

## BIOLOGY CONTRIBUTION

# DEVELOPMENT OF A PORCINE DELAYED WOUND-HEALING MODEL AND ITS USE IN TESTING A NOVEL CELL-BASED THERAPY

IVAN HADAD, M.D.,\* BRIAN H. JOHNSTONE, PH.D.,† JEFFREY G. BRABHAM, M.D.,‡  
MATTHEW W. BLANTON, M.D.,\* PAMELA I. ROGERS, RLATG,† CORY FELLERS,† JAMES L. SOLOMON, B.A.,†  
STEPHANIE MERFELD-CLAUSS, B.A.,† COLLEEN M. DESROSIERS, PH.D.,‡ JOSEPH R. DYNLACHT, M.D.,‡  
JOHN J. COLEMAN, M.D.,§ AND KEITH L. MARCH, M.D., PH.D.†

\*Department of Surgery and Divisions of †Radiation Oncology and ‡Plastic Surgery, Indiana University School of Medicine; and  
§Indiana Center for Vascular Biology and Medicine, Indianapolis, IN

**Purpose:** A delayed full-thickness wound-healing model was developed and used for examining the capacity of adipose-derived stem cells (ASCs), either alone or in platelet-rich fibrin gels, to promote healing.

**Methods and Materials:** Four pigs received electron beam radiation to the dorsal skin surface. Five weeks after radiation, subcutaneous fat was harvested from nonirradiated areas and processed to yield ASCs. Two weeks later, 28 to 30 full-thickness 1.5-cm<sup>2</sup> wounds were made in irradiated and nonirradiated skin. Wounds were treated with either saline solution, ASCs in saline solution, platelet-rich plasma (PRP) fibrin gel, ASCs in PRP, or non-autologous green fluorescence protein–labeled ASCs.

**Results:** The single radiation dose produced a significant loss of dermal microvasculature density (75%) by 7 weeks. There was a significant difference in the rate of healing between irradiated and nonirradiated skin treated with saline solution. The ASCs in PRP-treated wounds exhibited a significant 11.2% improvement in wound healing compared with saline solution. Enhancement was dependent on the combination of ASCs and PRP, because neither ASCs nor PRP alone had an effect.

**Conclusions:** We have created a model that simulates the clinically relevant late radiation effects of delayed wound healing. Using this model, we showed that a combination of ASCs and PRP improves the healing rates of perfusion-depleted tissues, possibly through enhancing local levels of growth factors. © 2010 Elsevier Inc.

Adult stem cells, Wound healing, Disease models, Radiation injuries.

## INTRODUCTION

The most challenging wounds to manage clinically are chronic wounds and wounds with poor healing potential because of pre-existing pathologies (*e.g.*, diabetes) or radiation-induced alterations of skin and subcutaneous tissues. In particular, postmastectomy breast reconstruction, neck dissections, and extremity sarcoma resections are fraught with complications such as slow healing, dehiscence, infections, excessive scarring, and poor cosmesis in up to 67% of procedures (1, 2). These problems likely result from late effects of radiation, such as fibrosis and loss of the skin microvascular network (3).

An improved understanding of normal and pathologic healing processes has resulted in implementation of a myriad of therapeutic modalities for improving wound healing. However, the beneficial effects of most of these agents have not been validated in late-stage clinical trials of efficacy. Development programs for novel therapies to treat complicated wounds are hindered by a paucity of preclinical animal models that closely mimic human pathophysiology, use current clinical practices, and most importantly, are predictive of the therapeutic potential of agents or devices. The anatomy and physiology of swine, particularly with respect to cutaneous blood supply and wound-healing characteristics, have made this species the

Reprint requests to: Ivan Hadad, M.D., Department of Surgery, Indiana University School of Medicine, Emerson Hall, Ste. 202, 545 Barnhill Dr., Indianapolis, IN 46202. Tel: (317) 417-3733; Fax: (317) 274-8769; E-mail: [ihadad@iupui.edu](mailto:ihadad@iupui.edu)

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standard model for plastic surgical and wound-healing studies (4, 5). Porcine skin, like that of humans, heals primarily through epidermal cell migration (4). Furthermore, it has also been shown that porcine skin responds to radiation in a similar time- and dose-dependent manner to human skin (6, 7).

The use of cell- and protein-based therapies, in particular, to enhance the healing process is rapidly expanding (4). Platelet-derived growth factor (PDGF), a well-known factor involved in normal wound healing, is approved in the recombinant form as a treatment for wounds (5). Vascular endothelial growth factor (VEGF-A) and stromal cell-derived growth factor 1 $\alpha$  (SDF-1 $\alpha$ ) have also been shown to accelerate wound healing in animal models (8, 9). A major shortcoming is that these treatments only supply individual factors in short boluses and do not take advantage of the complex interplay of the multitude of factors involved in wound repair. Theoretically, cell-based therapies are an advancement in that pluripotent stem or progenitor cells assist healing through both regenerating lost tissues at the wound site and providing a sustained source of many beneficial factors (10, 11).

Adipose-derived stem cells (ASCs) are abundant and easily isolated therapeutic cells present in the non-adipocyte (stromal) fraction of adipose tissues. In addition to their capacity to differentiate into cells of mesodermal, endodermal, and ectodermal origin (12, 13), ASCs secrete many potentially beneficial growth factors and cytokines (12, 14). Notably, though, ASCs do not produce PDGF, and therefore supplementation of this factor may enhance the beneficial effects of ASC, especially in wound healing.

Platelet-rich plasma (PRP) possesses multiple characteristics that make it an ideal adjuvant to ASCs for promoting repair of damaged tissues. In particular, PRP is a rich source of growth factors and cytokines involved in wound repair, including PDGF. After clotting, platelet concentrates form insoluble fibrin matrices that may be useful for containing ASCs at the wound site. Interestingly, there is a dose response between platelet concentration and proliferation of human adult mesenchymal stem cells and fibroblasts, as well as production of Type I collagen by these cells (15).

In a previous study, we used healthy, normal juvenile swine to show the safety and neovascularization benefit of ASCs embedded in PRP-derived fibrin matrix in a full-thickness wound model (16). Here, we extend those studies by showing a significant improvement in healing rates and repaired tissue quality of irradiated healing skin by ASCs embedded in a PRP matrix. In addition, we show that the porcine model of delayed wound healing developed for this study could be useful for evaluating the therapeutic potential of new treatment modalities in a system that is physiologically similar to human skin and uses modern clinical procedures.

