed as a mean ± standard deviation (SD).

Microscopic analysis. One animal from each group was killed every third day through Day 24, and wounds were harvested. The wound bed including a 0.5-cm margin of unwounded skin was removed en bloc and fixed in 10 percent neutral-buffered formalin. Wound tissues were processed and stained with hematoxylin and eosin. Stained sections were scanned at low power to identify areas with the most intense reepithelialization.

Statistical analysis

A t test and analysis of variance (ANOVA) were used to test differences between the groups. Differences were considered significant when p values were less than 0.05. Results are given as mean ± SD.

RESULTS

FDPOs retain the ability to promote cell proliferation and tube formation in vitro

The ability to promote metabolic activities in HUVECs as well as tube formation capacity (Fig. 1B), as measured by the MTT assay, remained similar between random-donor PLT concentrate units and FDPIs and FDPOs up to 9 days after collection (Fig. 1A). Moreover, FDPIs (processed 3 days after collection) and FDPOs (processed 9 days after collection) stimulated HUVEC metabolic activity in a manner comparable to fresh PLTs (Fig. 2). Visual inspection of the tissue culture plates showed that HUVECs that were treated with RT-PLTs, FDPIs, and FDPOs showed rearrangement and organization into tube-like structures (Fig. 3). Note that FDPOs prepared from RT-PLTs had expired but were 8 to 9 days old.

FDPOs retain PDGF-ββ and TGF-β1

PLTs contain a number of bioactive factors that contribute to the process of wound healing such as PDGF-ββ and TGF-β1. The freeze-drying process preserved growth factors essential for wound healing present in outdated RT-PLTs The outdated and indated FDPs, upon reconstitution, retained growth factors PDGF-ββ and TGF-β1 in quantities similar to fresh PLTs (Table 1).

TABLE 1. Analysis of growth factors content in various PLT preparations*

Source	PDGF-ββ (g/PLT)	TGF-β1 (g/PLT)
RT-PLTs	$1.35 \times 10^{-17} \pm 3.54 \times 10^{-19}$	$3.89 \times 10^{-18} \pm 1.82 \times 10^{-19}$
FDPI	$1.38 \times 10^{-17} \pm 1.09 \times 10^{-18}$	$3.83 \times 10^{-18} \pm 5.84 \times 10^{-20}$
FDPO	$1.29 \times 10^{-17} \pm 1.16 \times 10^{-18}$	$3.54 \times 10^{-18} \pm 6.39 \times 10^{-20}$

* Growth factors content was analyzed by ELISA. Liquid-stored RT PLTs (3 days after collection), FDPIs (3 days after collection), and FDPOs (9 days after collection) have similar levels of growth factors (PDGF-ββ and TGF-β1). Results represent mean ± SD of five different lots of RT-PLTs and FDPs.

Single application of indated or outdated FDPs accelerated wound closure

A single application of FDPIs and FDPOs to the wounds on Day 0 reached 90 percent closure in 17 days, whereas untreated wounds required 21 days to reach 90 percent closure ($p < 0.01$). Wound area (raw surface) was higher ($p < 0.01$) in the group of wounds left untreated than in wounds treated with indated FDPs on Day 7. Multiple applications of outdated FDPs induced a trend toward faster wound closure when compared to single applications of outdated FDPs with ANOVA statistical method of analysis (Fig. 4) and reached 90 percent closure in 12 days versus 21 days for the no-treatment group ($p < 0.01$). Wound area was larger ($p < 0.01$) in the wounds left untreated compared to wounds treated with multiple applications of FDPO treatments on Days 7 and 9 (Fig. 4).

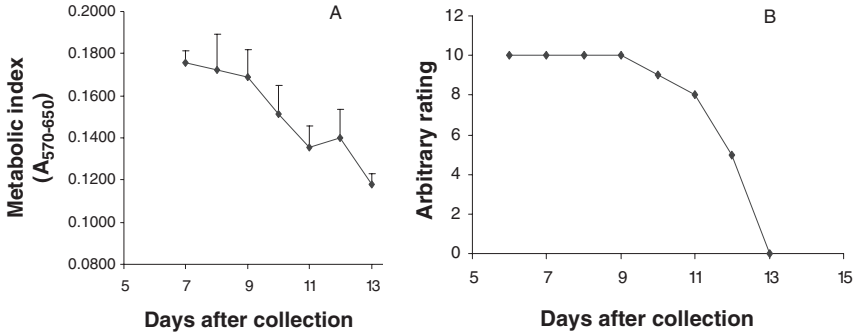

Fig. 1. Outdated PLTs were obtained from the blood bank at the dates indicated in the plot and stored at RT. PLTs (5×10^7/mL) were removed aseptically from the container and assayed for their ability to induce endothelial cell metabolic activities (A) and tube-forming capacity (B). Results represent mean ± SD of five random-donors.

