



Platelet-rich fibrin matrix improves wound angiogenesis via inducing endothelial cell proliferation

Sashwati Roy, PhD; Jason Driggs, BS; Haytham Elgharably, MD; Sabyasachi Biswas, PhD;
Muna Findley, BS; Savita Khanna, PhD; Urmila Gnyawali, RN; Valerie K. Bergdall MD, PhD;
Chandan K. Sen, PhD

Comprehensive Wound Center, Department of Surgery, Davis Heart and Lung Research Institute, The Ohio State University Medical Center, Columbus, Ohio

Reprint requests:

Dr. C.K. Sen, 473 West 12th Avenue, 512
DHLRI, The Ohio State University Medical
Center, Columbus, OH 43210, USA.
Tel: +614 247 7658;
Fax +614 247 7818;
Email: chandan.sen@osumc.edu

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ABSTRACT

The economic, social, and public health burden of chronic ulcers and other compromised wounds is enormous and rapidly increasing with the aging population. The growth factors derived from platelets play an important role in tissue remodeling including neovascularization. Platelet-rich plasma (PRP) has been utilized and studied for the last four decades. Platelet gel and fibrin sealant, derived from PRP mixed with thrombin and calcium chloride, have been exogenously applied to tissues to promote wound healing, bone growth, hemostasis, and tissue sealing. In this study, we first characterized recovery and viability of as well as growth factor release from platelets in a novel preparation of platelet gel and fibrin matrix, namely platelet-rich fibrin matrix (PRFM). Next, the effect of PRFM application in a delayed model of ischemic wound angiogenesis was investigated. The study, for the first time, shows the kinetics of the viability of platelet-embedded fibrin matrix. A slow and steady release of growth factors from PRFM was observed. The vascular endothelial growth factor released from PRFM was primarily responsible for endothelial mitogenic response via extracellular signal-regulated protein kinase activation pathway. Finally, this preparation of PRFM effectively induced endothelial cell proliferation and improved wound angiogenesis in chronic wounds, providing evidence of probable mechanisms of action of PRFM in healing of chronic ulcers.

The economic, social, and public health burden of chronic ulcers and other compromised wounds is enormous and rapidly increasing with the aging population.¹ Of the over 20 million individuals with diabetes in the United States, 15% or more can be expected to develop diabetic ulcers, while over 2 million individuals currently suffer from venous leg ulcers. The numbers of patients with arterial ulcers and pressure ulcers are equally staggering. Managing these wounds represents \$5–10 billion each year.¹ Consequently, there has been heightened interest in developing new advanced therapies to address the compromised wound.

Healing in acute wounds follows a progression through a series of complex but orderly physiological and molecular processes. These processes include coagulation, inflammation, cell recruitment, migration, proliferation, and connective-tissue production followed by matrix remodeling and maturation.² Chronic, compromised wounds are characterized as having stalled somewhere in this progression to healing due to a variety of systemic and local factors including poor perfusion and low oxygen tension, excess microbial burden, necrotic tissue, chronic venous insufficiency, wound bed cells that are unresponsive to normal cell signaling,³ and decreased growth factor production and response. Even with optimal wound bed preparation including adequate debridement, infection control, restoration of blood supply and establishment of a moist healing environment, significant progress toward healing is still not achieved in 30% or more of ulcers seen.⁴ In these cases,

advanced therapies must be considered.⁴ Current advanced therapies available include therapy to replace deficient components (autologous epidermis, allografts, and living skin equivalents), complimentary therapies (hyperbaric oxygen, negative pressure, ultrasound, and electrical stimulation), dermal matrix equivalents, and exogenous growth factors (purified single growth factors, autologous growth factor, and growth factor/fibrin preparations).³ Much renewed interest has recently been seen in this last category of autologous platelet/fibrin biologics.⁵ However, there still remain questions concerning their effectiveness compared to other advanced therapies, the extent of variability inherent in autologous biologics and, ultimately, questions concerning their mode of action in compromised, nonhealing wounds.

The role of platelets in wound healing is well described. However, there are few studies on platelet-induced changes in wounded skin at the molecular level. There is a need to characterize key platelet-derived factors in terms of levels of expression, viability, and functionality in order to better understand their role in the healing process. There are several animal wound models reported in the literature.^{6,7} As compromised blood supply and local tissue ischemia are often of paramount importance in chronic ulcers, the comparison of the wound healing process in ischemic and nonischemic wound models is of particular relevance.⁸ Growth factors derived from platelets play an important role in tissue remodeling including neovascularization.⁹ Two complimentary

studies are presented in this article. First, we identified and characterized representative factors present in an autologous human platelet-rich fibrin matrix (PRFM) that impact endothelial cell function in an endothelial cell culture system. Secondly, we examined the action of platelet-derived factors on wound healing coupled with an approach to a potential mechanism of action at the tissue molecular levels in an ischemic porcine wound model.

MATERIALS AND METHODS

Human subjects and sample collection

Blood samples were collected from healthy adult male and female donors aged 20–50 years. The subjects were not pregnant and/or on immunosuppressants. Protocols were approved by the Ohio State University's Institutional Review Board. Declaration of Helsinki protocol was followed and patients gave their written, informed consent.

PRFM preparation

PRFM was prepared using the FIBRINET® PRFM system (Cascade Medical, Wayne, NJ) as per protocol as shown in Figure 1A. In brief, blood (18 mL) was drawn into two collection tubes (yellow top tube), containing trisodium citrate as an anticoagulant and a proprietary separator gel. The tubes were centrifuged at $1,100 \times g$ for 6 minutes at room temperature. The platelet-rich plasma (PRP) layer was transferred to a 30-mL vial containing CaCl_2 . The clotting process was triggered by the presence of CaCl_2 , and the vial was immediately centrifuged at $4,500 \times g$ for 25 minutes at room temperature. The PRFM membranes were recovered and were used for either in vitro culture or porcine wound studies. For porcine wound studies, PRFM were prepared from autologous blood obtained from pigs used for the study.

Percent platelet recovery

Percent recovery of platelets in PRP was determined by counting platelets in whole blood and in PRP using Beckman Coulter LH 755 (Miami, FL). The Beckman Coulter LH 755 Workstation is an automated quantitative hematology analyzer that utilizes a refined electronic particle counting principle to quantitate white cells, red cells, and platelets. Percent platelet recovery was calculated using the following formula:

$$\frac{(\text{Platelet count in PRP} \times \text{Total PRP volume})}{\text{Whole blood platelet count} \times \text{Whole blood collected volume}} \times 100$$

Cell viability assay for platelets in PRFM

PRFMs were placed in 6-well plates with 2 mL of RPMI 1640 media supplemented with antibiotics solution (100 IU/mL penicillin and 0.1 mg/mL streptomycin) and were maintained in a standard cell culture incubator at 37 °C and 5% CO_2 . The media was collected from the wells at designated times.

Lactate dehydrogenase (LDH) leakage from PRFM platelets to the media was assessed using CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI). We have observed that presence of even very minute amounts of hemoglobin interferes with most colorimetric assays available to assay LDH-based cell viability. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay is a fluorometric method where hemoglobin does not interfere. This assay is routinely used for estimating the number of nonviable cells present in culture plates. The CytoTox-ONE™ Assay measures the release of LDH from cells with a damaged membrane. LDH released into the culture medium is measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into a fluorescent resorufin product. The amount of fluorescence produced is proportional to the number of lysed cells. The assay was performed as per manufacturer's instructions.

Enzyme-linked immunosorbent assay

Human platelet-derived growth factor (PDGF)-BB, transforming growth factor (TGF)- β , and vascular endothelial growth factor A (VEGF-A) contents from PRFM-conditioned media were measured at specified times using commercially available assay kits as per manufacturer's recommendation (R&D Systems Inc., Minneapolis, MN).

Human endothelial cell proliferation assay

Human microvascular endothelial cells (HMEC-1) were cultured under standard culture conditions (5% CO_2 at 37 °C) in MCDB-131 growth medium (GIBCO-BRL, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 2 mol/L L-glutamine.¹⁰ Cells were counted using a Coulter Z1 particle counter (Beckman Coulter Inc.). For proliferation assay, cells were seeded (5,000 cells per well) in 96-well plates. After 24 hours of seeding, the media was changed to serum-free media. Cells in serum-free media were treated with conditioned media from PRFM as indicated in figure legends. The proliferation of cells following 48 hours of PRFM-conditioned media treatment was determined using CyQUANT™ cell proliferation assay kit (Molecular Probes, Invitrogen).

