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Noncontact, Low-Frequency Ultrasound Therapy Enhances Neovascularization and Wound Healing in Diabetic Mice

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Abstract

Background—Chronic wounds are a major source of morbidity for patients and represent a significant health burden. Implementing noninvasive techniques that accelerate healing of these wounds would provide great benefit. Ultrasound appears to be an effective modality for the treatment of chronic wounds in humans. MIST Therapy is a noncontact, low-frequency ultrasound treatment delivered through a saline mist. A variety of mechanisms have been proposed to explain the efficacy of ultrasound therapy, but the underlying molecular and cellular pathways impacted by this technique remain unclear. The *in vivo* effect of noncontact, low-frequency ultrasound was therefore examined in a humanized excisional wound model.

Methods—The treatment group received noncontact, low-frequency ultrasound therapy three times per week, whereas the control group received a standard dressing change. Wounds were photographed at regular intervals to calculate healing kinetics. Wound tissue was harvested and processed for histology, quantitative polymerase chain reaction, and enzyme-linked immunosorbent assay.

Results—The MIST group demonstrated significantly accelerated wound healing, with 17.3 days to wound closure compared with 24 days in the controls ($p < 0.05$). This improvement became evident by day 9, with healing evidenced by significantly decreased mean wound area relative to original size (68 percent versus 80 percent; $p < 0.01$). Expression of markers of neovascularization (stromal cell-derived factor 1, vascular endothelial growth factor, and CD31) was also increased in the wound beds of noncontact, low-frequency ultrasound–treated mice compared with controls.

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Conclusion—Noncontact, low-frequency ultrasound treatment improves neo-vascularization and wound closure rates in excisional wounds for diabetic mice, likely because of the stimulated release of angiogenic factors.

Chronic wounds affect more than 6 million Americans, with an annual cost estimated at \$25 billion.¹ These wounds frequently manifest in the setting of diabetes mellitus¹ and are associated with diminished new blood vessel formation (neovascularization), leading to an impaired wound healing response.^{2,3} With an increase from 17.5 million to over 22 million diagnosed cases of diabetes between 2007 and 2012,^{4,5} this will be an increasing problem for U.S. health care. Therapeutic ultrasound has gained in popularity as a modality with significant efficacy in the treatment of chronic wounds.⁶ Several mechanisms for the efficacy of therapeutic ultrasound have been proposed. These include reduction of edema,⁷ inhibition of bacterial colonization,⁸ stimulation of neovascularization⁹ and subsequent tissue formation,^{7,9} leukocyte adhesion to endothelial cells,¹⁰ and macrophage stimulation of fibroblast proliferation.¹¹

Mechanotransduction, the conversion of mechanical energy into a biochemical response at the cellular level, has also recently emerged as an important modulator of the healing response in a variety of tissues.^{12–16} For example, microdeformational wound therapy, a subset of negative-pressure wound therapy that involves the application of a porous interface material between a wound and a semiocclusive dressing connected to suction, has been shown to facilitate the formation of granulation tissue and accelerate wound healing.¹⁷ With microdeformational wound therapy, direct mechanotransduction is used to generate microforces capable of stretching individual cells and ultimately stimulating cellular proliferation. Interestingly, therapeutic ultrasound can also be used to stimulate wound healing, with the mechanical energy in this technique derived from low-frequency sound waves.⁹

MIST Therapy (Celleration, Inc., Eden Prairie, Minn.) is a noncontact, low-frequency ultrasound treatment delivered through a saline mist to the wound bed. Noncontact, low-frequency ultrasound is effective in the treatment of chronic wounds in humans.^{18,19} Mechanistically, it has been shown to influence fibroblast physiology and bacterial load in wounds, which may partially explain its efficacy.^{8,20} However, despite the promising attributes of this method for tissue regeneration, the molecular and cellular mechanisms underlying its clinical benefit have yet to be fully elucidated. In this study, we aim to investigate whether the clinical efficacy of the ultrasound is the result of accelerated angiogenesis at the wound site, a well-described phenomenon wherein new blood vessels grow from existing vessels in regions of tissue ischemia, and whether noncontact, low-frequency ultrasound-activated cells release cytokines that promote angiogenic activity.

MATERIALS AND METHODS

Animals

All animal experiments were conducted in accordance with a protocol approved by the Stanford Administrative Panel on Laboratory Animal Care in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal care facility.

Ten-week-old, diabetic (BKS.Cg-*Dock7*^{m +/+} *Lepr*^{db/J}) male mice, obtained from The Jackson Laboratory (Bar Harbor, Me.), were used in all studies. The mice were housed individually in a room maintained at constant temperature with a 12-hour light/dark cycle and free access to food and water. Mice were allowed to rest and adapt after arrival for 2 weeks before experiments began.

