WOUND HEALING PROPERTIES OF RECONSTITUTED FREEZE-DRIED PLATELETS

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Abstract

The use of fresh platelets has gained value in medicine as an essential part of wound treatments. This is not surprising since platelets contain a number of bioactive factors that contribute to the process of wound healing such as: platelet derived platelet-derived growth factor (PDGF) and transforming growth factor (TGF). Fresh platelets' short shelf life limits platelet based therapies. If platelets can be stabilized in a freeze-dried form (FDP) then long-term storage and pathogen inactivation methods become possible.

Adlyfe and Oregon Freeze-Dry have been developing technologies to stabilize freeze-dried human platelets which can be subjected to gamma irradiation and stored for a long duration. Upon re-constitution, irradiated FDP retained growth factors PDGF-bb and TGF-b1 in quantities similar to fresh platelets as judged by capture ELISA. The rehydrated FDP promoted new DNA synthesis and cellular proliferation of primary human dermal fibroblasts and endothelial cells (HUVECs) similar to fresh platelets. The FDP also promoted remodeling of extracellular matrix by accelerating fibroblast mediated contraction of collagen gels and stimulated HUVECs to undergo angiogenesis and form capillary structures *in vitro*. Pre-clinical wound healing studies in diabetic mice indicated that FDP promoted rapid wound healing and closure in a manner comparable to fresh platelets and VEGF controls. Furthermore, wounds treated with FDP are active, granulated and saturated with blood vessels. Additional study using swine models are underway to solidify our contention that FDP are safe and well-suited alternative to fresh platelets for wound healing applications.

Materials and Methods

Materials:
Whole citrate or ACD blood was obtained from healthy screened donors at BRT laboratories; Baltimore, MD, and used within 3-4 hour of venipuncture. ELISA kits were obtained from Allevex Life Sciences (San Jose, CA). Primary human cell lines and media were obtained from Cambrex (Walkersville, MD). Non-Radioactive Cell Proliferation Assay kits were from ATCC (Manassas, VA).

Methods:
Freeze-dried platelet preparation: We used Adlyfe's proprietary technique to freeze dry human platelets. Briefly, platelets were collected into acid citrate dextrose (ACD) anticoagulant buffer (1.5 volumes + 8.5 volumes blood). Platelet Rich Plasma (PRP) was obtained by low speed centrifugation 135g for 15 minutes to remove red blood cells. The PRP was acidified to pH 6.5 by adding 1/14 volumes of ACD and then pelleted by centrifuge at 1000 g for 10min. The platelet pellet was resuspended in 1 ml of Cation-Free Tyrodes Buffer Containing 50 mM Trehalose, pH 6.8 and adjust to ~ 1.0x109/ml. The mixture was incubated for 2 hours at 37 °C, mixing once each half hour. Finally, adjusted the albumin concentration to 5% of platelet preparation for lyophilization.

Evaluate Biochemical Characteristics of FDP: We examined the ability to recover platelet growth factors, primarily TGF-beta and PDFG-bb from FDP and compared them against fresh platelets draw from the same platelets pool. This was done primarily using commercially available ELISA kits when possible. In order to determine the bioactivity of the growth factors present in FDP Matrix preparations, we employed cell based bioassay, such as the HUVEC proliferation and collagen contraction assays, to determine the activity of PDGF-bb and TGF-beta.

Proliferation assay: HUVECs (purchased from Cambrex Biosciences, Walkersville, MD)at passage 7 were starved for 24 hrs using starvation medium (without FBS). After 24 hrs, the cells were passaged to P8, seeded at 10,000 cells/well in a 96 well flat bottom, and allowed to attach for 2-3 hrs. Once the cells were attached, the samples were added and incubated in a 37°C, 5% CO2 humidified incubator for 48 hrs. At 48 hour, the proliferation was measured by MTT assay (ATCC), in which the cells reduced MTT dye that could be measured by the absorbance at 590-650nm. Briefly, MTT dye was added to the well at 1:10 ratio, and the plate was incubated at 37°C, 5%CO2 humidified incubator for 2-3 hrs. After incubation, 100 µl of detergent was added and the optical density was determined at 590-650nm. The numerical values obtained from A590-650 readings were used as referenced proliferation index.

