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Transdermal Delivery of Adipocyte-Derived Stem Cells Using a Fractional Ablative Laser

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Abstract

Background: Chronic wound healing problems can pose a significant clinical challenge. Transdermal delivery of adipose-derived stem cells (ADSC) may be a possible solution to healing these recalcitrant, debilitating wounds. Pretreatment of the skin with a fractionated laser has already been shown to assist transdermal drug delivery both in vitro and in vivo and may be an ideal approach to facilitating delivery of ADSC to the target tissue.
Objectives: The authors investigate in a porcine model whether ADSC can be delivered transdermally following pretreatment with a fractional laser.
Methods: After ethics approval was obtained, the abdomens of 2 adult female domestic pigs were pretreated with an erbium:YAG fractionated ablative laser. Following laser treatment, 20×10^6 bromodeoxyuridine (BrdU)–labeled ADSC were applied topically to the first animal for 4 hours. The same number of BrdU-labeled ADSC was applied to the second animal for 48 hours. The animals were euthanized at the end of their respective treatment periods, and the BrdU-labeled ADSC were counted after tissue harvest.
Results: At 4 hours, an average of 2.40×10^6 cells, or 12.0% of the total cells applied, were found in the tissue. At 48 hours, an average of 1.1×10^6 cells, or 5.5% of the total cells applied, were seen.
Conclusions: This pilot study demonstrates that ADSC can be delivered transdermally through skin that has been pretreated with a laser. Potential future applications of this approach might include wound-healing or aesthetic indications. Further studies need to be conducted to determine the optimal number of ADSC to use in this approach, the best methods of application, and the effect of transdermally delivered ADSC on wound healing.

Keywords

adipose-derived stem cells, ADSC, laser-assisted drug delivery, fractionated lasers, stem cell therapy, research

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Chronic wound-healing problems can be clinically challenging and cause significant morbidity. Multiple dressing changes over a protracted length of time also place an enormous drain on health care resources in terms of hospital admissions and long-term care. As we look for ways to improve recalcitrant, debilitating wounds, it is natural to consider the potential of stem cells as a possible solution.

The role of stem cells in cutaneous wound healing is the subject of great debate.[1-5] Stem cells have the ability to renew themselves as well as differentiate into specialized cell types. Their role has been demonstrated in tissue regeneration,[6,7] as well as in animal models of wound healing.[4,8-10] These studies have commonly utilized bone marrow–derived stem cells and intravenous administration for stem cell delivery. However, although we know that the introduction of stem cells can improve wound

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healing, the mechanism through which this occurs is currently not clearly understood.

Adipose-derived stem cells (ADSC) may be ideal to study for wound healing because these cells are able to differentiate into cells of various lineages and, since fat is abundant throughout the human body, it is therefore relatively easy to harvest large quantities of these cells compared with bone marrow cells.

Currently, the favored mechanisms for stem cell delivery are intravenous administration or subcutaneous injection. However, a more focused, local application may have benefits over systemic delivery in cutaneous wound healing. Although local application could be achieved with a subcutaneous injection beneath the wound, this may be particularly challenging in some cases due to poor skin quality, the depth of the wound, and/or the extensive nature of the wound and its location. Furthermore, if therapy is directed toward the dermis, intradermal deposition may be desirable. Topical application of ADSC could be a good alternative to subcutaneous injection. The permeability of the skin to transdermal applications is dependent on a number of factors, including the stratum corneum and composition of the applied drug. A fractional laser can be used to assist penetration of the stratum corneum and has been shown to facilitate the transdermal delivery of drugs both in vitro and in vivo.[11-14] We propose that a fractional, ablative, erbium laser can be used to create channels in the skin through which stem cells can be delivered into the dermis and subcutaneous tissue. The aim of this study, therefore, was to determine if ADSC can be delivered topically following pretreatment with a fractional erbium laser.

METHODS

Ethics approval for the study was sought and granted by the Institutional Animal Care and Use Committee (IACUC) board at the University of Texas Southwestern Medical Center in Dallas, Texas.