Surgical Wounding and Assessment

Wounding was carried out in accordance with a splinted excisional model of murine wound healing previously described by our laboratory,²¹ which minimizes wound contracture and allows for healing to occur through granulation and reepithelialization. Briefly, a sterile 6-mm punch biopsy tool is used to make duplicate full-thickness wounds, extending through the panniculus carnosus, on either side of the midline on the shaved dorsum of anesthetized mice. A donut-shaped silicone splint with a 10-mm diameter is centered on the wound and fixed to the skin using an immediate-bonding adhesive (Krazy Glue; Elmer's, Inc., Columbus, Ohio) and interrupted 6-0 nylon sutures (Ethicon, Inc., Somerville, N.J.). A semioclusive dressing (Tegaderm; 3M, St. Paul, Minn.) is then applied to the wound. After surgery, the mice were placed on warming pads and allowed to fully recover from anesthesia before being returned to the institutional animal facility in separate cages.

The animals were monitored daily for signs of infection or distress. Images of the wounds were taken on the day of surgery and every other day thereafter using digital photography. On treatment days, photographs were taken before treatment. The camera was kept at a fixed distance from the wound during photography. A blinded observer used ImageJ software (National Institutes of Health, Bethesda, Md.) to analyze the photographs and record wound area relative to the area of the silicone ring, mitigating the effects of interphotograph variability. The percentage change in wound size over time, relative to the initial margins, was used as a measure of wound healing kinetics. At predefined time points (days 7 and 14 and at complete closure) animals were killed and wounds were harvested ($n = 3$ mice and $n = 6$ total wounds per time point) and halved, for either fixation in 4% paraformaldehyde (12 hours at 4°C) for histologic and immunohistochemical analysis, or snap-frozen in dry ice and storage at -80°C for transcriptional and protein analysis.

Noncontact, Low-Frequency Ultrasound Treatment

The MIST noncontact, low-frequency ultrasound system consists of a transducer wand that generates ultrasonic pressure waves at 40 kHz with a displacement of 65 μ m, and a specialized saline applicator, which controls a saline flow. (See Figure, Supplemental Digital Content 1, A, which shows a MIST ultrasound system, consisting of a transducer wand and a saline applicator, which atomizes saline to propagate ultrasonic waves, <http://links.lww.com/PRS/B63>.) The saline is atomized by the ultrasonic waves (but not aerosolized) into a saline spray, which propagates the ultrasound wave and allows for a noncontact delivery.²² The MIST system calculates the treatment time based on wound area according to a predetermined algorithm. (See Figure, Supplemental Digital Content 1, B, which shows treatment time based on area of wound, <http://links.lww.com/PRS/B63>.)

During treatment, the entire wound surface was misted with the transducer held at a distance of 5 to 15 mm, creating an actual ultrasound distance of 15 to 25 mm (applicator adds 10 mm), which is the recommended treatment distance. (See Figure, Supplemental Digital Content 1, C, which shows that holding the ultrasound transducer at 5 to 15 mm from the wound maximizes the effectiveness of ultrasound delivery to the tissue, <http://links.lww.com/PRS/B63>.) Animals in the treatment group were administered noncontact, low-frequency ultrasound therapy for 3 minutes per wound three times per week. Animals in the control group received a change of dressing instead. During treatment, each mouse was positioned on an absorbent pad such that the transducer was positioned perpendicular to the wound and the adjacent wound was shielded to prevent inadvertent misting.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction and Enzyme-Linked Immunosorbent Assay

Total RNA was isolated from harvested wound tissue using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was eluted in RNase-free water and quantified using a spectrophotometer at 260 and 280 nm. On the same day, first-strand cDNA was synthesized by reverse transcription of 50 ng of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, Calif.) with random hexamers as primers. For polymerase chain reactions, we used Taq-Man Assays-on-Demand Gene Expression Products from Applied Biosystems (Foster City, Calif.): stromal cell-derived factor 1 (SDF-1), assay ID Mm00445552_m1; vascular endothelial growth factor (VEGF)- α , assay ID Mm01281447_m1; and CD31, assay ID Mm01242584_m1. Gene expression levels were normalized to B2M expression, assay ID Mm00437762_m1, and presented as relative values. Polymerase chain reaction was repeated three times for each assay.