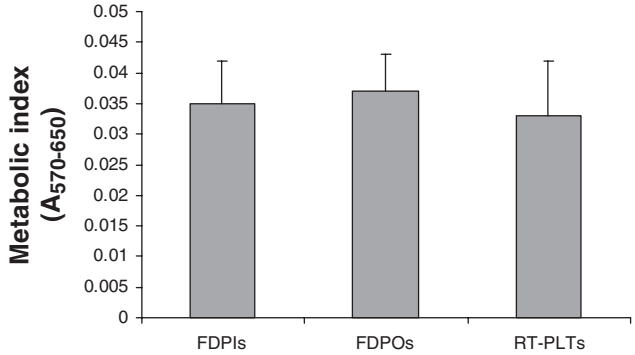

Fig. 2. Proliferation of endothelial cells induced by FDPOs. The ability of FDPOs processed 9 days after collection to induce endothelial cell metabolic activity was compared to FDPIs and RT-PLTs by adding 5×10^8 FDPIs, FDPOs, or RT-PLTs. RT-PLTs, FDPIs, and FDPOs induced comparable levels of proliferation. Results represent mean ± SD of five different lots of RT-PLTs and FDPs.

The wound beds of nine animals, three from each of the no-treatment group and the two groups that received single and multiple treatments of FDPOs, were harvested on Day 9 and were stained with hematoxylin and eosin. Figure 5 showed the representative panoramic composite of the wound tissues at 40× magnification. Whereas the no-treatment group showed lack of reepithelialization, the treatment groups showed robust granulation tissue and reepithelialization. Furthermore, the group that received multiple treatments of FDPO showed a thicker layer of reepithelialization and an area of hyperplasia at the edge of the wound bed.

DISCUSSION

This study explores the therapeutic effects of FDPOs for diabetic wound healing. PLT preparations supply a rich mixture of factors to the wound bed, but their use has been traditionally hampered by difficulties in preparation and storage.

Previous work in our laboratory showed that freeze-dried PLTs induced comparable effects to fresh PLT preparations used to treat wounds in diabetic mice (data submitted for publication). This study demonstrates that PLTs can be processed and freeze-dried up to 9 days after collection or 3 to 5 days after their RT shelf life for infusion has passed. Approximately 20 percent of the

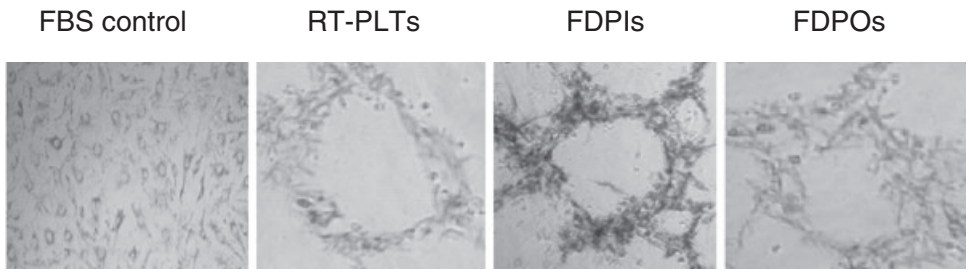

Fig. 3. Tube formation induced by RT-PLTs and FDP samples on uncoated wells. Both FDPIs and FDPOs stimulated endothelial cells to form tube (capillary-like) structures in the same manner as RT-PLTs in contrast to FBS control sample. At 40×, it is possible to see PLTs along the tube structure, suggesting an important role of PLTs for mechanical support for proliferating endothelial cells.