Small interfering RNA (siRNA) transfection

HMEC were seeded in antibiotic-free medium 24 hours prior to transfection. DharmaFECT™ 1 transfection reagent (Dharmacon RNA technologies, Lafayette, CO) was used to transfect cells with 100 nM of siRNA pool for extracellular signal-regulated protein kinase (ERK) 1/2 (Dharmacon RNA technologies) for 72 hours as described previously.¹¹ For controls, siControl nontargeting siRNA pool (mixture of four siRNAs, designed to have at least 4 mismatches with the corresponding gene) was used. After transfection with siRNA, HMEC were seeded in 96-well plates and followed by treatment with conditioned media from PRFM as described for the cell proliferation assay. For quantification of protein using Western blot, samples were collected after 72 hours of siRNA transfection. Protein extraction was done, and immunoblot analysis for ERK 1/2 was performed.

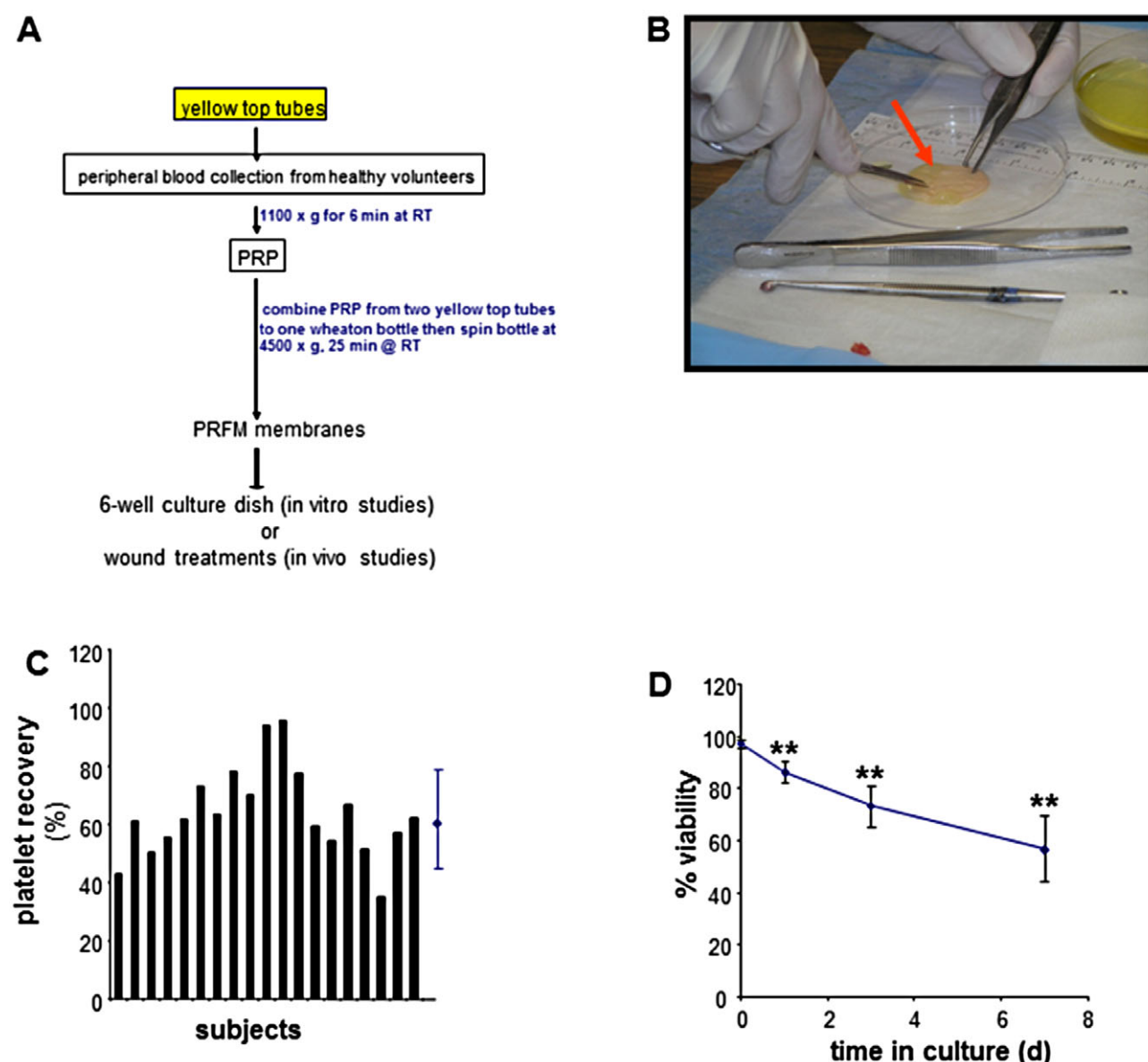


Figure 1. Preparation of platelet-rich fibrin matrix (PRFM), quantification of platelet recovery and viability. (A) An outline of the PRFM preparation protocol. (B) Red arrow indicates a ready PRFM membrane that was used for in vitro and in vivo wound studies. (C) Platelet recovery determined in the first 20 subjects enrolled. Individual % recovery data as well as average (error bar) recovery is shown. Average data shown are mean $\pm$ standard deviation (SD) ($n = 20$). (D) Changes in platelet viability in PRFM over time. Lactate dehydrogenase release from cells was used as measure of loss of platelet viability. Data shown are % change compared to 0 hour or baseline sample (100% viable). Mean $\pm$ SD ($n = 10$); ** $p < 0.001$ compared to baseline.

Immunoblot analyses

For ERK, phospho-ERK immunoblots, HMEC cellular protein extracts were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel under reducing conditions, transferred to nitrocellulose and probed with anti-ERK (1:1,000, Biosource, Invitrogen) and phospho-ERK (1:1,000, Biosource, Invitrogen) antibody. Immunoblotting was performed using standard procedures described previously.¹²

Porcine ischemic excisional wound model and PRFM treatment

All experiments were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. Pigs (70–80 lb) were anesthetized using Telazol® (Fort Dodge Animal Health, Fort Dodge, IA) followed by isoflurane. The dorsal region was shaved and was surgically prepared with

alternating Betadine® (Purdue Pharma L.P., Stamford, CT) and alcohol scrubs. Under such aseptic conditions, four full-thickness bipedicle skin flaps measuring 15×5 cm were developed on the back of each animal by means of parallel incisions as described previously.⁸ Ischemia of the flap tissue was verified by laser Doppler imaging of blood flow.⁸ Full-thickness excisional wounds were developed in the middle of each flap with an 8-mm disposable biopsy punch. Six more wounds were developed similarly on nonischemic skin to serve as controls. Blood from the wounds was blotted, and the PRFMs were placed in the treatment wounds such that the bulk of it was within the wound with a small margin around the wound edge. One thoracic and one lumbar wound were treated for both the ischemic and control wound groups. The incisions were dressed with VAC Drape (KCI Inc., San Antonio, TX). The dressing was changed every three days, and any accumulating wound fluid was drained. All wounds were digitally imaged. On day 14 post-wounding, the entire wound tissue (1.5×1.5 cm with the 8-mm wound at the center) was harvested for tissue analyses (RNA, protein, and histological analyses). The pigs were euthanized after the completion of experiments.

Histology

Formalin-fixed paraffin-embedded or optimum cutting temperature (OCT)-embedded frozen wound-edge specimens were sectioned. The paraffin sections were deparaffinized and stained with hematoxylin & eosin (H&E), Masson's trichrome, and picrosirius red staining using standard procedures. Immunohistochemical staining of paraffin or frozen sections was performed as described earlier⁸ using the following primary antibodies: anti-von Willebrand's factor (vWF) (1:400; BD Pharmingen, San Diego, CA) and anti-Ki67 (1:200; Neomarkers, Fremont, CA) after heat-induced epitope retrieval when necessary. Secondary antibody detection and counterstaining were performed as described previously.¹³

Image quantification

The mosaic images of whole wounds were collected under $20\times$ magnification guided by MosaiX software (Zeiss, Thornwood, NY) and a motorized stage. Each mosaic image was generated by combining a minimum of ~ 100 images. Between 7 and 10 high-powered representative areas from mosaic images were quantified for each data point from each animal. Quantification was performed employing Auto-measure software (Zeiss).