Total protein was isolated using 4°C radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Mo.). After homogenization of tissue in the supplemented radioimmunoprecipitation assay buffer, samples were centrifuged at 10,000 g for 10 minutes at 4°C. Protein was quantified using the Quick Start Bradford Protein Assay Kit (Bio-Rad, Hercules, Calif.). SDF-1 protein levels were measured using the Mouse CXCL12/SDF-1 alpha Quantikine ELISA Kit (R&D Systems, Minneapolis, Minn.)

Histology and Immunohistochemistry

Fixed tissue was dehydrated and embedded in paraffin blocks, from which 8- μ m-thick sections were serially cut. Sections were stained with hematoxylin and eosin for qualitative assessment of gross wound morphology, including regularity and density of the dermis. Masson trichrome and picrosirius red staining, performed according to the manufacturer's protocol (IHC World, Woodstock, Md.), were used to qualitatively and quantitatively assess collagen density and organization. Each section was visualized under light microscopy (Leica DM 4000B microscope; Leica Microsystems, Buffalo Grove, Ill.) for hematoxylin and eosin and trichrome stains and a light microscope (Leica 5000B) equipped with a polarization filter and camera (Leica DFC 500) for picrosirius red staining. At least five

high-power fields were analyzed for each wound by a blinded observer using ImageJ software as described previously.²³

Additional sections were used for immunofluorescence studies. Vascular density was assessed using a polyclonal rabbit anti-mouse anti-CD31 primary antibody (1:100) (ab28364; Abcam, Cambridge, United Kingdom) incubated overnight at 4°C, followed by Alexa Fluor 594 Goat Anti-Rabbit immunoglobulin G secondary antibody at room temperature (1:200) (Invitrogen). SDF-1 α expression was assessed using a polyclonal rabbit anti-mouse anti-SDF-1 α primary antibody (1:100) (ab25117; Abcam) incubated overnight at 4°C, followed by Alexa Fluor 488 Goat Anti-Rabbit immunoglobulin G secondary antibody at room temperature (1:200) (Invitrogen). VEGF expression was assessed using a polyclonal rabbit anti-mouse anti-VEGF primary antibody (1:100) (46154; Abcam) incubated overnight at 4°C, followed by Alexa Fluor 594 Goat Anti-Rabbit immunoglobulin G secondary antibody at room temperature (1:200) (Invitrogen). All samples were counterstained with 4',6-diamidino-2-phenylindole. Slides were mounted with the Vectashield Mounting Medium (Vector Laboratories, Burlingame, Calif.) and cover-slipped. A Zeiss Axioplan 2 fluorescence microscope was used to image the slides (Carl Zeiss, Inc., Thornwood, N.Y.). Quantification of fluorescence was performed by a blinded observer analyzing at least five high-power fields per wound using ImageJ software.

In Vitro Assessment of Noncontact, Low-Frequency Ultrasound

Dermal fibroblasts were harvested from the dorsal skin of db/db mice as described previously.²⁴ In passage 2, cells were cultured on Nunc Lab-Tek II Chamber Slides (Thermo Fisher Scientific, Rochester, N.Y.) and treated with noncontact, low-frequency ultrasound as described above for 3 minutes per slide three times per week. Six hours after the third treatment, cells were fixed and permeabilized.

Cell proliferation was assessed using a monoclonal rabbit anti-mouse anti-Ki67 primary antibody (1:50) (16667; Abcam) incubated overnight at 4°C, followed by Alexa Fluor 594 Goat Anti-Rabbit IgG secondary antibody at room temperature (1:200) (Invitrogen). All samples were counterstained with 4',6-diamidino-2-phenylindole. Slides were mounted, imaged, and analyzed as described above.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Statistical analyses were performed using an unpaired t test. All values of $p < 0.05$ were considered statistically significant. Matlab R2010a (MathWorks, Inc., Natick, Mass.) was used to perform a power analysis to determine the minimal sample size (independent wound pairs) required to obtain significance levels at each time point. Our analysis determined that three measurements per group would be sufficient to power these assays, and we elected to use $n = 3$ mice per group per time point to ensure adequate statistical precision.