## METHODS AND MATERIALS

### *Phase I study: Dose determination and microvasculature effects study*

*Radiation injury.* Animal care was performed according to the National Institutes of Health's Guidelines for the Care and Use of

Laboratory Animals, and the protocol was approved by the institutional animal care committee. In the first phase of the study, pigs were irradiated to determine dose tolerance and microvascular effects of a single dose of electron beam radiation on porcine skin. Three female Yorkshire pigs weighing 30 to 35 kg were used. Once anesthetized via inhaled isoflurane, the pigs were transported to the Department of Radiation Oncology, Indiana University Cancer Center. Each pig received a single fraction of 16, 18, or 20 Gy with 6-MeV electrons to an 18 × 40-cm field on each side of the paraspinal dorsal skin surface of the animal, generated by a Siemens Mevatron linear accelerator (Erlangen, Germany). The radiation level was calculated to ensure that greater than 90% of the prescribed dose would be limited to a maximum depth of 2 cm. Doses were verified by use of silicon diode dosimeters. The borders of the fields were marked out with pen and then subsequently tattooed to allow for precise delineation of the treated area as the pig grew in the weeks after treatment. Afterward, pigs were extubated and recovered before returning to their cages.

*Skin assessment.* The pigs were housed for 12 weeks to allow late effects of radiation to develop. Trained personnel examined the skin to determine the degree of erythema and the presence of moist or dry desquamation, as well as any untoward systemic effects. The pigs were fed a standard laboratory diet, cleaned, weighed, and monitored daily. Every 7 days, they were sedated with ketamine and xylazine, and two 8-mm punch biopsy specimens of skin in the irradiated fields were aseptically taken. Biopsy specimens were also taken from normal, nonirradiated skin.

*Immunohistochemical analysis of skin microvasculature.* All biopsy samples were fixed in 10% formalin, embedded in paraffin, sectioned, and then examined histologically. The 5- $\mu$ m sections from each punch biopsy specimen were stained with anti-smooth muscle  $\alpha$ -actin (clone 1A4; Sigma-Aldrich, St. Louis, MO) to determine vessel density according to a standard three-step immunohistochemical procedure (17). After washing, sections were incubated with conjugated antimouse IgG antibodies (Vector Elite Kit PK-6102; Vector Laboratories, Burlingame, CA), followed by development with diaminobenzidine, and counterstained with hematoxylin A. Adjacent sections were stained with nonimmune IgG from the same species by use of the same dilution as the primary antibody as negative controls. Sections from normal porcine skin served as positive controls. Each section was examined microscopically for the number of positively stained lumen-containing vessels in ten fields per slide, which were averaged together for sections. Within-group and between-group means were compared with respect to time, treatment, and irradiation. Significance was detected with a two-tailed *t* test.

### *Phase II study: Treatment of delayed-healing wounds with ASC and PRP*

*Delivery of 20 Gy radiation.* A similar protocol to that in Phase I was used. Female Yorkshire pigs (*n* = 4), weighing 30 to 35 kg, received a single 20-Gy dose. They were then maintained on a standard porcine laboratory diet without intervention for 7 weeks, allowing time for the late effects of radiation.

*Autologous adipose tissue harvest and processing.* Five weeks after the radiation treatment and 2 weeks before wound creation, the pigs were anesthetized for adipose harvest through a 10-cm-long × 3-cm-deep incision in the nonirradiated dorsal hump, followed by adipose tissue excision (approximately 30 g) with a No. 10 blade scalpel. Adipose tissue from transgenic Yorkshire pigs expressing green fluorescence protein (GFP) under control of the ROSA26 promoter (National Swine Resource and Research Center,

University of Missouri-Columbia) was also processed. Fat was transferred to sterile 50-mL conical tubes (Fisher, Pittsburgh, PA) and processed as described previously (18) within 15 minutes of harvest. Hemostasis was ensured, and a layered wound closure with absorbable suture was completed. Isolated cells (approximately  $10^6$  nucleated cells per gram of adipose tissue) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and frozen until used.

**Preparation of wound treatment combinations.** On the day of wound creation, 110 mL of blood was withdrawn from each pig and placed into two anticoagulant citrate dextrose solution A (Citra, Braintree, MA) primed Gravitational Platelet Separator devices (GPS II; Biomet Biologics, Warsaw, IN). A single 12-minute centrifuge spin produced approximately 12 mL of PRP. Autologous ASCs or allogeneic GFP-labeled ASCs ( $20 \times 10^6$ ) were mixed with either 4 mL of PRP (platelet count,  $998 \pm 39.8 \times 10^3/\mu\text{L}$ ) or normal saline solution, and each solution was placed into a FibriJet surgical sealant applicator (Micromedics, St. Paul, MN) with a dual-cannula applicator tip. A solution of 5,000 IU of thrombin (Jones Pharma, Bristol, VA) and 10% calcium chloride was added at a 10:1 volume ratio.

**Wound creation and treatment application.** Seven weeks after the radiation treatment, the 4 female Yorkshire pigs were anesthetized and prepared for wound creation as described previously (19). The pigs were quickly cleaned with tap water, and the hair was removed with Dark Honee Wax (GiGi Cosmetic Laboratories, Petah Tikva, Israel); the dorsal skin surface including all the irradiated skin and a 5-cm border was depilated and wiped briefly with a phenol solution and then 70% isopropyl alcohol to remove residual oils. The pig was then moved to the operating room suite, where the skin was prepared for sterile surgery. Areas to be undergo wound creation were marked with a sterile pen within a border of  $1.5 \text{ cm}^2$  caudal to the shoulder blades, 1.5 cm from the irradiated field borders and 2.5 cm away from any other wounds. The wounds were organized in four paraspinal rows, two on either side of the midline (Fig. 1). Four wounds, two cranial and two caudal to the irradiated areas, were also created as normal healing control wounds. Care was taken not to injure the underlying musculature. The wounds had a mean measured depth of approximately 4 mm.

Wounds treatments were randomly assigned to account for differences in anatomic locations. The surrounding skin was prepared with sterile skin adhesive (Nu-Hope Laboratories, Pacoima, CA). Each wound was completely filled (approximately 0.8 mL) with one of the following treatments: (1) saline solution, (2) ASCs in saline solution, (3) PRP, (4) ASCs in PRP, or (5) GFP-labeled ASCs in PRP. Wounds were quickly covered after each application with an occlusive dressing (Tegaderm; 3M Health Care, St. Paul, MN) to avoid leakage out of the wound site. The number of ASCs added to each wound was  $4 \times 10^6$  cells ( $1.8 \times 10^6$  cells/cm<sup>2</sup>), which is greater than 3-fold higher than the number used in our previous study (16) and in agreement with doses used in similar studies published by other groups (20).