The collagen contraction assay: Human dermal fibroblasts (purchased from Cambrex Biosciences, Walkersville, MD) cultured at 80% confluence were harvested by treatment with 0.05% trypsin / 0.02% EDTA. Trypsin was inactivated by addition of starvation medium (without FBS). The cells were washed twice with starvation medium and 60,000 cells were added into each well in 24-well plate. The fibroblasts were mixed quickly with 100 µl of freeze-dry stabilized platelet sample and 250 µl of 2mg/ml type I rat tail collagen (Upstate, NY). The collagen was allowed to polymerize at 37°C for 2-3 hrs. After incubation, the gels were gently detached from the plastic surface to allow contraction, 1.0 ml of starvation medium was added, and the gels were incubated overnight at 37°C in 5% CO2. The contraction of collagen gel was analyzed the next day.

In vivo wound healing study: Diabetic mice (male Lepr db +/+), 15 animals per group, were used for in vivo wound healing experiment. The hair on the back of the mice was removed and the animals were allowed to rest for 24 hrs. At day 0 a full-thickness wound (approximately 1cm2) was made on the back and test materials (occlusive dressing, freeze-dried platelets, fresh platelets or VEGF) were applied to the wound site. For FDP group, 1 time application of 5 x108 platelets; for fresh platelets group, 5 times application on 5 consecutive days of 1 x 108 platelets; for VEGF control group, 5 times application on 5 consecutive days of 1 µg VEGF. Wound measurements and pictures were taken every 3rd day for 24 days. The tissue at the wound bed was removed from one animal of each group every 3rd day for H&E analysis.

Results

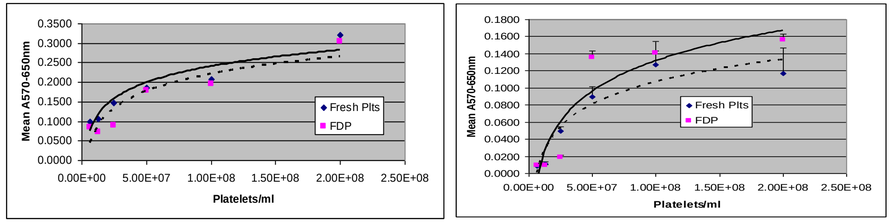

Figure 1: FDP and fresh platelets stimulate metabolic activities of fibroblasts and endothelial cells in a similar dose dependent manner.

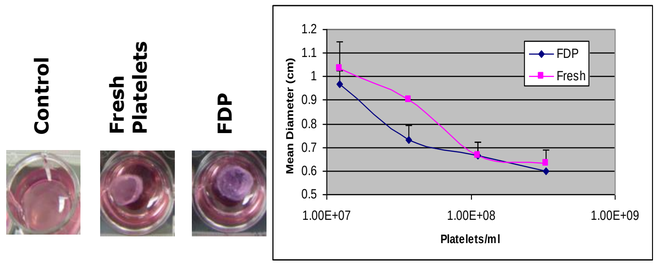

Figure 2: The collagen contraction assay. FDP caused fibroblasts populated collagen gel to contract in the same manner as fresh platelets.