RESULTS

Noncontact, Low-Frequency Ultrasound Accelerates Wound Healing in Diabetic Mice

Noncontact, low-frequency ultrasound therapy visibly improved cutaneous wound healing in db/db diabetic mice compared with the control group treated with changes of dressing only (Fig. 1, *above*). The effect of ultrasound treatment became evident by day 9 after wounding, with a significantly decreased mean wound area relative to original size at this time point (68 ± 3.4 percent versus 80 ± 3.2 percent; $p = 0.003$) (Fig. 1, *below, left*). Typical of db/db mice, the control group wounds closed at a mean of 24 ± 1.0 days, compared with 17.3 ± 1.5 days in the treatment group ($p < 0.05$) (Fig. 1, *below, right*).^{25–27}

Noncontact, Low-Frequency Ultrasound Improves Wound Architecture and Collagen Organization

Dermal thickness and cutaneous integrity in reepithelialized wounds are known to be reduced in db/db mice.^{25–27} Noncontact, low-frequency ultrasound therapy increased dermal thickness over healed wound beds (Fig. 2, *above, left*) and improved connective tissue regularity and density compared with controls (Fig. 2, *above, right*); it also significantly increased collagen deposition and organization, visualized using Masson trichrome (32.8 ± 1.5 versus 21.0 ± 3.2 ; $p < 0.05$) and picrosirius red staining (22.8 ± 2.4 versus 9.2 ± 1.3 , $p < 0.05$) (Fig. 2, *below*). (See Figure, Supplemental Digital Content 2, A and B, which displays the histology of collagen density and in vitro fibroblast proliferation in response to noncontact, low-frequency ultrasound. Representative images of Masson trichrome and picrosirius red staining showing increased collagen density in the ultrasound group, <http://links.lww.com/PRS/B64>.) Noncontact, low-frequency ultrasound increased fibroblast proliferation in vitro, assessed using Ki-67 index (42 ± 2 percent versus 22 ± 2 percent; $p < 0.001$), potentially contributing to the increased collagen deposition. [See Figure, Supplemental Digital Content 2, C and D, which shows increased Ki67-positive fraction of fibroblasts in the noncontact, low-frequency ultrasound group. Scale bar = $100 \mu\text{m}$ (** $p < 0.001$), <http://links.lww.com/PRS/B64>.]

Noncontact, Low-Frequency Ultrasound Enhances VEGF Expression and Neovascularization in Diabetic Wounds

Neovascularization in wound tissue of noncontact, low-frequency ultrasound-treated and control db/db mice was assessed by evaluating the vascular density of wound tissue by means of immunofluorescent staining for CD31 (platelet endothelial cell adhesion molecule-1) at day 7 and day 14, and measuring CD31 mRNA at day 14 using quantitative reverse-transcriptase polymerase chain reaction. Both CD31 mRNA levels (100 ± 12.1 versus 53 ± 9.7 ; $p < 0.05$) and immunofluorescent protein expression (1.2 ± 0.1 versus 0.8 ± 0.09 at day 7, $p = 0.01$; 3.6 ± 0.5 versus 1.4 ± 0.3 at day 14, $p = 0.01$) were significantly increased in the noncontact, low-frequency ultrasound treatment group compared with controls (Fig. 3, *above, right*, and *below*). VEGF expression was also assessed, using immunofluorescent staining and quantitative reverse-transcriptase polymerase chain reaction, as it is critical for neovascularization.²⁸ Both VEGF mRNA levels (100 ± 15.4 versus 41.4 ± 5.7 , $p = 0.008$) and immunofluorescent protein expression (2.2 ± 0.1 versus 0.8 ± 0.09 at day 7, $p < 0.05$; 2.2 ± 0.1 versus 1.1 ± 0.3 at day 14, $p = 0.009$) were

significantly increased in the ultrasound treatment group compared with untreated controls (Fig. 3, *above, left, and center*). These data suggest that this method induces VEGF expression and increases vascular density in diabetic wounds.

Noncontact, Low-Frequency Ultrasound Increases SDF-1 Expression in Diabetic Wounds

The expression of SDF-1, a potent chemotactic and proangiogenic cytokine, known to be reduced in the setting of diabetes,²⁹ was assessed using quantitative reverse-transcriptase polymerase chain reaction, enzyme-linked immunosorbent assay, and immunofluorescent staining of wound tissue sections. SDF-1 mRNA levels (100 ± 7.7 versus 53 ± 3.3 , $p = 0.003$), protein concentration (4.4 ± 0.2 versus 3.5 ± 0.3 at day 7, $p < 0.05$; 4.2 ± 0.1 versus 3.5 ± 0.2 at day 14, $p < 0.01$), and immunofluorescent protein expression (18.4 ± 2.3 versus 5.6 ± 1.6 at day 7, $p = 0.004$; 7.9 ± 0.5 versus 5.2 ± 0.5 at day 14, $p = 0.006$) were all significantly increased in the noncontact, low-frequency ultrasound treatment group compared with untreated controls (Fig. 4). These data suggest that this method restores a specific cytokine that is deficient in diabetic wounds, and therefore directly addresses a pathologic mechanism for poor wound healing in this setting.