Two adult female domestic pigs (pig A and pig B) were obtained and acclimatized for 1 week prior to the study. Both animals were kept nil by mouth the night before surgery. Under general anesthesia, the abdomen of each pig was shaved and prepped. On the abdomen of pig A, four 3 × 3-cm areas were marked: area 1 was the control and did not receive laser treatment, whereas areas 2, 3, and 4 were treated with the laser. Labeled stem cells were then applied topically to all 4 areas and covered with an occlusive dressing for 4 hours with the animal kept under anesthesia. Approximately 20 million cells in 1.5 mL of medium (20×10^6 cells) were applied topically to each of the 4 marked areas. At the conclusion of 4 hours, the dressings were removed from the skin and the marked areas were cleansed with normal saline to remove any residual fluid, excised in toto, and submitted for immunohistochemistry and hematoxylin and eosin (H&E) staining. Pig A was then euthanized.

For pig B, three 3 × 3-cm areas were marked on the animal's abdomen: area 1 was the control (no laser treatment), and areas 2 and 3 were treated with the laser. Approximately 20 million stem cells in 1.5 mL of medium (20×10^6 cells) were applied topically to areas 1 and 2. In area 3, the same volume of stem cells was injected subcutaneously under the laser-treated area to provide a comparison of administration methods. After stem cell application, all areas were covered with occlusive dressings and the animal was allowed to recover from anesthesia. The dressings were left in place for 48 hours, after which the animal was euthanized and areas 1 to 3 were excised in toto for immunohistochemistry and H&E staining.

Laser Settings

An erbium:YAG (Er:YAG) laser (Profile; Sciton, Inc, Palo Alto, California) was used in this study. The settings were 1000 µm depth, at 22% density (the equivalent of 375 J/cm^2). A 1-cm^2 spot size was used to deliver the laser energy. The depth of 1000 microns was arbitrarily chosen to ensure that the columns would extend into the deep dermis, allowing for better observation of the channels on histology and facilitating subsequent immunohistochemistry.

Stem Cell Preparation

Allograft ADSC were extracted from pigs that had undergone prior surgery and according to Lequeux et al[15] and the Björntorp protocol.[16] The adipose tissue was minced, digested with collagenase (0.120 U/mL; Roche Diagnostics, Indianapolis, Indiana) at 37°C for 30 minutes, and subjected to constant shaking. Digestion was stopped by adding Dulbecco's modified Eagle's medium (DMEM) (Gibco GlutaMAX; Life Technologies Corporation, Carlsbad, California) containing 10% fetal calf serum (Thermo Scientific HyClone, Logan, Utah). Floating adipocytes were discarded and cells from the stromal vascular fraction (SVF) were pelleted, rinsed with media, centrifuged at 1000 rpm, and incubated in an erythrocyte lysis buffer for 10 minutes at 37°C. The cell suspension was then centrifuged.

The cells were counted using trypan blue, seeded at a density of 2×10^4 cells/cm^2, and cultured in preadipocyte expansion media containing DMEM, Ham's F12 nutrient mix with L-glutamine (Gibco; Life Technologies Corporation), 10% fetal calf serum, 10 ng/mL basic fibroblast growth factor (Sigma-Aldrich, St Louis, Missouri), and antibiotics (20 µg/mL gentamycin and 100 IU/mL penicillin). The medium was changed every 3 days until confluence was reached at 37°C, 5% CO_2, and 95% air humidity.

At 60% confluence, the ADSC were labeled with bromodeoxyuridine (BrdU) (Invitrogen; Life Technologies Corporation). BrdU competes with thymidine and is substituted into DNA. It, therefore, can be used to label stem cells by tracking the DNA as the cells divide and replicate. This allows for assessment of cell viability and

to determine if the labelled cells have incorporated into surrounding tissues. To prepare the solution, 7.65 mg BrdU was dissolved in 2.5 mL phosphate-buffered saline (PBS). The solution was then sterilized by filtration using a 70-µm cell filter and further diluted to a 10-µM concentration with expansion media containing fibroblast growth factor. The media were then added to the culture flasks and the cells left to proliferate for 48 hours. The action of the BrdU was stopped by removal of the medium. The cells were washed 3 times with PBS and trypsinized prior to use by washing the culture flasks twice with PBS and adding 4 mL trypsin containing 0.01% EDTA (Life Technologies Corporation) to each flask. After incubation at 37°C for 5 minutes, the cells were detached and the action of the enzyme was stopped using DMEM. The cells were then centrifuged at 1200 rpm for 5 minutes, the supernatant removed, and the cell pellet resuspended in 5 mL DMEM. The cell suspension was centrifuged a second time at 1200 rpm for 5 minutes, the supernatant again discarded, and the cell pellet resuspended in DMEM. The cells were counted with a hemocytometer prior to use.