The thorax and abdomen were then covered with a snug-fitting, nonadherent tube gauze dressing and then a modified cotton vest (Four Flags Over Aspen, Jamesville, MN) to further protect the wounds. All animals received cephalexin 30 minutes before wound creation and a fentanyl patch (25  $\mu\text{g}$ ) for 72 hours. Antibiotic prophylactic coverage was continued for the duration of the study (21 days).

**Wound contraction assessments.** The wounds were examined by persons blinded to treatment of specific wounds on Days 4, 8, 12, 16, and 21 after wound creation. All dressings were re-

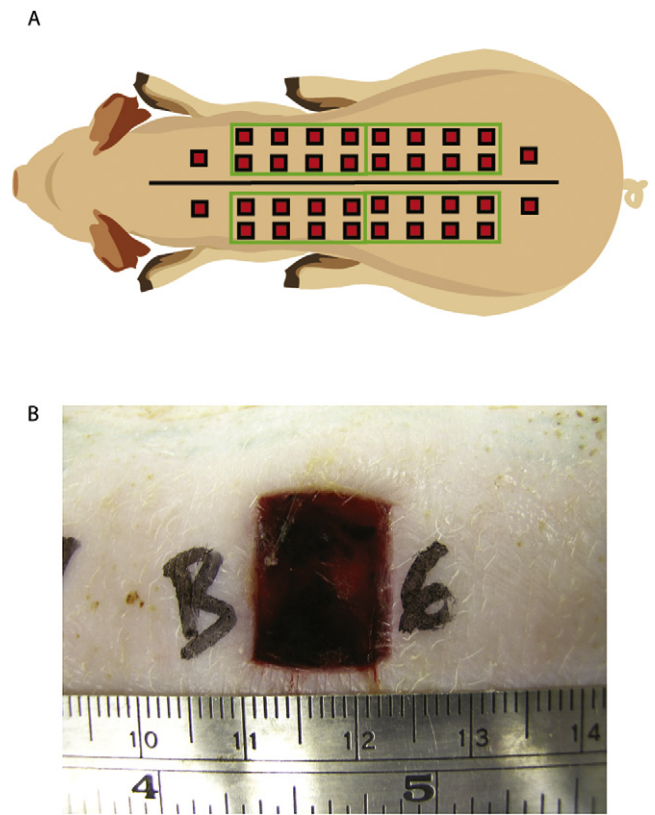


Fig. 1. (A) Schema of dorsal, paraspinal wound location and pattern in irradiated (border) and nonirradiated skin. Treatments were applied randomly to the wounds. (B) Representative study wound 3 days after wound creation.

moved, and the wound was cleaned with saline solution, without debridement within the wound bed, so as not to affect the healing rate. Qualitative assessments were recorded, and individual digital photos of the wounds were taken. Length and width were measured by a ruler to the nearest millimeter; the open surface area was calculated, and contraction was expressed as a percentage of the original wound area on the day of wound creation. In addition, on Days 12 and 16, after all noninvasive assessments were completed, one wound from each treatment group was biopsied and removed from further assessments. The biopsy specimens were full-thickness incisional biopsy specimens, 5 to 6 mm in width, mediolaterally oriented, and they included approximately 3 to 4 mm of unwounded skin on both sides of the wound. All wounds were then redressed, and the protective jackets were replaced. All remaining wounds were biopsied on Day 21 (the day on which the pigs were sacrificed humanely). Samples were fixed in 10% neutral-buffered formalin.

**Histologic analysis of wound tissue.** For histologic analysis, hematoxylin-eosin stain was used to determine re-epithelialization. Each wound section was scanned across its length to determine the extent of re-epithelialization. Each wound section evaluated was scored as either positive or negative for complete re-epithelialization. The statistical difference over time and between each dose in nonirradiated skin and irradiated skin was determined by a Fisher exact test.