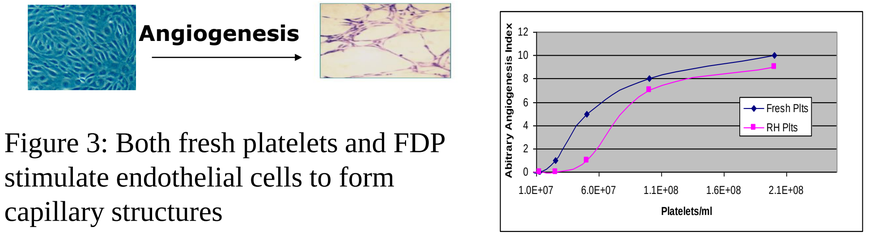

Figure 3: Both fresh platelets and FDP stimulate endothelial cells to form capillary structures

Growth factor contents within the FDP are the same as fresh platelets and literature reported values

Source	PDGF-bb ng/platelet	TGF-b1 pg/platelet
Zimmerman R. et. al.	$1.61x10^{-8} \pm 1.35 x10^{-8}$	$1.07x10^{-4} \pm 1.25 x10^{-4}$
Eppley B.L. et. al.	$1.06x10^{-8} \pm 5.00 x10^{-9}$	$7.50x10^{-5} \pm 2.63 x10^{-5}$
Fresh Platelets	$1.35x10^{-8} \pm 6.35 x10^{-6}$	$6.45x10^{-6} \pm 2.77 x10^{-7}$
FDP	$1.64x10^{-8} \pm 4.49 x10^{-7}$	$3.83 x10^{-6} \pm 5.84 x10^{-8}$

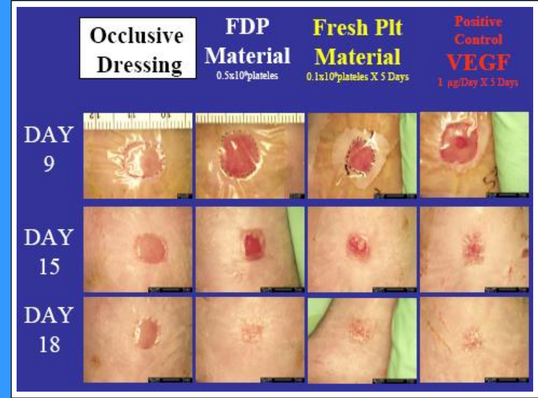

Figure 4. Effect of Freeze-dried platelets on wound healing. Clinical pictures were taken on days 9, 15 and 18. While the Occlusive Dressing group is clearly in an early phase of healing, the other groups have undergone re-epithelization. For the FDP, Fresh platelets and VEGF samples, complete wound closure is nearly accomplished at day 18

Figure 5. Microscopic view of the Wound bed of the Occlusive Dressing, FDP, fresh platelets and the VEGF treated groups at days 12 and 15. The deposition of granulation tissue and relative vascularity is more evident in FDP, fresh platelets and the VEGF treated groups than in the control group.

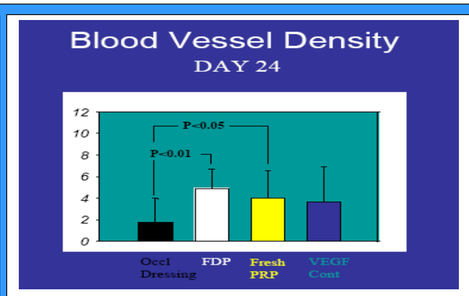

Figure 6: The H&E stained sections were scanned at low power to identify areas with the most intense neovascularization. To evaluate neovascularization, 3 fields per slide at 40x magnification were systematically taken, one in the middle of the lesion and two at wound edges. The pictures obtained were viewed with Adobe Photoshop CS Software (Adobe Systems Incorporated, San Jose, CA) and blood vessels per field were marked and counted.

CONCLUSIONS

- **Adlyfe's proprietary freeze-drying process produces stabilized freeze-dried platelets that have physical and biochemical characteristics of fresh platelets.**
- ***In vitro*, freeze-dried platelets stimulate cell proliferation, tissue remodeling and angiogenesis in the same manner as fresh platelets.**
- ***In vivo*, freeze-dried platelets accelerate wound healing and promote wound closure in a similar manner as fresh platelets and VEGF control.**
- **Freeze-dried platelets promote angiogenesis at the wound bed in a similar manner as fresh platelets and VEGF control.**
- **Freeze-dried platelets can be used as a safe and well-suited alternative to fresh platelets for wound healing applications.**