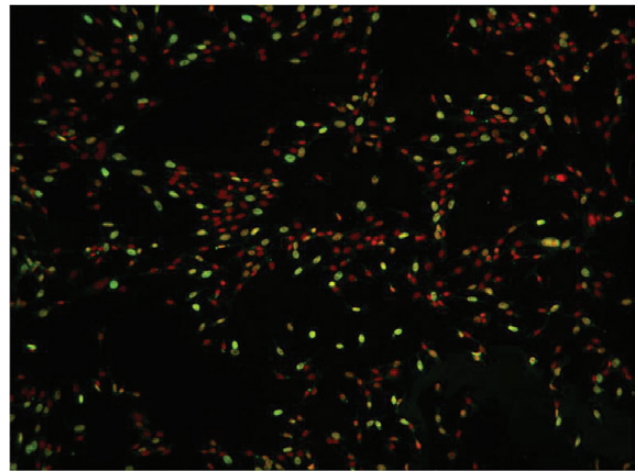

Figure 1. Bromodeoxyuridine-labeled porcine adipocyte stem cells in vitro counterstained with propidium iodide. The labelled cells (BrdU) fluoresces green/yellow; the counterstain for all nuclei is red (propidium iodide).

Tissue Analysis and Immunohistochemistry

For tissue analysis, each biopsy specimen was fixed in a 10% buffered formaldehyde solution at a pH of 7.4 for at least 48 hours, embedded into paraffin wax, cut into 6-µm sections, and stained with immunohistochemistry counterstains and H&E standard staining. For tissue immunohistochemistry, the 6-µm paraffin-embedded sections were deparaffinized and then incubated in 2N HCl for 60 minutes at 37°C for DNA denaturation. The acid was neutralized using 0.1% borate acid, and the sections were then washed with PBS, incubated with Triton 0.5% for 10 minutes, and washed again with PBS. The cells were counterstained with propidium iodide (Life Technologies Corporation).

BrDU Immunohistochemistry and Stem Cell Counts

Porcine ADSC on glass cover slips were fixed with 2% paraformaldehyde in PBS for 5 minutes, followed by incubation in 2N HCl for 10 minutes at 37°C for DNA denaturation. Cells were washed twice with PBS, incubated with Triton 0.5% for 10 minutes, and washed again. For nonspecific antibody reactions, ADSC were blocked with 1.5% normal horse serum (Vector Laboratories, Burlingame, California) in PBS and then incubated with mouse anti-BrdU antibody (Sigma-Aldrich) with 0.1% bovine serum albumin (Sigma-Aldrich) in PBS for 16 to 18 hours at 25°C. Cells were washed, incubated with biotinylated horse anti-mouse IgG (Vector Laboratories) for 30 minutes at 25°C, washed again, and incubated with FITC streptavidin for 30 minutes at 25°C. After being washed with PBS, the cells were counterstained with propidium iodide (Life Technologies Corporation). The cover slips were then mounted on slides using Vectashield mounting media (Vector Laboratories) and viewed under a fluorescence microscope (Figure 1).

Two 5-µm sections per laser-treated area were analyzed by 3 independent observers under fluorescence microscopy to obtain counts of the labeled stem cells. All visible fluorescent stem cells were counted and averages were taken across the observers. To calculate the total number of stem cells found in the 3 × 3-cm tissue areas, the following formula was used:

$$\text{Number of cells} \times 30\,000 \text{ (length of specimen in µm)/5 (thickness of section)} = \text{total number of cells in specimen.}$$

RESULTS

In the 4-hour study of pig A, no positively labeled cells were found in the epidermis or dermis of the control area (area 1, no laser pretreatment). In contrast, positively stained BrdU cells were found in the laser-pretreated areas within microchannels that had been created by the laser (Figure 2). Found in different distributions within the microchannels, some of these stem cells were suspended within the channel, some were adherent to the walls of the channel, and some were at the base of the channel (Figure 3). No positively labeled stem cells were seen outside of the channel.