**Histologic localization of GFP-labeled ASCs in wounds.** A mouse antihuman monoclonal GFP antibody (Clontech, Mountain

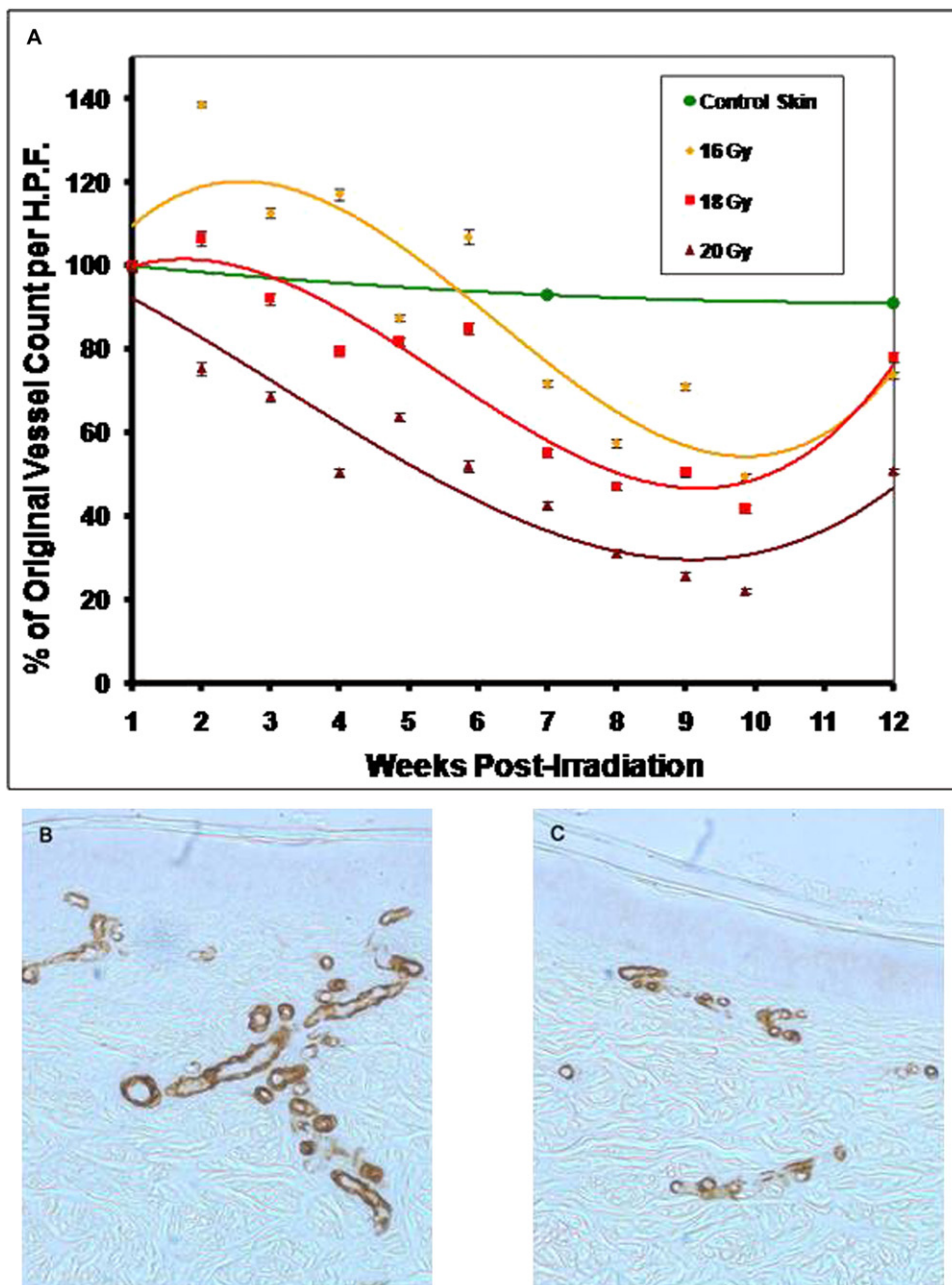


Fig. 2. Relationship between radiation dose and effects on skin microvasculature. Both paraspinous dorsal surfaces of 3 pigs were exposed to a single fraction of 16, 18, or 20 Gy of radiation with a calculated penetration of 2 cm for 90% of the radiation dose. (A) Punch biopsy specimens from normal and irradiated skin were taken weekly and preserved for histology. Thin sections were analyzed histologically for the presence of smooth muscle  $\alpha$ -actin (SMA)-containing vessels (arterioles). The density of small arterioles was determined by microscopically examining the SMA-stained section and recording the number of lumen-containing vessels in each high-powered field (HPF). The mean density for each section was compared with the baseline value obtained on Day 1 for each pig and expressed as a percentage. The trend lines are third-order polynomials. (B) Representative section from nonirradiated skin after immunohistochemical detection of SMA-containing arterioles (brown). (C) Representative section from irradiated skin (20 Gy) after immunohistochemical detection of arterioles (Hematoxylin stain with anti-SMA, Magnification  $\times 100$ ).

View, CA) at 1:800 dilution was used to detect GFP by immunohistochemistry. The immune-stained slides were analyzed qualitatively at low magnification ( $\times 40$ ) to determine the location and presence of the delivered cells within the wound boundaries. Negative control slides were also made by processing tissues not treated with GFP-labeled ASCs, to ensure the specificity of the GFP antibody on porcine skin.

## RESULTS

### *Phase I: Determination of radiation dose and subsequent chronic effects on microvasculature*

*Gross effects of radiation injury.* Irradiation was well tolerated by all animals in the study, with the only acute effects observed at the skin at any doses being mild erythema,

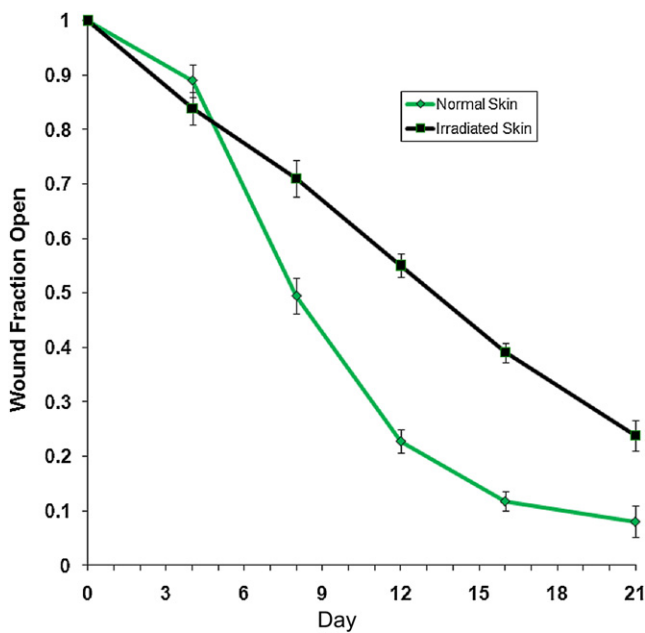


Fig. 3. Comparison of wound-healing rates in normal skin and skin exposed to 20 Gy of irradiation. At 7 weeks after focal exposure of the skin to 20 Gy of irradiation, full-thickness wounds were created within and outside (normal) the irradiated fields. The open wound areas were measured for each wound (4 pigs and 4–9 wounds per pig) and expressed as a fraction of the original wound area. Data are expressed as mean  $\pm$  SEM.

corresponding to Grade 1 radiation dermatitis by the National Cancer Institute Common Toxicity Criteria v. 2.0 (21). Gross gastrointestinal abnormalities leading to bloody stools or other pathologies associated with bystander or abscopal effects were not observed. Furthermore, no effect on food intake or weight gain was observed in any of the pigs. It was noted that during the first 6 weeks, hair regrowth did not occur in irradiated areas of the skin after shaving for biopsy, but it resolved thereafter. Grossly visible chronic radiation effects were limited to increased skin dryness and decreased hair density within the irradiated fields only.

**Effects on skin microvasculature.** After an initial increase in densities of microvessels possessing distinct lumens (*e.g.*, functional arterioles) in skin exposed to 16 and 18 Gy of irradiation, microvascular densities after all doses declined dramatically over the course of the study (Fig. 2), reaching a nadir at 7 weeks. The early transitory increase may be explained by vasodilatation produced by an acute inflammatory response with moderate levels of irradiation (22) causing expansion of collapsed vessels to yield smooth muscle  $\alpha$ -actin-positive vessels with visible lumens. Both the rate and extent of vessel density decline correlated with the intensity of radiation administered. The reduction of microvasculature was significant for all groups ( $p < 0.05$ ), with the 20-Gy dose having the most profound effect of a 75% loss in vessel density (Fig. 2). Between 7 and 10 weeks, vessel density was relatively stable; thus 7 weeks was selected as the optimal time to initiate wound-healing studies and was therefore used in the subsequent phase of the study.