Laser Doppler blood flow imaging

The MoorLDI™-Mark 2 laser Doppler blood perfusion imager (resolution: 256×256 pixels in the region of interest, each pixel being an actual measurement) employs a visible red laser beam (633 nm) to map tissue blood flow and enable quantification (Figure 12).⁸

Laser microdissection pressure catapulting

Tissue sectioning and fixation

Frozen tissue blocks were cut into 8- μ m sections. The sections^{2,3} were mounted on each RNAZap-treated thermo-

plastic (polyethylene naphthalate)-covered glass slide (PALM Technologies, Bernreid, Germany) and kept at -80°C until use. Sections were fixed and treated as described.¹⁴

Granulation tissue staining

After rinsing slides in diethyl pyrocarbonate (DEPC)-treated water for 2 minutes, the sections were stained using a quick hematoxylin QS for granulation tissue identification.¹⁵ The sections were rinsed in DEPC phosphate buffer saline solution for 2 minutes, and sequentially dehydrated as described.¹⁴ Sections were stained using a quick hematoxylin QS protocol.¹⁵ The granulation tissue was identified as hypercellular region (Figure 11A–C). Furthermore, $1 \times 10^6 \mu\text{m}^2$ granulation tissue was captured in Trizol for mRNA extractions.

Laser microdissection pressure catapulting

Laser microdissection pressure catapulting was performed using the laser microdissection system from PALM Technologies containing a PALM MicroBeam and RoboStage for high-throughput sample collection and a PALM RoboMover (PALM RoboSoftware version 4.0).^{14,15} Typical settings used for laser cutting were ultraviolet (UV)-Energy of 75–85 and UV-Focus of 52. The granulation tissue was cut and captured under a $10\times$ ocular lens.

mRNA extraction and CD31 quantification was performed as described before.¹⁴

Statistics

Data are reported as mean $\pm$ standard deviation of at least three experiments. Difference between means was tested using Students *t* test or analysis of variance as appropriate. A value of $p < 0.05$ was considered statistically significant.

RESULTS

In this study, PRFMs were prepared as described,⁵ from 20 human donors. The PRFM consists of a cross-linked fibrin matrix containing platelets. The system of preparation employed here separates a PRP fraction from whole blood by centrifugation ($1,100 \times g$) for 6 minutes in a BD Vacutainer® tube (Becton, Dickinson and Company, Franklin Lakes, NJ) (Figure 1A). The heavier red blood and white blood cells reside in the lower layer of the tube, and a thixotropic separator gel separates this layer from the upper fraction containing the platelets. The transfer of the PRP fraction to a second "membrane vial," containing calcium chloride, is facilitated by a transfer device. The calcium ions initiate thrombin-catalyzed cleavage of fibrinogen to fibrin. The process is further accelerated by a further centrifugation of the second tube at a higher *g* force ($4,500 \times g$) for 25 minutes. This results in a PRFM disc or membrane, which adopts the contour of the base of the vial. The conditions of preparation are such that the membrane contains a concentrated platelet population, which are intact. In the wound care application described above, this membrane, which can be readily removed from the Cascade® membrane vial, is applied directly to the wound (Figure 1B).

The recovery of platelets in PRP using the FIBRINET® system varied in subjects between 35 and 95% with mean

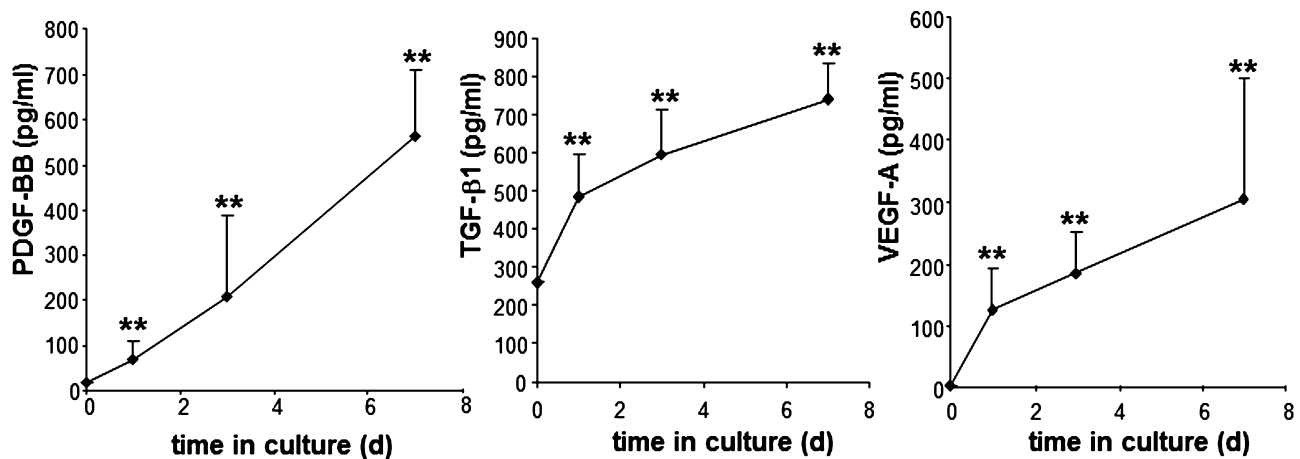


Figure 2. Release of growth factors from platelet-rich fibrin matrix (PRFM) in culture. PRFM was prepared and placed in 6-well culture plates with RPMI 1640 media. Growth factors (platelet-derived growth factor [PDGF-BB], transforming growth factor [TGF]- β 1, and vascular endothelial growth factor [VEGF-A]) released in media were measured using enzyme-linked immunosorbent assay. Data shown are actual growth factor levels (ng/mL) in media bathing PRFM. Data are mean $\pm$ standard deviation ($n = 10$ for PDGF and $n = 5$ for VEGF and TGF- β 1); ** $p < 0.01$ compared to 0 hour (baseline) sample.

recovery was 63% (Figure 1C). The freshly prepared PRFMs were incubated in cell culture media under standard culture conditions, and the viability of platelets in PRFM was determined using LDH system. Under these conditions, a gradual loss of viability of platelets in PRFM was observed (Figure 1D). The growth factor levels in media overlying PRFM (conditioned media) were determined using enzyme-linked immunosorbent assay (Figure 2). An increase in the levels of PDGF-BB, TGF- β 1, and VEGF-A was noted in culture media indicating a gradual release of growth factors by platelets embedded in PRFM (Figure 2).

To determine whether the growth factors released by PRFM are biologically active, the PRFM-conditioned media was tested in a HMEC proliferation assay. The HMEC were cultured in 96-well plates followed by serum starvation to restrict the proliferating HMEC in G1 phase. PRFM-conditioned media was collected on day 7 after culture. The HMEC were treated either with varying percentage (v/v) of day 7 PRFM-conditioned media and serum-free HMEC culture media or 10% (v/v) FBS with HMEC culture media. As anticipated, serum starvation resulted in growth suppression compared to cells cultured using standard 10% FBS (Figure 3A). Increasing the concentration of day 7 PRFM-conditioned media in serum-free HMEC culture media resulted in progressive increase in HMEC proliferation (Figure 3A). Treatment of serum-starved HMEC with days 1, 3, or 7 PRFM-conditioned media (25% v/v) resulted in significant but similar increase in cell proliferation (Figure 3B). Treatment of serum-starved HMEC with conditioned media from platelet-poor plasma gel did not change the rate of cell proliferation, suggesting that the factors released by platelets is required for endothelial cell proliferation (Figure 3C). The growth factor levels (especially PDGF and TGF- β) in conditioned media increased gradually (Figure 2), whereas the extent of cell proliferation induced by the media collected on these days was comparable. These data show that the cell proliferation is not correlated to PDGF or TGF- β levels

in PRFM-conditioned media. Treating PRFM-conditioned media with neutralizing antibody against PDGF and VEGF resulted in depletion (>90%) of the levels of these growth factors from the media (data not shown). Depletion of PDGF from conditioned media did not affect HMEC proliferation. A small but significant decrease in HMEC proliferation was noted in cells treated with VEGF-depleted PRFM-conditioned media (Figure 4A), suggesting that VEGF released by PRFM is at least, in part, involved in HMEC proliferation.

VEGF is a strong activator of ERKs 1 and 2 via its major receptor, kinase insert domain-containing receptor (KDR), resulting in endothelial cell mitogenesis. The treatment of HMEC with day 7 PRFM-conditioned media resulted in an increase in ERK 1/2 phosphorylation, while the total levels of ERK in the cells remained unchanged (Figure 4B–C). A pharmacological inhibitor of ERK UO126 significantly inhibited day 7 PRFM-conditioned media-induced endothelial cell proliferation (Figure 4D). To determine a specific role of ERK 1/2 in PRFM-conditioned media-induced endothelial cell proliferation, we used the siRNA approach. Transfection of HMEC with ERK 1 or 2 siRNA either alone or in combination resulted in knockdown of the respective proteins (Figure 5A–C). ERK 1/2 knockdown using siRNA significantly inhibited day 7 PRFM-conditioned media-induced endothelial cell proliferation (Figure 5D).