Total cell counts for the 4-hour study are summarized in Table 1. ANOVA was performed using GraphPad Prism (GraphPad Software Inc, La Jolla, CA). The average counts for the 3 laser-pretreated areas (areas 2, 3, and 4) were 2.18×10^6, 2.27×10^6 and 2.74×10^6 cells, respectively ($P = .751$). This corresponds to a respective 10.9%, 11.4%, and 13.7% of the 20 million cells that were

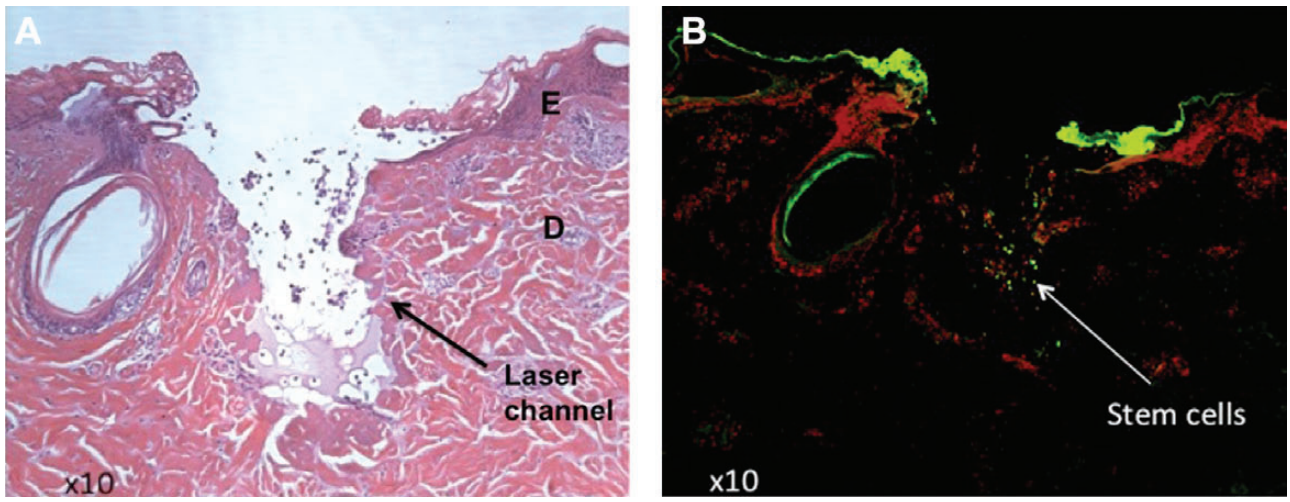

Figure 2. Tissue from pig A (4-hour study) showing the results of laser pretreatment plus topical application of the stem cells. (A) Hematoxylin and eosin–stained section showing the laser channel penetrating the dermis. (B) The same section under fluorescence microscopy showing the bromodeoxyuridine-labeled stem cells within the laser channel. D, dermis; E, epidermis.

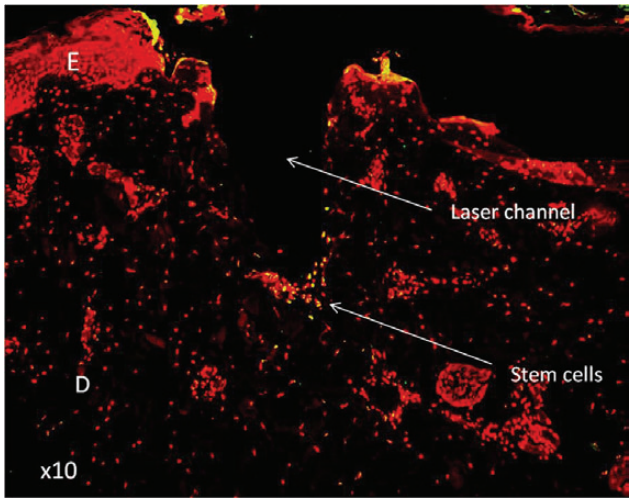

Figure 3. Bromodeoxyuridine-labeled stem cells (yellow) at the base of the laser channel in a section from pig A (4-hour study). D, dermis; E, epidermis.