DISCUSSION

In addition to the societal burden associated with nonhealing wounds, there is also a significant impact on the quality of life of afflicted patients. Chronic wounds result in pain, poor sleep quality, and deficits in mobility,^{30,31} further limiting the daily activities of diabetic and elderly patients.^{1,32,33} Given the significant morbidity associated with nonhealing wounds, considerable efforts have focused on developing improved wound healing therapies. Neovascularization—new blood vessel growth in response to ischemia—plays a critical role in tissue repair and regeneration^{2,34} and is therefore an important therapeutic target to better address the problem of chronic wounds.

Noncontact, low-frequency ultrasound provides an attractive therapeutic modality to address poor wound healing. It is noninvasive and noncontact, preventing any undesired disturbance of the wound bed, and is a relatively straightforward technology to implement after minimal training. In addition, it is a safe technology that has long been in medical use and has wide acceptance among both clinicians and the general public. A number of clinical trials have also established its efficacy in treating ischemic, diabetic, and nonhealing wounds, without any apparent side effects.^{6,35–40} One of the larger clinical trials examining the MIST Therapy system demonstrated that noncontact, low-frequency ultrasound almost doubled the percentage of chronic wounds that healed within 90 days and that it accelerated the rate of healing in these wounds.³⁵

In this study, noncontact, low-frequency ultrasound significantly accelerated wound healing in diabetic mice, and the increase in collagen deposition and dermal thickness, potentially occurring secondary to increased fibroblast proliferation, suggests that this technique improves the integrity of healed diabetic wounds. This is in contrast to the typically fragile reepithelialization, prone to breakdown, which occurs in diabetic healing. Extrapolating on these data, if noncontact, low-frequency ultrasound is effective in improving healed wound integrity, it may also decrease recurrence of diabetic wounds, and thereby significantly

reduce associated morbidity, mortality, and health care costs. Nonetheless, further studies specifically assessing diabetic wound recurrence following noncontact, low-frequency ultrasound treatment are necessary before any conclusions regarding the secondary effects of this method can be made.

As an additional point of interest, the decreased wound size in noncontact, low-frequency ultrasound-treated diabetic mice during the latter two-thirds of the healing process, combined with the ability of the ultrasound to reduce biofilm in infected wounds,⁸ may allow for “neoadjuvant” application of this method to minimize wound size and prepare the wound site before the use of a local flap or a free tissue transfer for definitive treatment. For this indication, noncontact, low-frequency ultrasound may be preferred to established modalities, such as negative-pressure wound therapy, which requires the constant presence of a drain and a vacuum pump⁴¹ and has been associated with pain and decreased quality of life for patients.⁴²

By increasing the vascularity of the wound bed, noncontact, low-frequency ultrasound may also facilitate the use of split-thickness and full-thickness skin grafts for wound closure, an approach that has traditionally failed because of the paucity of vasculature in the wound beds of diabetic patients. This increased vascularity is likely attributable to the increased expression of VEGF and SDF-1, induced through a mechanoresponsive component in the transcriptional regulation of these genes. Specifically, the mechanoresponsive expression of these angiogenic cytokines may be fibroblast mediated, as ultrasound has been shown to influence extracellular signal-regulated kinase phosphorylation in fibroblasts,²⁰ and extracellular signal-regulated kinase activation can increase the expression of angiogenic cytokines.^{43,44} Furthermore, our study has demonstrated that noncontact, low-frequency ultrasound can influence fibroblast physiology, stimulating proliferation in vitro. Moreover, although the phosphorylation of extracellular signal-regulated kinase may be mediated by a variety of cell-surface or cytoplasmic proteins that become activated in response to mechanical stimuli, focal-adhesion kinase has been shown to be mechanoresponsive and to activate the extracellular signal-regulated kinase signaling pathway⁴⁵ and is therefore a likely candidate. Interestingly, the effect of non-contact, low-frequency ultrasound may also be partially mediated by increasing cell membrane permeability, allowing for calcium influx⁴⁶ and activation of signaling pathways controlling gene expression.^{47,48}

The noncontact, low-frequency ultrasound stimulation of VEGF expression is likely a major contributor to its efficacy, as VEGF is the primary stimulus for local endothelial cell proliferation and motility resulting in angiogenesis.²⁸ Stimulation of SDF-1 expression is also of particular interest in this setting, as SDF-1 has been shown to be down-regulated in the setting of diabetes, with a subsequently decreased capacity of diabetic wounds to recruit circulating progenitors thought to play an important role in the healing process.²⁹