To determine effects of PRFM on wound healing, we used a porcine ischemic wound model (Figure 6). These ischemic wounds show impairment in angiogenesis and subsequent closure.⁸ PRFM was applied to ischemic excisional wounds (Figure 6A–B), and the quality of the regenerated tissue was determined using the Masson-trichrome and H&E staining of the wound tissue on day 14 post-wounding. Histological evaluation of the wounds sections shows that PRFM-treated ischemic wounds at day 14 show presence of mature collagen fibers in the treated wounds compared to untreated wounds (Figure 7). vWF is a large multimeric gly-

coprotein produced constitutively in the endothelium. On day 14 post-wounding, PRFM-treated ischemic wounds displayed increased number of vWF+ cellular structures indicative of vascular formation (Figure 8).

Based on the data derived from the effect of PRFM on endothelial cell proliferation under cell culture conditions, we asked whether the increased number of blood vessels in wound treated with PRFM is attributable to increased proliferation of the cells. Cell proliferation in wounds was detected using antibody against Ki67 antigen. Ki67 is found in growing, dividing cells but is absent in the resting phase of cell growth. PRFM-treated ischemic wounds displayed greater numbers of Ki67+ compared to nontreated control ischemic wounds (Figure 9). Colocalization studies show that many of these proliferating Ki67+ cells were also positive for vWF (Figure 10). To quantitatively assess that PRFM treatment to ischemic wounds increases endothelial cell numbers in the granulation of the healing wounds, we utilized the laser capture microdissection (LCM) approach. Granulation tissues were captured using LCM near (within 500 μ m) the epithelial margins; CD31 gene expression was measured in the granulation tissue as a marker for endothelial cells (Figure 11). Laser Doppler imaging is a useful technique for measuring microvascular perfusion in wounds because it involves no contact and produces a color image representing flow distri-

bution over an area of tissue.¹⁶ Finally, to show that more CD31 and vWF positive endothelial cells were actually functional vessels, we performed laser Doppler blood flow imaging (Figure 12). Taken together, the LCM, laser Doppler data and immunohistochemical data indicate presence of functional vessels and angiogenesis in PRFM-treated wounds.

DISCUSSION

The study characterized recovery and viability of as well as growth factor release from platelets in a novel preparation of platelet gel and fibrin matrix, namely PRFM. The growth factors released by platelets in PRFM induced endothelial cell proliferation and promoted wound angiogenesis in porcine ischemic wounds. PRP has been utilized and studied since the 1970s.¹⁷ PRP is used clinically in humans for its healing properties attributed to the increased concentrations of autologous growth factors and secretory proteins that may enhance the healing process on a cellular level.¹⁷ Conventional PRP has been defined as plasma with a platelet concentration above the "normal" physiologic levels found in whole blood.¹⁸ Currently, several methods and systems are available for the preparation of PRP, most producing a liquid end product. Platelet gel and fibrin sealant derived from PRP mixed with

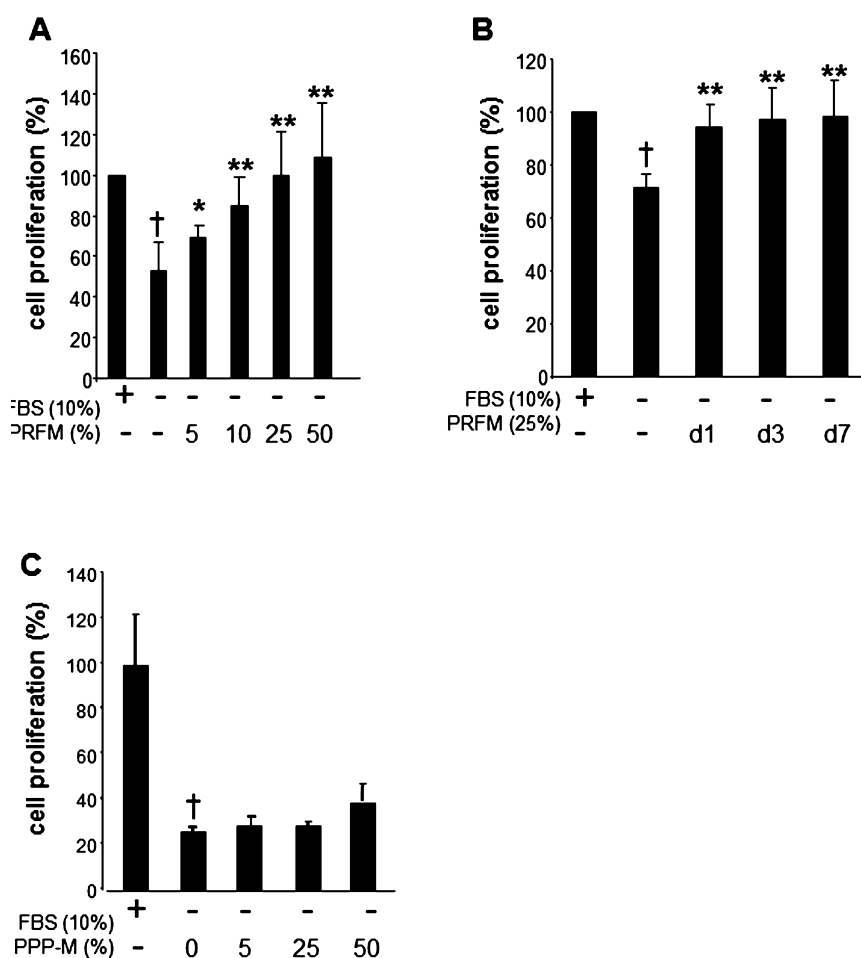


Figure 3. Conditioned media from platelet-rich fibrin matrix (PRFM)-induced human endothelial cell proliferation. Human microvascular endothelial cells (HMEC) were cultured in 96-well plates. The cells were serum starved and simultaneously treated with conditioned media from PRFM. The cells were cultured with 10% fetal bovine serum (FBS) in culture media served as positive control. (A) Cell proliferation in the presence of PRFM-conditioned media (dilution = % conditioned media of the total culture media). Data are mean $\pm$ standard deviation (SD) ($n=5$); $\dagger p < 0.01$ compared to positive control; $*p < 0.05$; $**p < 0.01$ compared to conditioned media untreated group. (B) HMEC cells cultured in serum-free conditions were treated with 25% (i.e., one part condition media added to three parts of cell culture media) PRFM-conditioned media collected on days 1, 3, or 7 post-PRFM formation. Data are mean $\pm$ SD ($n=4$); $\dagger p < 0.01$ compared to positive control; $**p < 0.01$ compared to conditioned media-untreated group; (C) Cell proliferation in the presence of platelet-poor plasma gel-conditioned media (PPP-M, dilution = % conditioned media of the total culture media). Data are mean $\pm$ SD ($n=5$); $\dagger p < 0.01$ compared to positive control.