Table 1. Total Stem Cell Counts in the 4-Hour Study

Area	Cell Counts (× 10^6)			Average Total Cell Count (× 10^6)
	1	2	3	
Area 1[a] A	0	0	0	0
B	0	0	0	
Area 2[b] A	2.08	3.80	2.25	2.18
B	1.33	1.94	1.67	
Area 3[b] A	3.37	6.42	2.92	2.27
B	1.51	1.93	0.98	
Area 4[b] A	3.93	3.99	3.55	2.74
B	2.15	2.44	1.97	

Two 5-µm sections (A and B above) were analyzed for each laser-treated area.
[a]Control; topical stem cell application only; no laser pretreatment of the skin.
[b]Topical stem cell application after laser pretreatment of the skin.

applied. Therefore, at 4 hours, an average of 2.40 × 10^6 cells were found in the laser-pretreated tissue, equating to 12.0% of the total cells applied. In the 48-hour study of pig B, no positively labeled cells were found in the epidermis or dermis of the control area (area 1, no laser pretreatment). In area 3, where labeled ADSC were injected after laser pretreatment of the skin, numerous stem cells were found in discrete areas in the subcutaneous tissue. The cells were too dense to count (Figure 4). Few positively stained BrdU cells were found within the tissue where the stem cells were applied topically after laser pretreatment (area 2) (Figure 5A). H&E staining demonstrated that, at 48 hours, the microchannels had re-epithelialized (Figure 5B). Scant BrdU-labeled cells were found within the channels in the dermis of the skin, with some cells found along the wall of the channel and others at the base. No positively labeled stem cells were found outside of the channel.

Cell numbers in the tissue were much reduced at 48 hours compared with 4 hours. Total cell counts are recorded in Table 2. The average cell counts for the laser-pretreated areas were 0.73 × 10^6 and 1.47 × 10^6 cells,