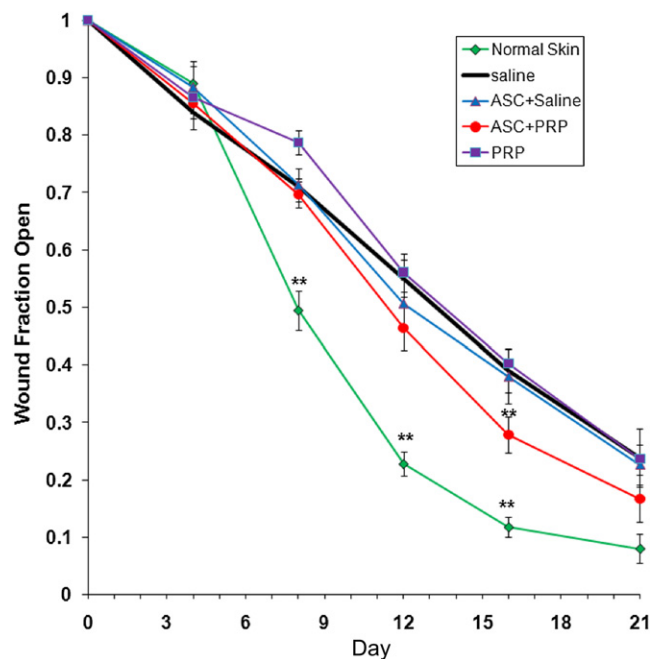


Fig. 4. Effects of treatments on wound healing. Wounds created 7 weeks after irradiation were treated with saline solution (negative control), adipose-derived stem cells (ASCs) in saline solution (ASC+Saline), ASCs in platelet-rich plasma (PRP) (ASC+PRP), and PRP alone. The fraction of wound closure was measured for each wound (4 pigs, with 4–9 wounds per pig). Data are expressed as mean  $\pm$  SEM. Two asterisks,  $p < 0.01$ .

#### Phase II: Treatment of delayed-healing wounds with ASCs and PRP

**Effect of 20 Gy of irradiation on wound contraction rates.** A baseline rate of healing for a full-thickness wound in irradiated skin was performed by comparing saline solution-treated wounds in normal skin and irradiated skin (Fig. 3). The irradiated skin showed a significant delay in healing, especially when compared with the linear phase of healing within normal skin ( $p < 0.01$  for Days 8 and 12). The mean time to achieve 50% closure was approximately 8 days for normal skin compared with approximately 13 days for irradiated skin. At Day 16, which was found to be the mean healing time for a 1.5-cm<sup>2</sup> wound in normal skin in this study, as well as previous studies (19), open wound areas of irradiated skin were nearly three times larger ( $p < 0.01$ ).

**Effects of various treatments on wound contraction.** There was no difference in the contraction rates of wounds

Table 1. Flow cytometry analysis of porcine specimens

	CD90	CD45	CD31	SWC-3a
SVF	65.3 $\pm$ 5.9	14.5 $\pm$ 8.4	34.7 $\pm$ 10.4	64.3 $\pm$ 2.7
ASCs	19.7 $\pm$ 4.6	0.6 $\pm$ 0.8	2.5 $\pm$ 2.0	91.4 $\pm$ 1.8
MNCs	25.2 $\pm$ 17.9	99.0 $\pm$ 0.4	11.4 $\pm$ 1.6	11.4 $\pm$ 1.6

The antibodies used for staining are listed in the top row. The mean percentages ( $\pm$  SD) of positively staining cells in each specimen or cultured cell type are shown.

**Abbreviations:** SVF = stromal vascular fraction; ASCs = adipose-derived stem cells; MNCs = mononuclear cells (isolated from peripheral blood).

Table 2. Re-epithelialization of wounds at 21 days after injury

	Extent of wound re-epithelialization		No. of wounds analyzed	% of completely re-epithelialized wounds
	Complete	Not complete		
Saline solution	6	8	14	43%
ASCs + saline solution	4	7	11	36%
PRP	9	13	22	41%
ASCs + PRP	12	8	20	60%
Normal skin	6	2	8	75%

The entire transection of each wound was scanned microscopically. Each wound received a binary score of either completely healed with total re-epithelialization or partially open (and thus not completely healed).

*Abbreviations:* ASCs = adipose-derived stem cells; PRP = platelet-rich plasma.

in irradiated skin that were treated with either PRP or saline solution (Fig. 4). Compared with wounds in normal, nonirradiated skin, the mean time to reach 50% closure was delayed by approximately 4.5 days. There was a significant delay in early healing of the PRP group up to Day 8 ( $p < 0.01$ ), which resolved during later stages of healing.

The application of ASCs, either alone or mixed with PRP, on wound closure rates was also assessed. The cultured porcine ASCs used in this study were a morphologically homogeneous population devoid of CD45<sup>+</sup> leukocytes and CD31<sup>+</sup> endothelial cells and were enriched in cells expressing the stromal cell marker CD90 (Table 1) (16). Thus, by use of the limited antibodies available for labeling porcine cells, the preparation of cells used in this study appeared to be similar to passaged human ASCs (23, 24).

The combination of ASCs and PRP was superior to all treatments in accelerating the rate of wound contraction (Fig. 4). The mean time to 50% closure of wounds treated with this combination was accelerated by approximately 2 days over saline solution. On Day 16, this corresponded to 11.2% greater reduction in open wound area when compared with irradiated wounds treated with saline solution ( $p < 0.01$ ).

When time to complete re-epithelialization was evaluated histologically at Day 21, only 43% of the saline solution-treated wounds (6 of 14) had completely re-epithelialized (Table 2). There was a greater (though not significant) percentage of re-epithelialized wounds (60%) among those treated with the combination of ASCs and PRP ( $p = 0.09$ ). For control, nonirradiated skin, 75% of the wounds had totally re-epithelialized by the same time point. The percentage of re-epithelialized irradiated wounds treated with only PRP and ASCs in saline solution was 36% and 41%, respectively.