Looking ahead, advances in our understanding of the mechanisms underlying the efficacy of noncontact, low-frequency ultrasound will likely allow for the optimization of parameters and potentially uncover novel applications for this technology. Although the treatment parameters used in this study were recommended by the manufacturer of the device based on their efficacy in clinical trials, these data provide proof of principle that by measuring the

neovascular response, and in particular the expression of growth factors and cytokines such as VEGF and SDF-1, the ideal ultrasound frequency and treatment schedule can be identified. Moreover, the stimulation of a neovascular response may also provide therapeutic benefit in the setting of cardiac or neurologic ischemia, and adaptation of the technology to address these clinical entities could be of considerable benefit. Within the setting of cutaneous healing, future studies should address the potential for “neoadjuvant” application of noncontact, low-frequency ultrasound, including comparisons to existing modalities such as negative-pressure wound therapy, and the impact of noncontact, low-frequency ultrasound on progenitor cell recruitment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Sen CK, Gordillo GM, Roy S, et al. Human skin wounds: A major and snowballing threat to public health and the economy. *Wound Repair Regen.* 2009; 17:763–771. [PubMed: 19903300]
2. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. *J Clin Invest.* 2007; 117:1219–1222. [PubMed: 17476353]
3. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature.* 2008; 453:314–321. [PubMed: 18480812]
4. American Diabetes Association. Economic costs of diabetes in the U.S 2007. *Diabetes Care.* 2008; 31:596–615. [PubMed: 18308683]
5. American Diabetes Association. Economic costs of diabetes in the U.S in 2012. *Diabetes Care.* 2013; 36:1033–1046. [PubMed: 23468086]
6. Johannsen F, Gam AN, Karlsmark T. Ultrasound therapy in chronic leg ulceration: A meta-analysis. *Wound Repair Regen.* 1998; 6:121–126. [PubMed: 9776854]
7. Houghton PE, Campbell KE. Choosing an adjunctive therapy for the treatment of chronic wounds. *Ostomy Wound Manage.* 1999; 45:43–52. quiz 53. [PubMed: 10655867]
8. Seth AK, Nguyen KT, Geringer MR, et al. Noncontact, low-frequency ultrasound as an effective therapy against *Pseudomonas aeruginosa*-infected biofilm wounds. *Wound Repair Regen.* 2013; 21:266–274. [PubMed: 23421692]
9. Young SR, Dyson M. The effect of therapeutic ultrasound on angiogenesis. *Ultrasound Med Biol.* 1990; 16:261–269. [PubMed: 1694604]
10. Maxwell L, Collicutt T, Gledhill M, Sharma S, Edgar S, Gavin JB. The augmentation of leucocyte adhesion to endothelium by therapeutic ultrasound. *Ultrasound Med Biol.* 1994; 20:383–390. [PubMed: 8085295]
11. Young SR, Dyson M. Macrophage responsiveness to therapeutic ultrasound. *Ultrasound Med Biol.* 1990; 16:809–816. [PubMed: 2095011]
12. Lu C, Saless N, Hu D, et al. Mechanical stability affects angiogenesis during early fracture healing. *J Orthop Trauma.* 2011; 25:494–499. [PubMed: 21738063]
13. Rustad KC, Wong VW, Gurtner GC. The role of focal adhesion complexes in fibroblast mechanotransduction during scar formation. *Differentiation.* 2013; 86:87–91. [PubMed: 23623400]