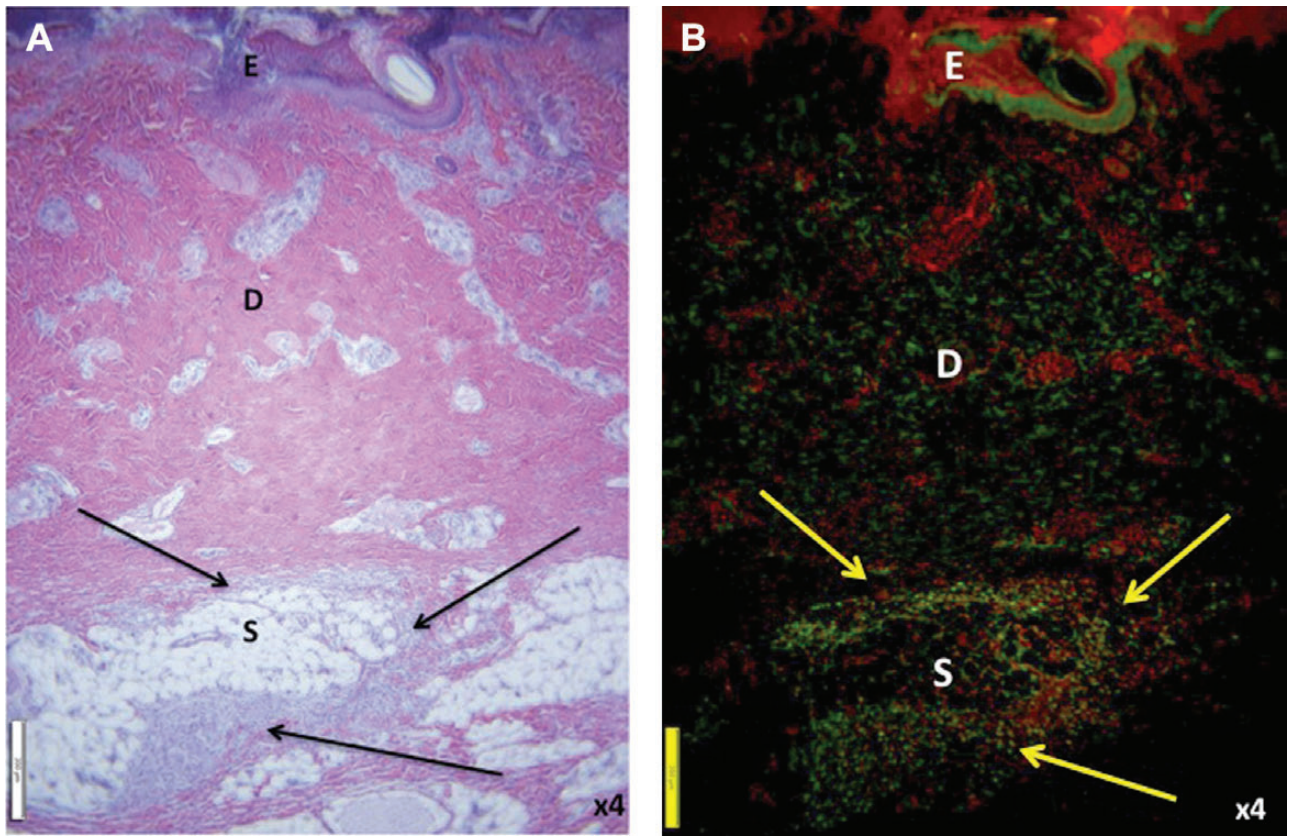

Figure 4. Tissue from pig B (48-hour study) showing results of laser pretreatment plus subcutaneous injection of the stem cells (area 3). (A) Hematoxylin and eosin–stained section showing numerous adipose-derived stem cells deposited in the subcutaneous tissue (arrows). Black arrows demonstrate the adipose derived stem cells in the subcutaneous tissues. (B) Bromodeoxyuridine immunohistochemistry of the same section showing dense clusters of stem cells (yellow arrows), with the cells too numerous to count. D, dermis; E, epidermis; S, subcutaneous tissue.

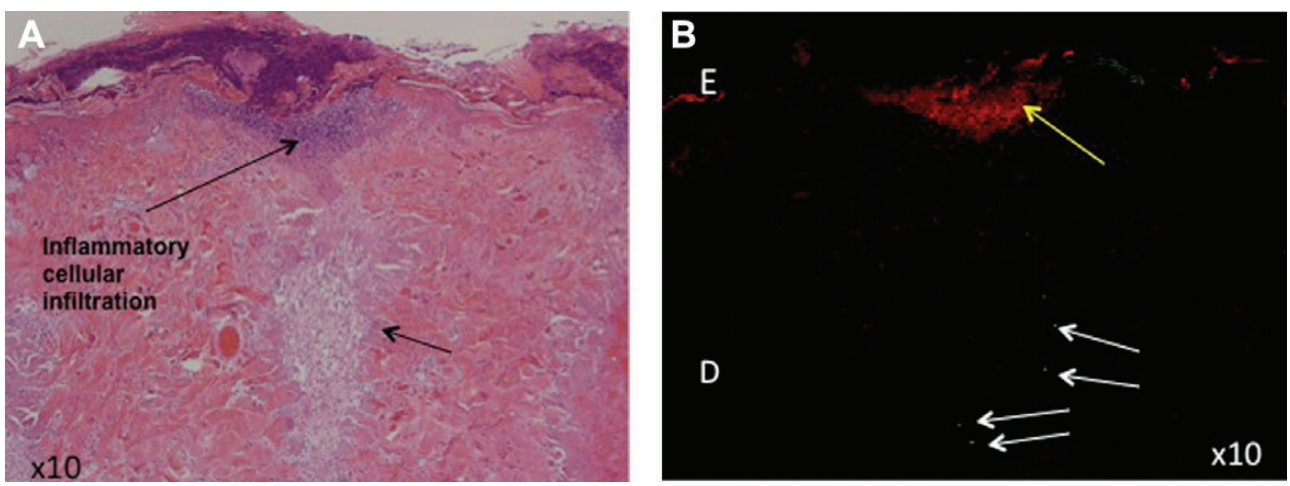

Figure 5. Tissue from pig B (48-hour study) showing results of laser pretreatment plus topical application of the stem cells (area 2). (A) Hematoxylin and eosin–stained section showing large inflammatory cellular infiltration and re-epithelialization of the laser channel. The re-epithelialised laser channel is demonstrated by the short black arrow. (B) Bromodeoxyuridine (BrdU) immunohistochemistry of the same section showing scant BrdU-labeled cells (white arrows) towards the base of the channel. The yellow arrow is pointing to the corresponding area of inflammatory cellular infiltrate as on the H&E image. D, dermis; E, epidermis.