*Determination of effects of treatment on microvasculature of healed wounds.* Thin sections of wound biopsy specimens taken at 21 days after injury were immunohistologically stained by use of a smooth muscle  $\alpha$ -actin-specific antibody. There was a significant ( $p < 0.05$ ) increase in the microvessel

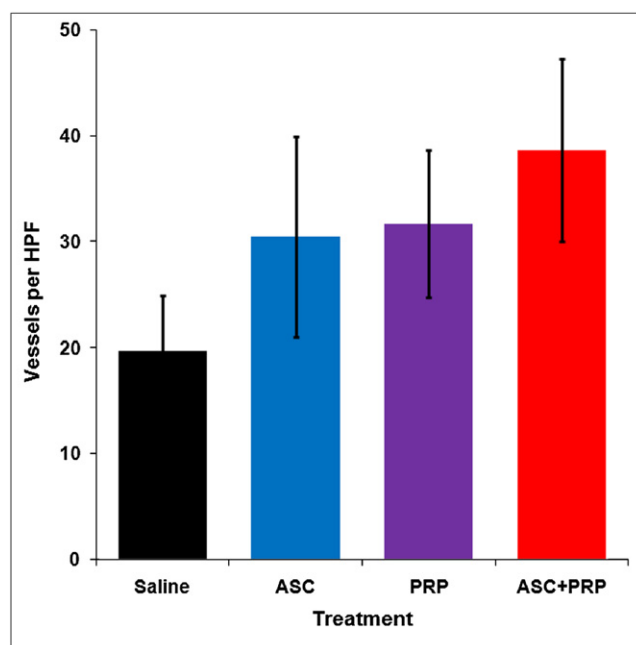


Fig. 5. Microvascular densities in healed wounds. Thin sections of wounds treated as indicated were probed with smooth muscle  $\alpha$ -actin to detect arterioles. The mean number of lumen-containing vessels per 20 high-powered fields (HPF) (magnification  $\times 200$ ) was calculated and plotted. Error bars are SEM. ASC = adipose-derived stem cell; PRP = platelet-rich plasma.

density of healed wounds treated with the combination of ASCs and PRP compared with saline solution (Fig. 5).

*Evaluation of non-autologous GFP-labeled ASCs on delayed wound healing.* Non-autologous ASCs combined with PRP were evaluated to determine the feasibility of this approach. To allow for tracking of cells after application, ASCs were isolated from swine carrying the transgene for GFP, which is expressed in all tissues, including adipose. The combination of PRP and GFP-expressing ASCs was essentially ineffective in promoting healing, with contraction being substantially worse than autologous ASCs in PRP ( $p < 0.02$  at Days 12 and 16) (Fig. 6A). Even though these non-autologous ASCs did not enhance healing, they did persist in the wound site (Fig. 6B). At Day 21, the cells were homogeneously dispersed throughout the wound. Cells positively staining for GFP were found in every biopsy specimen taken at each time point. No positively staining cells were found in the biopsy specimens of untreated wounds (Fig. 6C).

## DISCUSSION

Establishment of sufficient blood flow to a wound site is critical to proper healing (25). Studies in patients with vascular disease have shown that large-vessel arterial bypass and improvement of lower-limb blood flow improves wound-healing rates (26). Diffuse microvascular insufficiency in surface tissues, as observed in territories of irradiation, is not amenable to vascular bypass; therefore additional measures must be developed to overcome complications in wound healing.

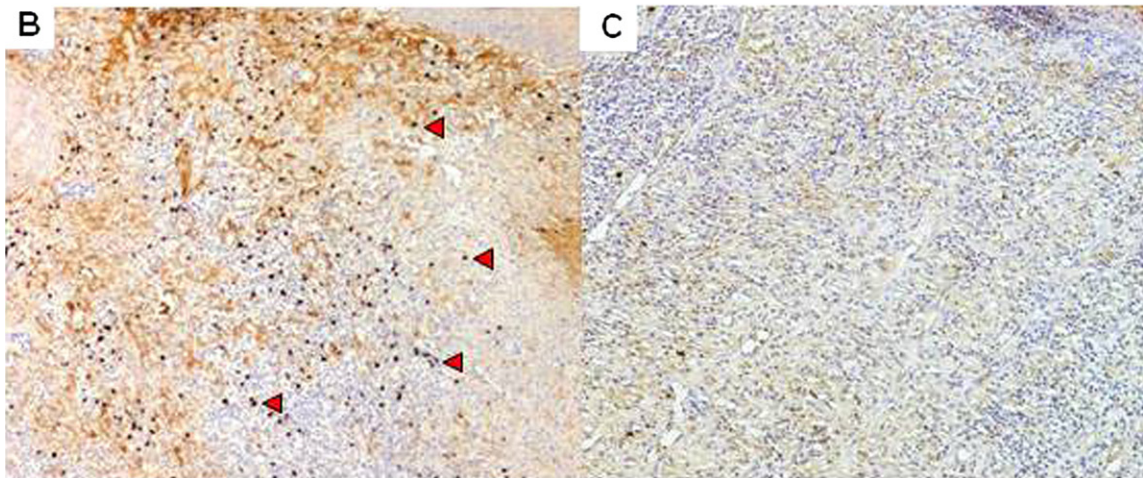
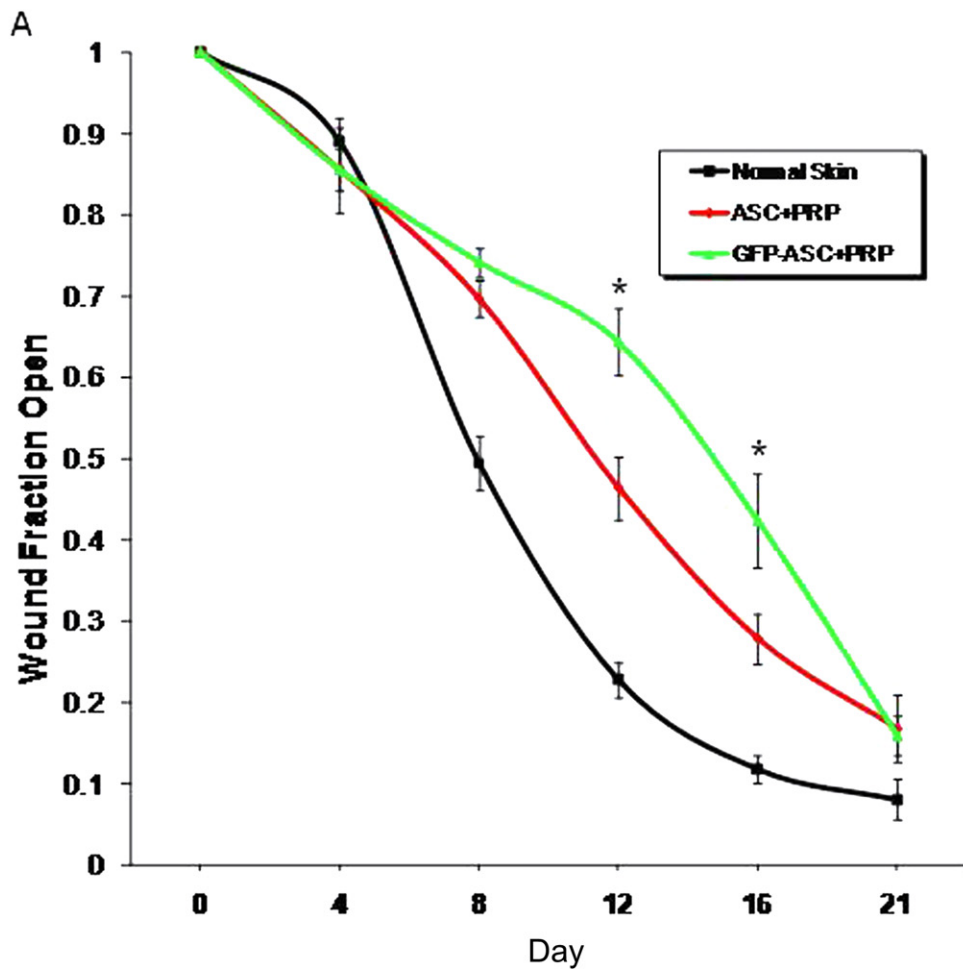