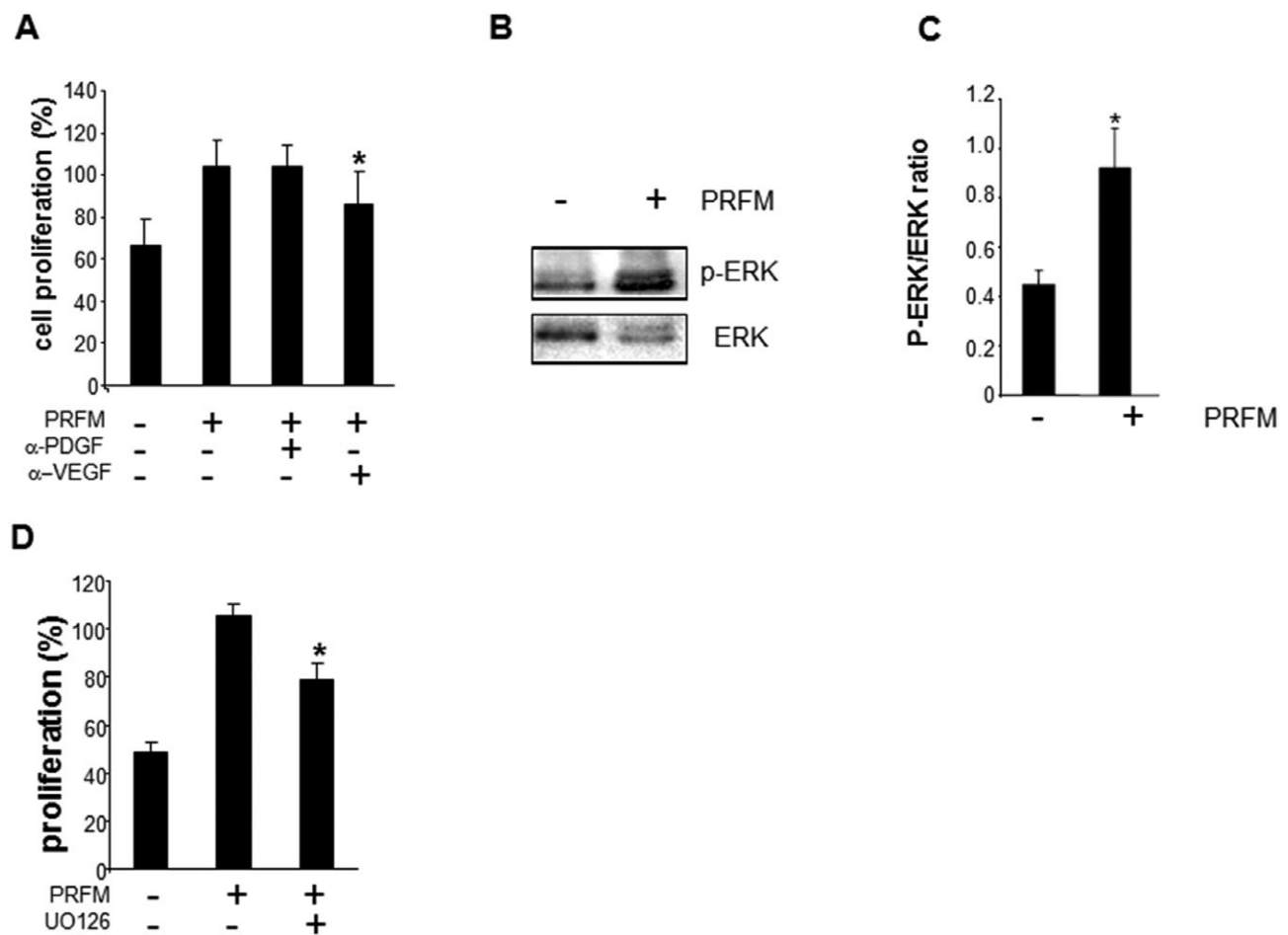


Figure 4. Role of platelet-derived growth factor (PDGF-BB) and extracellular signal-regulated protein kinase (ERK) activation in human microvascular endothelial cells (HMEC) proliferation induced by platelet-rich fibrin matrix (PRFM)-conditioned media. (A) PRFM-conditioned media was treated or not with anti-PDGF or anti-vascular endothelial growth factor (VEGF) (20 µg/mL) blocking antibody to sequester PDGF-BB or VEGF in PRFM-conditioned media. Cells cultured in serum-free conditions were treated with 25% PRFM for 48 hours. Data are mean $\pm$ standard deviation (SD) ($n = 4$). * $p < 0.05$ compared to PRFM-treated and blocking antibody-untreated group. (B) PRFM-conditioned media (25%) induced rapid (30 minutes) ERK phosphorylation (p-ERK) in HMEC. (C) Quantification of the immunoblot data (B) using densitometry. Ratio of p-ERK/ERK is shown. * $p < 0.05$ compared to PRFM-untreated group. (D) ERK inhibition attenuated HMEC proliferation induced by PRFM (25%). ERK inhibition was achieved by pretreatment of cells with the ERK inhibitor UO126 (10 µM) 5 minutes prior to PRFM exposure. Data are mean $\pm$ SD ($n = 5$). * $p < 0.05$ compared to PRFM-treated and inhibitor-untreated group.

thrombin and calcium chloride have been exogenously applied to tissues to promote wound healing, bone growth, hemostasis, and tissue sealing.¹⁹ PRFM was prepared as a membrane-based preparation of PRP, that is, without the use of exogenous thrombin.⁵ Ultrastructural analysis of PRFM with confocal scanning fluorescence microscopy and scanning electron microscopy revealed a dense highly cross-linked fibrin matrix with the intact platelets localized on one side of the membrane.²⁰

A large number of in vitro studies have been performed characterizing PRP and products derived from PRP.^{19,21} This study reports, for the first time, the viability of platelets within PRFM. Determination of viability of platelets in PRFM is critical to understanding the dynamics of the growth factor

release by these cells while on fibrin matrix. Viability assay shows that under in vitro conditions, platelets gradually lose viability over a period of time. It is also important to note that the platelets produced with this system immediately following production are intact.⁵ The loss of cell viability is expected to be more rapid in the harsh environment of wounds where high levels of proteases are known to be present.²² Studies are required to understand the fate of platelets' viability in a wound environment. A significant difference between the platelet number in the whole blood samples and their experimental PRP has been reported.²¹ In the majority of the studies reported, the data presented are difficult to interpret because of lack of reference value of the whole blood platelet numbers.^{23,24} The recovery of platelets in this study was of the order of 65% with

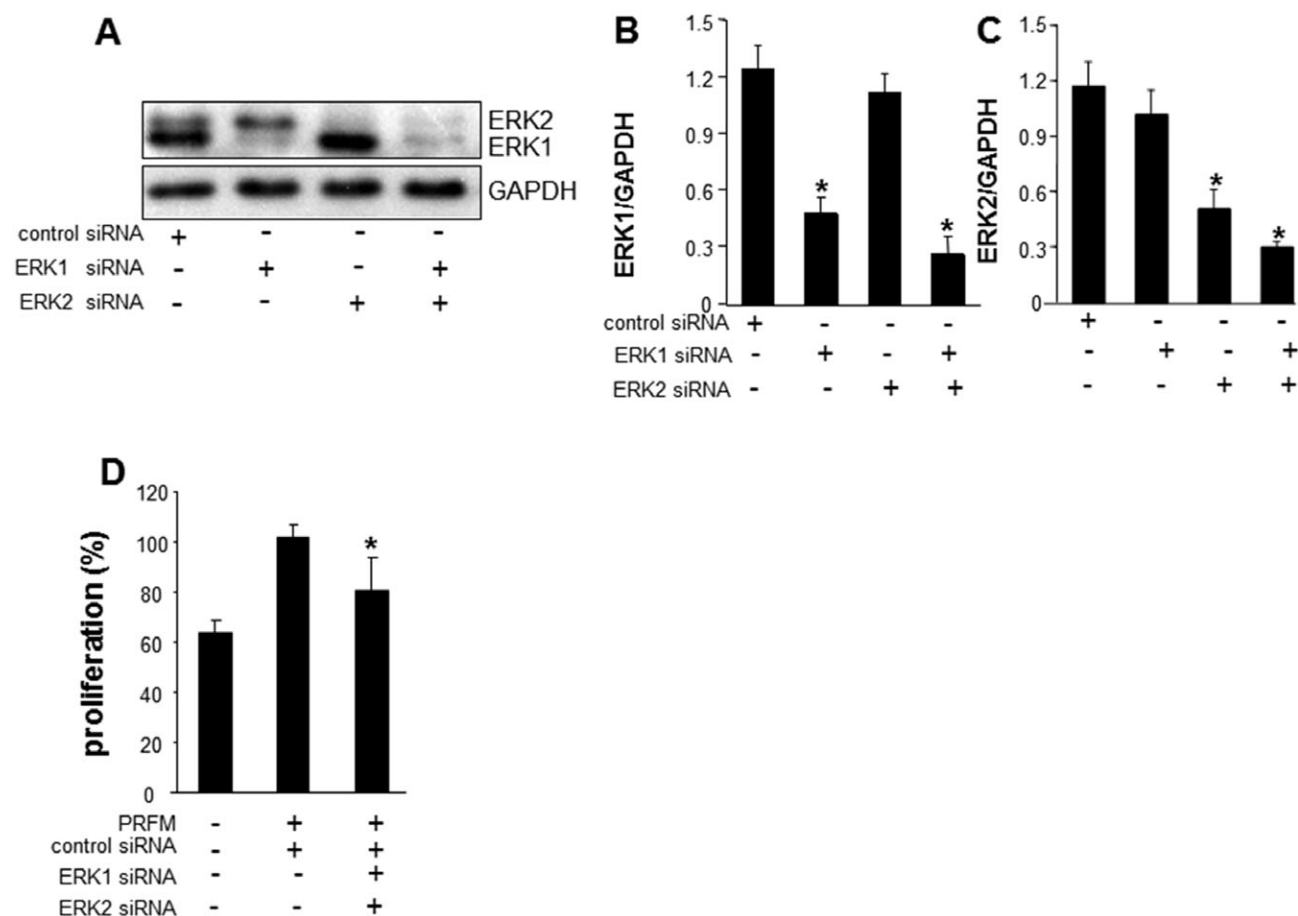


Figure 5. Extracellular signal-regulated protein kinase (ERK) knockdown inhibited platelet-rich fibrin matrix (PRFM)-conditioned media-induced proliferation of human microvascular endothelial cells (HMEC). Cells were transfected with siRNA to knockdown ERK 1 and 2. Control cells were transfected with scrambled (control) siRNA. (A) ERK immunoblot showing effective knockdown of ERK following siRNA transfection. (B,C) Quantification of the immunoblot densitometry data of ERK1 (B) and ERK2 (C) shown in (A). Ratio of ERK/GAPDH is shown. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein standardization of sample loading. Data are mean $\pm$ standard deviation (SD) ($n = 3$); * $p < 0.05$ compared to control siRNA-treated group. (D) ERK knockdown attenuated PRFM (25%)-induced proliferation of HMEC. HMEC were transfected with ERK 1 and 2 siRNA to knockdown ERK levels in cells. Control cells were transfected with scrambled (control) siRNA. ERK siRNA and control siRNA transfected cells were activated with 25% PRFM as described earlier. Results are mean $\pm$ SD. * $p < 0.05$ compared to scrambled (control) siRNA-treated group.