14. Wong VW, Longaker MT, Gurtner GC. Soft tissue mechanotransduction in wound healing and fibrosis. *Semin Cell Dev Biol.* 2012; 23:981–986. [PubMed: 23036529]
15. Wong VW, Paterno J, Sorkin M, et al. Mechanical force prolongs acute inflammation via T-cell-dependent pathways during scar formation. *FASEB J.* 2011; 25:4498–4510. [PubMed: 21911593]
16. Zhang H, Landmann F, Zahreddine H, Rodriguez D, Koch M, Labouesse M. A tension-induced mechanotransduction pathway promotes epithelial morphogenesis. *Nature.* 2011; 471:99–103. [PubMed: 21368832]
17. Lancerotto L, Bayer LR, Orgill DP. Mechanisms of action of microdeformational wound therapy. *Semin Cell Dev Biol.* 2012; 23:987–992. [PubMed: 23036531]
18. Ennis WJ, Foremann P, Mozen N, Massey J, Conner-Kerr T, Meneses P. Ultrasound therapy for recalcitrant diabetic foot ulcers: Results of a randomized, double-blind, controlled, multicenter study. *Ostomy Wound Manage.* 2005; 51:24–39. [PubMed: 16234574]
19. Wollina U, Heinig B, Naumann G, Scheibe A, Schmidt WD, Neugebauer R. Effects of low-frequency ultrasound on microcirculation in venous leg ulcers. *Indian J Dermatol.* 2011; 56:174–179. [PubMed: 21716543]
20. Lai J, Pittelkow MR. Physiological effects of ultrasound mist on fibroblasts. *Int J Dermatol.* 2007; 46:587–593. [PubMed: 17550557]
21. Galiano RD, Michaels JV, Dobryansky M, Levine JP, Gurtner GC. Quantitative and reproducible murine model of excisional wound healing. *Wound Repair Regen.* 2004; 12:485–492. [PubMed: 15260814]
22. Keltie K, Reay CA, Bousfield DR, et al. Characterization of the ultrasound beam produced by the MIST therapy, wound healing system. *Ultrasound Med Biol.* 2013; 39:1233–1240. [PubMed: 23562019]
23. Nauta AC, Grova M, Montoro DT, et al. Evidence that mast cells are not required for healing of splinted cutaneous excisional wounds in mice. *PLoS One.* 2013; 8:e59167. [PubMed: 23544053]
24. Lerman OZ, Galiano RD, Armour M, Levine JP, Gurtner GC. Cellular dysfunction in the diabetic fibroblast: Impairment in migration, vascular endothelial growth factor production, and response to hypoxia. *Am J Pathol.* 2003; 162:303–312. [PubMed: 12507913]
25. Michaels JV, Churgin SS, Blechman KM, et al. db/db mice exhibit severe wound-healing impairments compared with other murine diabetic strains in a silicone-splinted excisional wound model. *Wound Repair Regen.* 2007; 15:665–670. [PubMed: 17971012]
26. Callaghan MJ, Chang EI, Seiser N, et al. Pulsed electromagnetic fields accelerate normal and diabetic wound healing by increasing endogenous FGF-2 release. *Plast Reconstr Surg.* 2008; 121:130–141. [PubMed: 18176216]
27. Thangarajah H, Yao D, Chang EI, et al. The molecular basis for impaired hypoxia-induced VEGF expression in diabetic tissues. *Proc Natl Acad Sci USA.* 2009; 106:13505–13510. [PubMed: 19666581]
28. Connolly DT. Vascular permeability factor: A unique regulator of blood vessel function. *J Cell Biochem.* 1991; 47:219–223. [PubMed: 1791186]
29. Gallagher KA, Liu ZJ, Xiao M, et al. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1 alpha. *J Clin Invest.* 2007; 117:1249–1259. [PubMed: 17476357]
30. Franks PJ, Morgan PA. Health-related quality of life with chronic leg ulceration. *Expert Rev Pharmacoecon Outcomes Res.* 2003; 3:611–622. [PubMed: 19807395]
31. Gorecki C, Brown JM, Nelson EA, et al. Impact of pressure ulcers on quality of life in older patients: A systematic review. *J Am Geriatr Soc.* 2009; 57:1175–1183. [PubMed: 19486198]
32. Frykberg RG. Epidemiology of the diabetic foot: Ulcerations and amputations. *Adv Wound Care.* 1999; 12:139–141. [PubMed: 10655794]
33. Wieman TJ, Smiell JM, Su Y. Efficacy and safety of a topical gel formulation of recombinant human platelet-derived growth factor-BB (becaplermin) in patients with chronic neuropathic diabetic ulcers: A phase III randomized placebo-controlled double-blind study. *Diabetes Care.* 1998; 21:822–827. [PubMed: 9589248]