Fig. 6. Effectiveness of non-autologous green fluorescence protein (GFP)-labeled adipose-derived stem cells (ASCs) on delayed wound healing. (A) Graph depicting wound closure compared with original wound area in normal skin and irradiated skin treated with ASCs and platelet-rich plasma (PRP) and with transgenic GFP-labeled ASCs. (B) Immunohistochemical image of thin section from healed tissue at skin surface in wounds treated with GFP-labeled ASCs (dark brown staining noted by arrowheads). The even distribution of the GFP-labeled ASCs in the granulation tissue should be noted. (C) Thin section of untreated wounds (negative control) (Hematoxylin stain with anti-GFP, Magnification  $\times 100$ ). The lack of positive uptake should be noted.

In this study we sought to determine the potential therapeutic value of accelerating wound healing through the combined application of ASCs and PRP. First, it was necessary to de-

velop a new animal model that closely follows current clinical practices and scenarios and provides a surrogate for human pathology. Swine was chosen because of the similarity to human

skin anatomy, physiology, and biochemistry (27). The delayed-healing model described in this study closely simulates the common and difficult clinical problems experienced with postradiation reconstructive surgery (1, 2). As previously mentioned, swine skin is anatomically and physiologically similar to human skin, and it also heals similarly (4, 5). In addition, it responds to radiation similarly (6, 7). Moreover, the model incorporates the current practice of irradiation with electron beams for focused radiation exposure.

The single fraction of 20 Gy with 6-MeV electrons approximates the total dose given to postmastectomy patients during standard 30-fraction therapy over a period of 6 weeks (28). We realize that the linear–quadratic model used to determine equivalent unfractionated dose may not be appropriate for extrapolating to large doses; thus further research is needed to determine whether any of these treatments induce effects in skin exposed to fractionated radiation doses. It should be noted, though, that exposures used in this study are similar to hypofractionated doses, which are currently used in the clinic to treat certain tumors (29). Regardless of the dosing scheme, we did find that the single 20-Gy dose of electron beam irradiation significantly decreased skin microvasculature, which agrees with previous studies with X-rays on porcine skin (30, 31).

After validation, we used the model to test the effects of external agents on the wound-healing rate and the resulting qualities of the wounds. We chose ASCs because of the emerging understanding of the normal biologic role of these cells in supporting the vascular system in adipose (32). In addition to supporting structural integrity of vessels, ASCs also participate in new vessel growth or expansion through the secretion of proangiogenic factors, such as Vascular Endothelial Growth Factor and Hepatocyte Growth Factor, and vessel-stabilizing factors, such as Angiopoietin-1 (33). We have recently shown that ASCs cooperate with endothelial cells to form stable vessels *in vitro* and *in vivo* when co-implanted in three-dimensional matrices (32, 33). Indeed, ASCs in combination with PRP promoted greater vascularity of healed irradiated wounds in this study. Together, these findings support our results showing that a proangiogenic cell can improve the healing of wounds in poorly vascularized tissues.

Despite their function in promoting vascularization and stabilizing vessels, ASCs supplied alone did not improve closure rates or qualities in the irradiated wound-healing model. It is possible that the absence of a trapping agent, such as fibrin, allowed the ASCs to migrate away from the wound site.

Another possibility is that ASCs lack key factors required to promote wound healing, such as PDGF. Activation of PRP with thrombin produces large amounts of PDGF and transforming growth factor  $\beta$ 1, and ASC proliferation is significantly enhanced when co-cultured in activated PRP (16, 34). The complementarity between ASCs and PRP is also demonstrated by the finding that PRP by itself did not accelerate wound healing. This was an unexpected result, given the current clinical use of PDGF in wound-healing products.

Also noted in this study was the different effect of autologous ASCs and non-autologous ASCs in PRP. The non-autologous ASCs expressing the GFP transgene failed to impact the healing rate even though the cells were detectable in the wound sites 3 weeks after administration. It is possible that these cells, which were derived from nonmatched donors, possess less potency in an allogeneic environment, because of either inactivation or clearance to subtherapeutic numbers by the host immune response. Nonetheless, GFP staining allowed verification of ASC persistence in the wounds.

Future studies will examine dose and frequency in the application of each factor in isolation or combination. The model has proven to be useful in validating the therapeutic potential of ASCs within PRP for use in postradiation surgeries, which—if ultimately translated to clinical practice—will have a large impact on the quality of life of our patients.

## CONCLUSIONS

We have shown that the combination of ASCs in PRP promotes healing of full-thickness wounds in previously irradiated skin. To accomplish this, we first developed a novel porcine model of radiation-induced delayed wound healing that should be generally useful for assessing the efficacy of wound repair agents, as well as the biology of delayed wound healing. In addition, this model could be used to evaluate acute treatments for combined radiation–wound injuries, such as would be sustained by persons within the blast zone of radioactive explosive devices.

Future studies will address the applicability of this finding in aged pigs of both genders. There are well-documented differences in skin physiology and wound healing between genders and in relation to aging (35–37). Such a study would be more representative of the variety of postirradiation surgical patients.