some interdonor variation. The platelet yields and recoveries (~60%) are comparable to studies reported previously with other commercially available systems.²⁵

The alpha granules are the storage site for a plethora of factors notably among which are growth factors.²⁶ The level of PDGF and VEGF released in conditioned media from platelets embedded in PRFM is comparable to the levels of PDGF and VEGF released from platelet gels.^{21,25} A steady release of all three growth factors over a period of 7 days suggests that the release kinetics are distinct compared to the PRP gel preparations where use of thrombin causes an immediate release of growth factors as a result of degranulation.^{25,27} Endogenous release of these mediators by local tissue at the site of delivery is known to result in a slower release of growth factors and chemical mediators.²⁷ Earlier studies using PRFM

and “washed-out” protocol showed that the conditioned media from PRFM cultures produced high levels of PDGF, TGF- β 1, VEGF, and basic fibroblast growth factor, and epidermal growth factor (EGF) supports the finding of the present study on the release of growth factors over a period of 7 days from PRFM cultures in vitro.

The supernatants from PRP gels stimulated with thrombin and calcium chloride have been shown to be mitogenic for endothelial cells.^{25,28,29} The current study provides evidence that conditioned media derived from PRFM cultures for 7 days show potent mitogenic activity. Proliferation of endothelial cells, treated with day 1, 3, or 7 conditioned media, was comparable. The growth factor levels in the conditioned media increased in period from 1 to 7 days, suggesting that the increasing concentration of growth factors did not

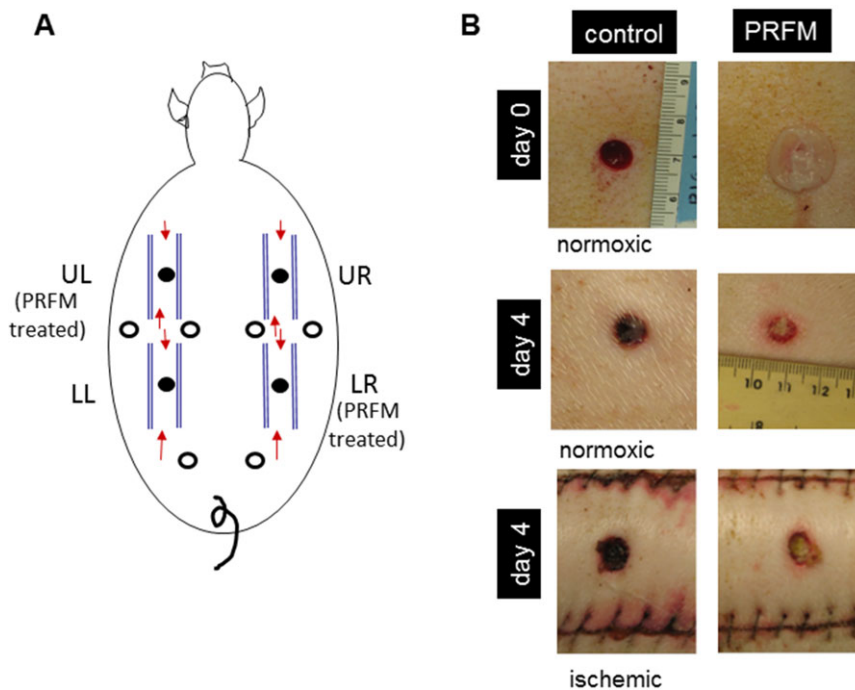


Figure 6. Treatment of porcine ischemic wound with platelet-rich fibrin matrix (PRFM). (A) Four ischemic wounds (solid circles) were developed on the back of pigs as described in the Methods section. Six additional non-ischemic wounds were developed (open circles). Arrows indicate direction of blood flow in flaps containing ischemic wounds. The upper left and lower right ischemic wounds were treated with PRFM at the time of wounding. (B) Representative digital images of excisional wounds treated or not with PRFM on days 0 and 4 post-wounding.

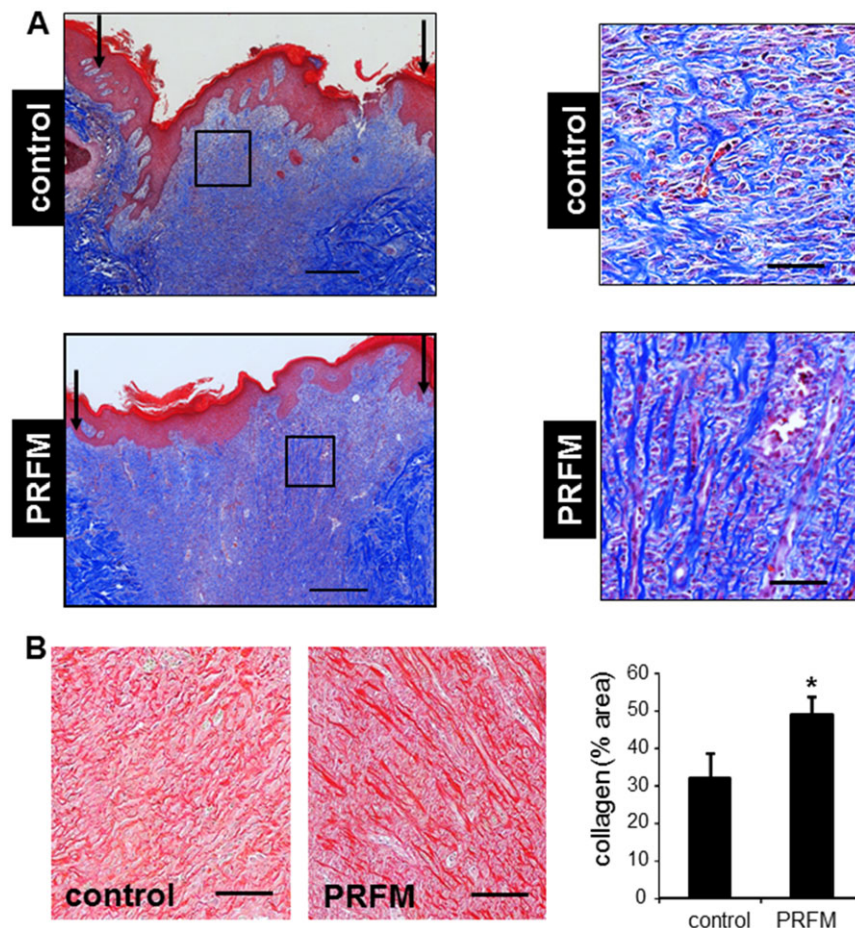


Figure 7. Ischemic wound histology following treatment with platelet-rich fibrin matrix (PRFM) membranes. Wound-edge tissues were collected on 14 days post-wounding. Formalin-fixed paraffin-embedded biopsy tissues were sectioned (5 μ m) and stained using (A) Masson's trichrome staining. This staining results in blue-black nuclei, blue collagen, and cytoplasm. Epidermal cells appear reddish. The arrows indicate the start and the end of wound. Scale bar = 500 μ m. Right panels are the zoom of boxed area of images shown in left panel. Scale bar = 50 μ m. (B) Picrosirius red staining. The red staining is the birefringence of collagen fibers, which is largely due to co-aligned molecules of type I collagen. Bar graph shows quantitation of the collagen fibers in PRFM-treated or -untreated wounds. Scale bar = 50 μ m. Data are mean $\pm$ standard deviation ($n = 3$); * $p < 0.05$ compared to untreated wounds.