34. Hamou C, Callaghan MJ, Thangarajah H, et al. Mesenchymal stem cells can participate in ischemic neovascularization. *Plast Reconstr Surg*. 2009; 123(2 Suppl):45S–55S. [PubMed: 19182663]
35. Kavros SJ, Liedl DA, Boon AJ, Miller JL, Hobbs JA, Andrews KL. Expedited wound healing with noncontact, low-frequency ultrasound therapy in chronic wounds: A retrospective analysis. *Adv Skin Wound Care*. 2008; 21:416–423. [PubMed: 18769168]
36. Ramundo J, Gray M. Is ultrasonic mist therapy effective for debriding chronic wounds? *J Wound Ostomy Continence Nurs*. 2008; 35:579–583. [PubMed: 19018197]
37. Waldrop K, Serfass A. Clinical effectiveness of noncontact, low-frequency, nonthermal ultrasound in burn care. *Ostomy Wound Manage*. 2008; 54:66–69. [PubMed: 18579927]
38. Ennis WJ, Foremann P, Mozen N, Massey J, Conner-Kerr T, Meneses P. Ultrasound therapy for recalcitrant diabetic foot ulcers: Results of a randomized, double-blind, controlled, multicenter study. *Ostomy Wound Manage*. 2005; 51:24–39. [PubMed: 16234574]
39. Ennis WJ, Valdes W, Gainer M, Meneses P. Evaluation of clinical effectiveness of MIST ultrasound therapy for the healing of chronic wounds. *Adv Skin Wound Care*. 2006; 19:437–446. [PubMed: 17008814]
40. Kavros SJ, Miller JL, Hanna SW. Treatment of ischemic wounds with noncontact, low-frequency ultrasound: The Mayo clinic experience, 2004–2006. *Adv Skin Wound Care*. 2007; 20:221–226. [PubMed: 17415030]
41. Isaac AL, Armstrong DG. Negative pressure wound therapy and other new therapies for diabetic foot ulceration: The current state of play. *Med Clin North Am*. 2013; 97:899–909. [PubMed: 23992900]
42. Upton D, Stephens D, Andrews A. Patients' experiences of negative pressure wound therapy for the treatment of wounds: A review. *J Wound Care*. 2013; 22:34–39. [PubMed: 23299356]
43. Clarke DN, Al Ahmad A, Lee B, et al. Perlecan Domain V induces VEGF secretion in brain endothelial cells through integrin $\alpha 5 \beta 1$ and ERK-dependent signaling pathways. *PLoS One*. 2012; 7:e45257. [PubMed: 23028886]
44. Treins C, Giorgetti-Peraldi S, Murdaca J, Van Obberghen E. Regulation of vascular endothelial growth factor expression by advanced glycation end products. *J Biol Chem*. 2001; 276:43836–43841. [PubMed: 11571295]
45. Wong VW, Rustad KC, Akaishi S, et al. Focal adhesion kinase links mechanical force to skin fibrosis via inflammatory signaling. *Nat Med*. 2012; 18:148–152. [PubMed: 22157678]
46. Mortimer AJ, Dyson M. The effect of therapeutic ultrasound on calcium uptake in fibroblasts. *Ultrasound Med Biol*. 1988; 14:499–506. [PubMed: 3227573]
47. Reher P, Doan N, Bradnock B, Meghji S, Harris M. Effect of ultrasound on the production of IL-8, basic FGF and VEGF. *Cytokine*. 1999; 11:416–423. [PubMed: 10346981]
48. Doan N, Reher P, Meghji S, Harris M. In vitro effects of therapeutic ultrasound on cell proliferation, protein synthesis, and cytokine production by human fibroblasts, osteoblasts, and monocytes. *J Oral Maxillofac Surg*. 1999; 57:409–419. discussion 420. [PubMed: 10199493]

Fig. 1.

Representative images at time intervals demonstrating the (*above*) increased rate of wound closure and increased vascularity of the wound bed in the noncontact, low-frequency ultrasound (NLFU) group. (*Below, left*) Wound healing kinetics demonstrating significantly decreased wound size in the noncontact, low-frequency ultrasound group relative to controls starting at day 9 and (*below, right*) shorter time to wound closure in the noncontact, low-frequency ultrasound group (* $p < 0.05$).

Fig. 2.

Histology of noncontact, low-frequency ultrasound-treated and control wounds. *Black vertical dotted lines* identify the original wound margin. (*Above, left*) Hematoxylin and eosin stain demonstrating a more organized architecture and (*above, right*) increased dermal thickness and cutaneous integrity in noncontact, low-frequency ultrasound treatment group with (*below*) significantly increased collagen density on Masson trichrome and picrosirius red staining. Scale bar = 100 μ m (* $p < 0.05$). NLFU, noncontact, low-frequency ultrasound.

Fig. 3. Immunohistochemistry and quantitative polymerase chain reaction for neovascularization in noncontact, low-frequency ultrasound-treated and control wounds. (*Above, left, and center*) Increased VEGF mRNA and protein levels in the noncontact, low-frequency ultrasound group, (*above, right, and below*) with subsequently increased CD31 mRNA and protein levels. *VEGF*, vascular endothelial growth factor; *NLFU*, noncontact, low-frequency ultrasound. Scale bar = 100 μ m (* $p < 0.05$).

Fig. 4. SDF-1 regulation in noncontact, low-frequency ultrasound-treated and control wounds. SDF-1 mRNA levels, protein concentration, and immunofluorescent protein expression were significantly enhanced by noncontact, low-frequency ultrasound treatment. *Scale bar* = 100 μ m (* p < 0.05).