## REFERENCES

- Girod D, McCulloch T, Tsue T, *et al.* Risk factors for complications in clean-contaminated head and neck surgical procedures. *Head Neck* 1995;17:7–12.
- Habel D. Surgical complications in irradiated patients. *Arch Otolaryngol* 1965;4:382–386.
- Tibbs MK. Wound healing following radiation therapy. A review. *Radiother Oncol* 1997;42:99–106.
- Hom DB, Unger GM, Parnell KJ, *et al.* Improving surgical wound healing with basic fibroblast growth factor after radiation. *Laryngoscope* 2005;115:412–422.
- Landry Y, Gies JP. Drugs and their molecular targets: An updated overview. *Fundam Clin Pharmacol* 2008;22:1–18.
- Fowler JF, Morgan RL, Silvester JA, *et al.* Experiments with fractionated x-ray treatment of the skin of pigs. *Br J Radiol* 1963;36:188–196.
- Morris GM, Hopewell JW. Epidermal cell kinetics of the pig: A review. *Cell Tissue Kinet* 1990;23:271–282.
- Galiano RD, Tepper OM, Pelo CR, *et al.* Topical vascular endothelial growth factor accelerates diabetic wound healing through

- increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. *Am J Pathol* 2004;164:1935–1947.
9. Badillo AT, Chung S, Zhang L, *et al*. Lentiviral gene transfer of SDF-1 $\alpha$  to wounds improves diabetic wound healing. *J Surg Res* 2007;143:35–42.
  10. March KL, Johnstone BH. Cellular approaches to tissue repair in cardiovascular disease: The more we know, the more there is to learn. *Am J Physiol Heart Circ Physiol* 2004;287:H458–H463.
  11. Urbich C, Aicher A, Heeschen C, *et al*. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. *J Mol Cell Cardiol* 2005;39:733–742.
  12. Rehman J, Traktuev D, Li J, *et al*. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004;109:1292–1298.
  13. Zuk PA, Zhu M, Mizuno H, *et al*. Multi-lineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Eng* 2001;7:211–228.
  14. Nakagami H, Maeda K, Morishita R, *et al*. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Arterioscler Thromb Vasc Biol* 2005;25:2542–2547.
  15. Eppley BL, Woodell JE, Higgins J. Platelet quantification and growth factor analysis from platelet-rich plasma: Implications for wound healing. *Plast Reconstr Surg* 2004;114:1502–1508.
  16. Blanton MW, Hadad I, Johnstone BH, *et al*. Adipose stromal cells and platelet-rich plasma therapies synergistically increase revascularization during wound healing. *Plast Reconstr Surg* 2009;123(Suppl.):56S–64S.
  17. Lamme EN, de Vries HJ, van Veen H, *et al*. Extracellular matrix characterization during healing of full-thickness wounds treated with a collagen/elastin dermal substitute shows improved skin regeneration in pigs. *J Histochem Cytochem* 1996;44:1322.
  18. Da Silva Meirelles L, Sand TT, Harman RJ, *et al*. MSC frequency correlates with blood vessel density in equine adipose tissue. *Tissue Eng Part A* 2009;15:221–229.
  19. Svensjo T, Pomahac B, Yao F, *et al*. Accelerated healing of full-thickness skin wounds in a wet environment. *Plast Reconstr Surg* 2000;106:602–612.
  20. Falanga V, Iwamoto S, Chartier M, *et al*. Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. *Tissue Eng* 2007;13:1299–1312.
  21. Trotti A, Byhardt R, Stetz J, *et al*. Common toxicity criteria: Version 2.0. An improved reference for grading the acute effects of cancer treatment: Impact on radiotherapy. *Int J Radiat Oncol Biol Phys* 2000;47:13–47.
  22. Friedman E. Immune modulation by ionizing radiation and its implications for cancer immunotherapy. *Curr Pharm Des* 2002;8:1765–1780.
  23. Mitchell JB, McIntosh K, Zvonice S, *et al*. Immunophenotype of human adipose-derived cells: Temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 2006;24:376–385.
  24. McIntosh K, Zvonice S, Garrett S, *et al*. The immunogenicity of human adipose-derived cells: Temporal changes in vitro. *Stem Cells* 2006;24:1246–1253.
  25. Saaristo A, Tammela T, Farkkila A, *et al*. Vascular endothelial growth factor-C accelerates diabetic wound healing. *Am J Pathol* 2006;169:1080–1087.
  26. Treiman GS, Oderich GS, Ashrafi A, *et al*. Management of ischemic heel ulceration and gangrene: An evaluation of factors associated with successful healing. *J Vasc Surg* 2000;31:1110–1118.
  27. Qiao GL, Brooks JD, Baynes RE, *et al*. The use of mechanistically defined chemical mixtures (MDCM) to assess component effects on the percutaneous absorption and cutaneous disposition of topically exposed chemicals. Studies with parathion mixtures in isolated perfused porcine skin. *Toxicol Appl Pharmacol* 1996;141:473–486.
  28. Barton M. Tables of equivalent dose in 2 Gy fractions: A simple application of the linear quadratic formula. *Int J Radiat Oncol Biol Phys* 1995;31:371–378.
  29. Stuschke M, Pottgen C. Altered fractionation schemes in radiotherapy. *Front Radiat Ther Oncol* 2010;42:150–156.
  30. Archambeau J, Ines A, Fajardo LF. Response of swine skin microvasculature to acute single exposures of x-rays: Quantification of endothelial changes. *Radiat Res* 1984;98:37–51.
  31. Patterson TJ, Berry RJ, Hopewell JW. The effect of radiation in survival of experimental skin flaps. In: Grabb WC, Myers MB, editors. *Skin flaps*. Boston: Little, Brown; 1975. p. 39–46.
  32. Traktuev D, Merfeld-Clauss S, Li J, *et al*. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. *Circ Res* 2008;102:77–85.
  33. Traktuev DO, Prater DN, Merfeld-Clauss S, *et al*. Robust functional vascular network formation in vivo by cooperation of adipose progenitor and endothelial cells. *Circ Res* 2009;104:1410–1420.
  34. Kakudo N, Minakata T, Mitsui T, *et al*. Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. *Plast Reconstr Surg* 2008;122:1352–1360.
  35. Dao H, Kazin RA. Gender differences in skin: A review of the literature. *Gen Med* 2007;4:308–328.
  36. Giacomoni PU, Mammone T, Teri M. Gender-linked differences in human skin. *J Dermatol Sci* 2009;55:144–149.
  37. Farage MA, Miller KW, Elsner P, *et al*. Functional and physiological characteristics of the aging skin. *Aging Clin Exp Res* 2008;20:195–200.