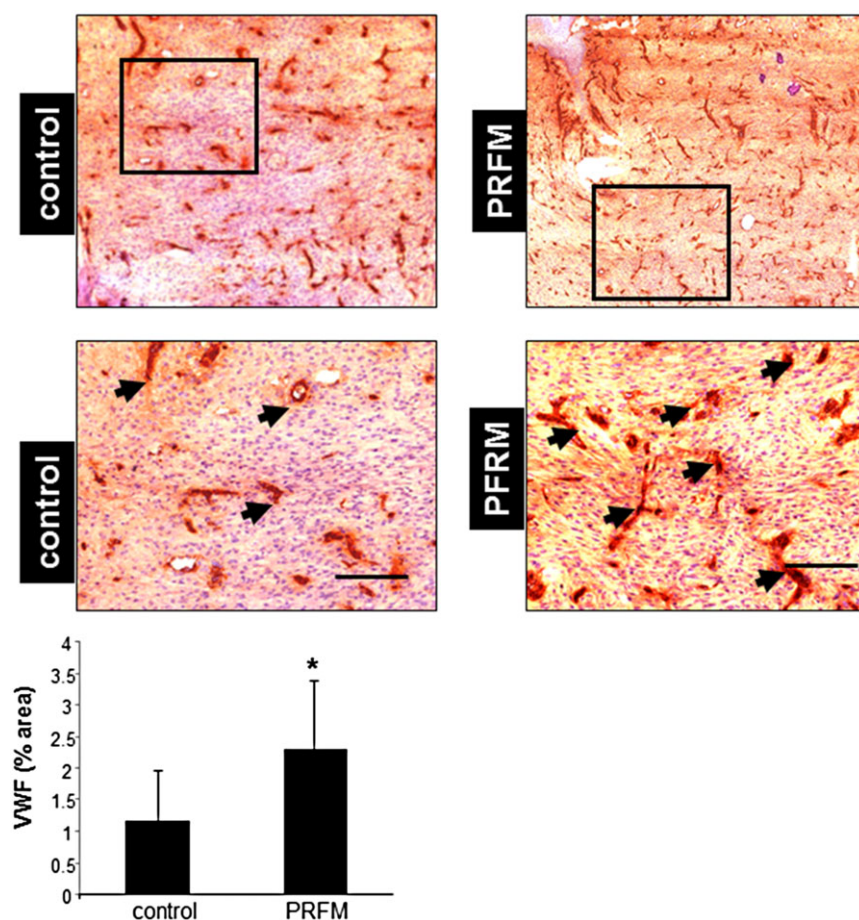


Figure 8. Increased vascularization of platelet-rich fibrin matrix (PRFM)-treated ischemic wounds. Wound-edge tissues were collected 14 days post-wounding. Formalin-fixed paraffin-embedded biopsy tissues were sectioned (5 μ m) and stained using von Willebrand's factor (vWF) (brown) and counterstained with hematoxylin (blue). Compared to nontreated ischemic wounds, the treated wounds showed increased vascular endothelial cell staining. Arrows indicate vWF-positive vessels. Right panel: Zoom of the boxed area shown in corresponding images to the left. Scale bar = 50 μ m. Bar graph shows quantitation of the blood vessels in PRFM-treated or -untreated wounds. Data are mean $\pm$ standard deviation ($n = 3$); * $p < 0.05$ compared to untreated wounds.

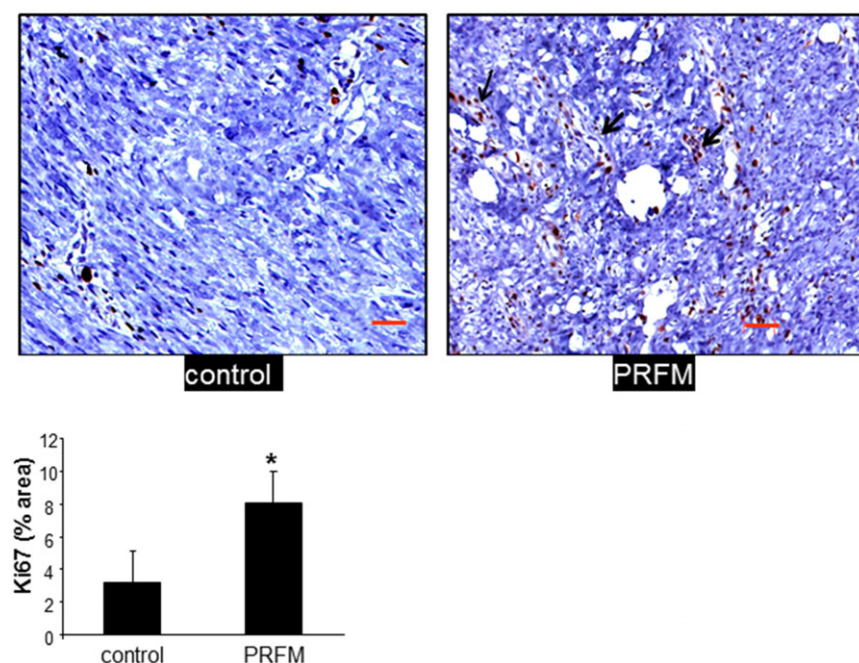


Figure 9. Increased number of Ki67 positive cells in platelet-rich fibrin matrix (PRFM)-treated porcine wounds. Wound-edge tissues were collected 14 days post-wounding. Formalin-fixed paraffin-embedded biopsy tissues were sectioned (5 μ m) and stained using Ki67 (brown), a marker of proliferating cells. The sections were counterstained with hematoxylin (blue). Compared to nontreated ischemic wounds, the treated wounds showed increased number of proliferating cells. Arrows indicate the Ki67-positive proliferating cells. Lower panel: Scale bar = 50 μ m. Bar graph shows quantitation of the Ki67-positive cells in PRFM-treated or -untreated wounds. Data are mean $\pm$ standard deviation ($n = 3$); * $p < 0.05$ compared to untreated wounds.

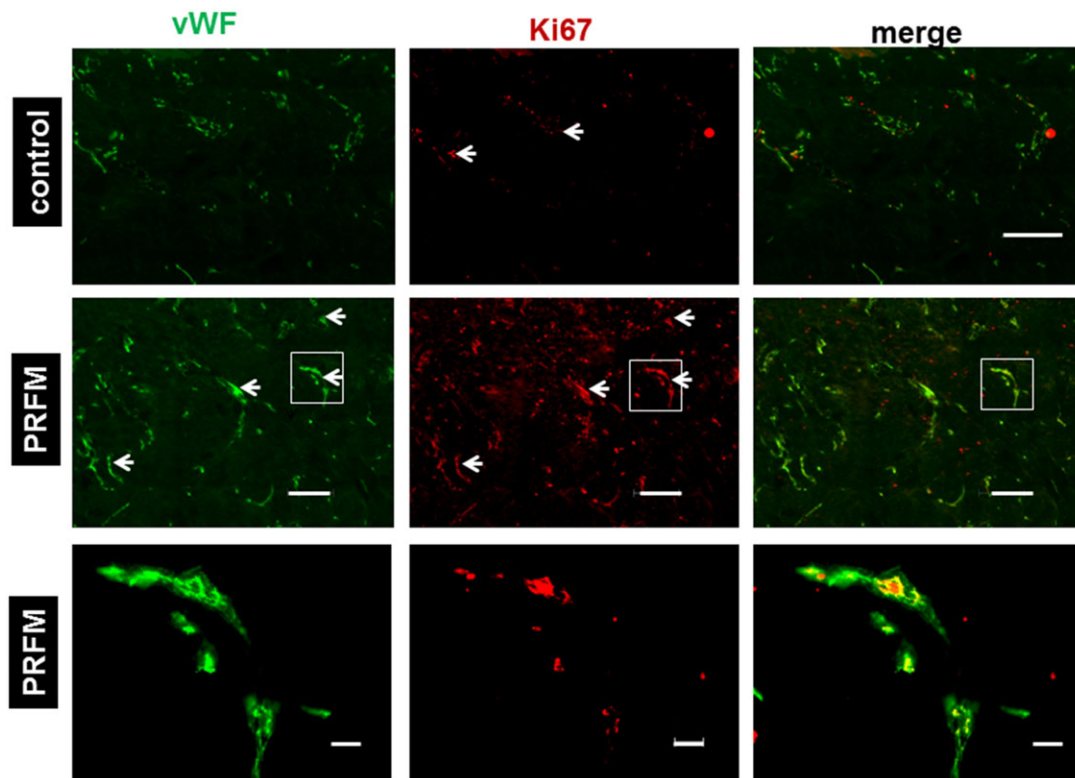


Figure 10. Colocalization of Ki67 and von Willebrand's factor (vWF)-positive cells in platelet-rich fibrin matrix (PRFM)-treated porcine wounds. Wound-edge tissues were collected 14 days post-wounding. Formalin-fixed paraffin-embedded biopsy tissues were sectioned (5 μ m) and stained using Ki67 (red) and vWF (green). Compared to nontreated ischemic wounds, the treated wounds showed increased number of vWF-positive proliferating cells. Arrows indicate Ki67 and vWF-positive blood vessels. Top two panels: Scale bar = 200 μ m. Bottom panel: Scale bar = 20 μ m.