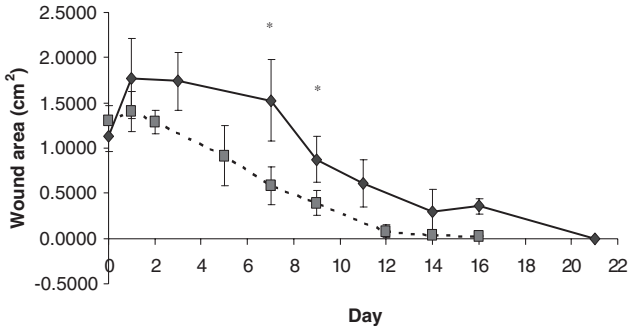

Fig. 4. Wound closure analysis for FDPO in multiple applications. Two groups of animals (n = 10 per group), either not treated (◆) or treated with 5×10^8 FDPOs on Days 0, 2, 5, 9, and 12 (■), were compared. Multiple applications of FDPOs induced more than 95 percent of wound closure 12 days faster than the nontreated group (*$p < 0.01$). Wound closure is the measurement of wound areas that had been epithelialized as described under Materials and Methods. Each data point represents mean ± SD of six observations.

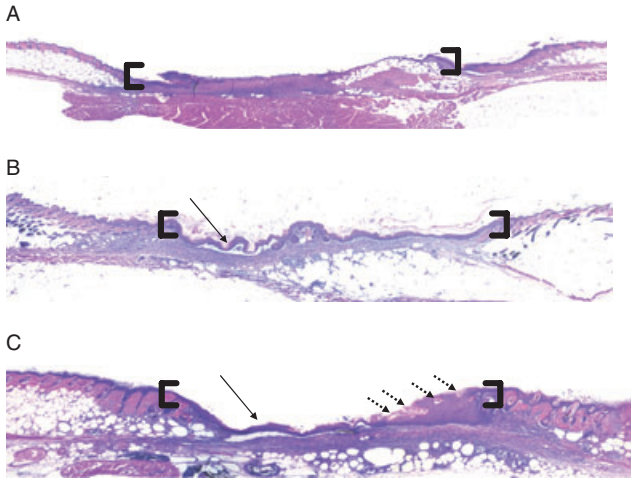

Fig. 5. Representative wound beds of the no-treatment group (A) and the two groups that received single (B) and multiple (C) treatments of FDPOs were stained for hematoxylin and eosin. Wounds were harvested at Postoperation Day 9 to demonstrate reepithelialization. Brackets mark the actual edges of the wounds and arrows point to the reepithelialized layers in the treatment groups. Dashed arrows point to the highly hyperplastic epithelium on one wound edge of the group that received multiple treatments.

total PLT supply becomes outdated and is discarded each year (National Blood Data Resources Center). Our investigation demonstrates that outdated PLTs can be salvaged, stabilized, freeze-dried, and used for the treatment of acute or chronic wound healing significantly increasing the availability of PLT-derived factors far beyond the time that autologous PLT-rich plasma preparations can be used. Upon reconstitution, we were able to recover PLT growth factors and biologic activity, with comparable results both in vivo and in vitro from FDPIs and FDPOs and fresh RT-PLTs. This opens the way to the use of a discarded product for wound healing with obvious advantages in terms of costs and availability.

The in vitro and in vivo results here presented show the important role of PLTs in supporting the growth of granulation tissue and the proliferation of epithelial cells to cover the wounds. PLTs have an established angiogenic effect, and in vitro, PLT fragments were seen along the extensions of endothelial cells, supporting the idea that PLTs may play a mechanical role of support for the formation of new blood vessels, in addition to the contribution of their metabolically active growth factor.[9]

Multiple applications of FDPOs induced a trend toward faster wound closure, when compared to single applications. This may derive from the delivery of additional growth factors during the repair process of wound healing. It is also possible that multiple applications of FDPO stimulated the proliferative phase of wound healing, accelerated the process of tissue granulation and reepithelialization, and thus sped up the overall wound closure process.

The long-term dry stabilization of outdated PLTs can dramatically change the practice of the use of PLT concen-

trates for wound-healing applications, as it will enable wider use of PLTs for wound repair in a number of clinical settings. Moreover, appropriate terminal sterilization methods will be studied in the near future to select a method that will inactivate bacteria and viruses and not affect the biologic activity of the PLT preparation. Additional studies are needed to optimize the concentration of PLTs to be used for wound healing and to investigate the possibility of embedding the freeze-dried material in a bandage or skin substitute to accelerate wound healing.

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