Table 2. Total Stem Cell Counts in the 48-Hour Study

Area on Pig B[a]	Observer Cell Counts[b] ($\times 10^6$)			Average Total Cell Count ($\times 10^6$)
	1	2	3	
1	0	0	0	0
2a	0.65	0.83	0.72	0.73
2b	1.16	1.66	1.59	1.47
3	UTC	UTC	UTC	UTC

Abbreviation: UTC, unable to count; stem cells too dense to count.
[a]Areas on pig: Area 1, control; Area 2a + b, laser pretreatment; Area 3, injected.
[b]Each column represents a separate observer.

respectively ($P = .025$). This corresponds to a respective 3.64% and 7.34% of the 20 million cells applied. Therefore, after 48 hours, an average of 1.1×10^6 cells were observed in the tissue, equating to 5.5% of the total cells applied or injected, a smaller percentage than in the 4-hour study.

DISCUSSION

Treatment using ADSC is currently an area of great interest in the scientific community. This is, in part, related to the fact that fat is an abundant and therefore renewable source from which to harvest stem cells. ADSC are pluripotent, having the ability to differentiate into numerous cell lines, including adipose tissue, nerve, bone, and cartilage.

Many of the animal studies to date using adipose-derived and other stem cells to address wound healing have focused on intravenous or local injection of the mesenchymal stem cells to exert effects on distant targets. Several of these studies have shown success in the acceleration of wound healing.[17-23] Other methods of stem cell administration have included fat grafts augmented with SVF from collagenased fat. This technique, which was first described by Yoshimura et al[24] and termed *cell-assisted lipotransfer* (CAL), has been shown to have a beneficial effect on radiation injuries[25] and longevity of the fat grafts themselves.[26]

In addition to cutaneous wound-healing applications, there has been considerable interest in the role of stem cells in skin rejuvenation, particularly in skin wrinkle improvement. Mojallal et al described the anecdotal improvement of skin texture following fat grafting to the face clinically,[19] which they investigated further using an athymic mouse model.[27] They found that the animal had a thickened dermis after grafting, with increased synthesis of collagen I fibers. The authors concluded that this was due to the presence of mesenchymal stem cells in the adipose tissue. Park et al[28] also demonstrated improvement in skin wrinkles after injecting a micro pig with autologous ADSC. Histological findings from this study showed an increase in dermal thickness and collagen expression.

To date, most stem cell delivery methods described in the literature have been fairly invasive or require some form of intravenous or subcutaneous injection. The aim of this study was to ascertain whether ADSC can be delivered less invasively, via the transdermal route into the dermis, following the creation of microchannels with a laser. The findings from this study showed that approximately 10% of topically applied cells were still present at 4 hours and 5% were present at 48 hours. It is important to note that the cells remained within the microchannels and were not seen within the surrounding tissue.

There may be several reasons why the cell count was lower in the 48-hour study compared with the 4-hour study. Although both studies were performed on the abdominal skin, the animal in the 48-hour study was allowed to recover from anesthesia for the duration of the study period. Once the animal was awake and ambulating, the abdominal skin was subjected to gravitational forces, which may have affected the results. In addition, at higher energy fluences, the microchannels can become partially occluded with normal inflammatory exudate that accompanies a laser injury, potentially preventing the cells from further entering the channel. Cell death due to lack of nutritional support as the media evaporated could also account for a decrease in cell count from the 4-hour to the 48-hour time point. Pig A was sacrificed at 4 hours whereas pig B was sacrificed at 48 hours; therefore, the process of inflammation could have affected absorption in pig B over a longer period of time. Finally, our cell count may be limited by the number of histological samples taken.

In the present study, subcutaneously injecting the cells was superior to topical administration in terms of cell numbers found in the tissues, but this application method poses some interesting questions. It is still unclear how many stem cells are required to exert an effect, and over what area. Further investigation needs to be performed in this area. Moreover, many patients find injection unpleasant. If they are already undergoing a laser treatment—for example, for facial rejuvenation—then a topical application of stem cells would be more appropriate and patient-friendly than a subcutaneous or intradermal injection. In addition, intradermal injections may be less precise than a predetermined laser depth unless some sort of imaging is used at the same time.