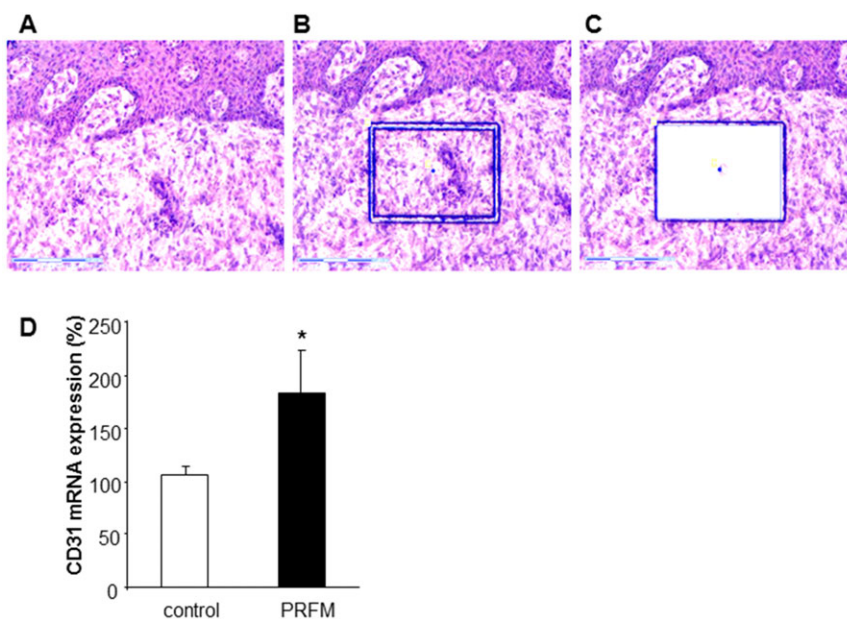


Figure 11. Increased CD31 expression in laser capture microdissection (LCM) captured granulation tissue from platelet-rich fibrin matrix (PRFM)-treated wounds. Wound-edge tissues were collected 14 d post-wounding. LCM capture was performed on hematoxylin & eosin-stained OCT-embedded tissue. (A) Visualization of section; (B) demarcation (in blue) of region of interest; (C) post-dissection and catapulting. Scale bar, 150 μ m; (D) real-time polymerase chain reaction was performed on LCM-captured granulation tissue from wounds treated or not with PRFM to quantify CD31 expression normalized against cyclophilin A levels. Data are mean $\pm$ standard deviation ($n = 4$); * $p < 0.05$.

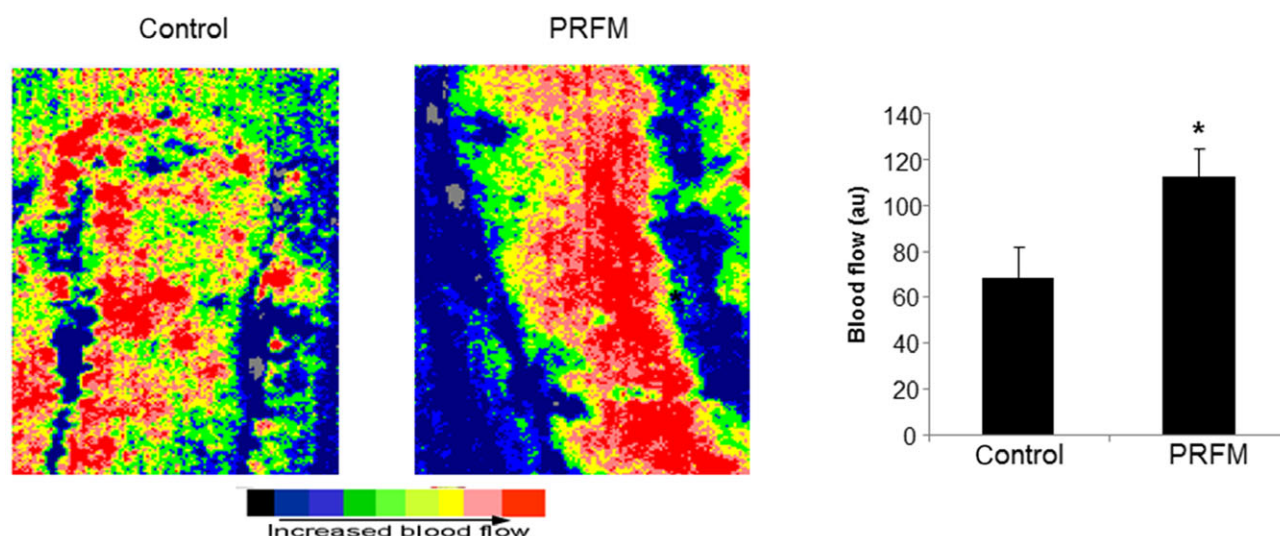


Figure 12. Increased blood flow in platelet-rich fibrin matrix (PRFM)-treated wounds. Doppler images at 14 days post-wounding of the back of swine with ischemic flaps treated or not with PRFM. Bar graph presents the quantitative data from the Doppler images. Data are mean $\pm$ standard deviation ($n = 3$); * $p < 0.05$.

further stimulate the endothelial cell proliferation. The effect of individual growth factor on endothelial cell proliferation was investigated using neutralizing antibodies against growth factors. The sequestering of PDGF using anti-PDGF antibody did not affect endothelial cell proliferation induced by PRFM-conditioned media. Anti-VEGF antibody, however, significantly inhibited the proliferative response. VEGF, a well-known endothelial cell-specific mitogen, promotes many of the events necessary for angiogenesis.^{30,31} PRP supernatants have been shown to be highly mitogenic for endothelial cells, at least in part, to the presence of EGF in media.³² Thus, a number of factors present in PRFM-conditioned medium may be responsible for endothelial cell proliferation. We therefore investigated the net response of the conditioned medium on key signaling pathways that are involved in endothelial cell proliferation. Mitogen-activated protein kinase/ERK activity has been shown to be important to the induction of endothelial cell proliferation by VEGF via VEGF receptor 2.³³ This study provides maiden evidence that PRFM-derived medium potentially activates ERK phosphorylation and that knockdown of this signaling mediator inhibits the conditioned media-induced endothelial cell proliferation.

Finally, to correlate the *in vitro* findings of endothelial cell proliferation in an experimental model of delayed wound angiogenesis in pigs was studied. Vascular complications commonly associated with problematic wounds are primarily responsible for wound ischemia. Ischemia limits the supply of bloodborne products, including nutrients, oxygen, and circulating cells to the wound site, thereby severely impairing the healing response.³⁴ Thus, clinically presented ischemic wounds do not readily lend themselves to the study of biological mechanisms because the collection of tissue biopsies at multiple time points from the same wound poses ethical challenges. The need for preclinical models of ischemic wounds is therefore compelling.⁸ We recently characterized a porcine ischemic wound model. Wound closure in the

ischemic pig skin tissue was severely impaired, resulting in wounds that persisted for 30 days or longer. Impaired angiogenesis was reported in these experimental wounds.⁸ Angiogenesis, or the formation of new blood vessels from existing ones, is a critical step in the regeneration of hard and soft tissues.⁹ Increased wound angiogenesis in ischemic wounds was comparable to a recently reported study where a slow and sustained release of growth factors from PRP via hydrogel approach increased neovascularization in a murine model of hindlimb ischemia.³⁵ These observations suggest that a slow, sustained release of growth factor in this preparation because of the absence of thrombin-induced stimulation may be one of the critical factors in promoting wound angiogenesis in PRFM-treated ischemic wounds. This preparation has shown effectiveness in the treatment of hard-to-heal wounds.⁵ Other clinical applications that have been reported using PRFM include rotator cuff repair,³⁶ bony repair in the oral cavity³⁷ and aesthetics.³⁸

In summary, the study, for the first time, shows the kinetics of the viability of platelets embedded in fibrin matrix. A slow and steady release of growth factors from PRFM was observed because of the use of non-thrombin activation approach. The VEGF released from PRFM was primarily responsible for endothelial mitogenic response via ERK activation pathway. Finally, the preparation effectively induced endothelial cell proliferation in wounds and improved wound angiogenesis in ischemic wounds indicating potential mechanisms of action of PRFM in healing of chronic ulcers.

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