Previous animal studies from our group have shown that the microchannels remain patent for up to 90 minutes, depending on the fluences used (data not shown). In this study, we were able to demonstrate that the pig skin re-epithelializes within 48 hours of laser treatment at the fluence used (375 J/cm^2), with a strong, acute inflammatory infiltrate both within and outside of the channel.

In addition to some of the limitations noted in our discussion of the superior results at 4 hours compared with 48 hours, there are other limitations to this study. The ADSC used in this study were allogeneic, not autologous. This may have had an effect on cell viability in vivo as the immune system of the animal would have identified these cells as foreign bodies. The use of autologous ADSC would be preferable and is recommended for further investigations. Autologous ADSC were not used in the current study as it was a proof-of-principle investigation and

would have entailed a 2-stage procedure, first for adipose tissue harvest and second for ADSC application. Further studies investigating the effect of ADSC on wound healing should consider the immunological ramifications of autologous versus allogeneic cells.

The variations in distribution of the stem cells in this study suggest that there was good penetration of the ADSC; however, they may have been disrupted during the process of removing the dressings and during tissue staining. A method that allows continuous delivery of the stem cells over time, such as an impregnated scaffold (eg, acellular dermal matrix) or fibrin glue applied topically, would be preferable to applying the cells freely in solution.

Autologous platelet-rich plasma applied topically following fractionated laser resurfacing has already been shown to improve wound healing.[29,30] A small study consisting of 4 patients injected intradermally with autologous platelet-rich fibrin matrix (PRFM) suggested that dermal collagenesis, angiogenesis, and adipogenesis were all induced by the PRFM.[31] Combining PRFM with ADSC may be worth investigating in future studies, as it could have an additive stimulatory effect with benefits not only within the realm of wound healing but also possibly within the realm of aesthetic skin rejuvenation therapies.

Although we were able to demonstrate the presence of cells in the channels at 48 hours, there was no evidence of migration into the tissues. A longer study period would be necessary to see if this occurs. As a nuclear stain, the BrdU label becomes diluted as the cells divide. This makes it difficult to use this stain to track the cells over an extended period of time. Lequeux et al[15] found that the detectable BrdU signal declines after 2 weeks in an in vitro study, although it is present for up to 30 days. There is also some debate as to whether the BrDU label persists even after the cell is dead, before it is phagocytosed. In future studies, other methods of stem cell tracking may be more efficient, such as fluorescence-activated cell sorting (FACS) or RNA evaluation. These methods are more objective and do not rely on counts by the human eye, which can be confounded by clerical error.

This study investigated only the physical location of the labeled stem cells and not what effect they exerted by being present. Further investigations could be undertaken to see if the stem cells secrete cytokines or other factors implicated in wound healing. It would also be of interest to find out whether the stem cells have an effect on the amount of inflammatory infiltrate present, utilizing a method that more adequately quantifies it.

The potential benefits of the combined laser and topical stem cell application described in the current study range from wound healing to skin rejuvenation procedures. A laser could be used to "reactivate" a chronic dormant wound and the stem cells applied topically to stimulate angiogenesis and other pro-healing growth factors found in acute wounds such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). It may be possible that a gel formulation containing stem cells could be applied following the laser procedure. This would accelerate healing and shorten downtime, which can be considerable following procedures such as facial resurfacing. Alternatively, depths of injury could be set for the laser, allowing for a more precise delivery of ADSC to a specified target within the skin.

CONCLUSIONS

This study in a porcine model demonstrates that it is possible to topically deliver ADSC following pretreatment with an ablative laser. Further studies need to be conducted to determine whether those stem cells are viable and, if so, what their mode of action is once delivered into the skin. In addition, further investigations are required to look at enhancing cell delivery, perhaps by seeding them first on an acellular dermal matrix or PRFM to increase the number of cells found within the dermis. This study is the first step of a pathway that could potentially enhance wound healing through the introduction of stem cells derived from adipose tissue.

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Disclosures

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