Changes in S100 Proteins Identified in Healthy Skin following Electrical Stimulation: Relevance for Wound Healing

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ABSTRACT

OBJECTIVE: Targeted electrical energy applied to wounds has been shown to improve wound-healing rates. However, the mechanisms are poorly understood. The aim of this study was to identify genes that are responsive to electrical stimulation (ES) in healthy subjects with undamaged skin.

METHODS: To achieve this objective, study authors used a small, noninvasive ES medical device to deliver a continuous, specific, set sequence of electrical energy impulses over a 48-hour period to the skin of healthy volunteers and compared resultant gene expression by microarray analysis.

MAIN RESULTS: Application of this specific ES resulted in differential expression of 105 genes, the majority of which were down-regulated. Postmicroarray analyses revealed there was commonality with a small number of genes that have previously been shown to be up-regulated in skin wounds, including venous leg ulcers.

CONCLUSIONS: The specific sequence of ES applied continuously for 48 hours to the skin of healthy patients has the effect of modifying expression in a number of identified genes. The identification of the differential expression in this subset of genes in healthy subjects provides new potential lines of scientific inquiry for identifying similar responses in subjects with slow or poorly healing wounds.

KEYWORDS: electrical stimulation, epidermis, gene expression, healthy skin, microarray, wound healing

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INTRODUCTION

The use of externally applied electrical energy to promote the healing of complex wounds was introduced more than 40 years ago.[1] There are various types of electrical stimulation (ES) devices, for example, the PosiFect RD DC device (BioFiscia, Odiham, Hampshire, United Kingdom) and the woundEL LVMPC device (Göteborg, Sweden). Despite variation in application mode, dose, and duration of therapy, the majority of trials show significant improvement in wound healing or wound area reduction with ES therapy compared with control treatment or standard care.[2,3] The use of continuous direct current[4–7] of 200 to 800 μA and pulsed current[8,9] improved healing of chronic wounds in a number of studies. However, the mechanisms of ES-mediated wound healing in vivo are poorly understood; a number of different explanations have been provided for the clinical outcomes reported.

Clinical Problem Addressed

Identifying how ES can regulate or modify gene expression in skin is critical in improving the understanding of why ES treatment improves healing rates in chronic wounds in some individuals and not in others. Further, identifying ES-responsive genes might provide new potential lines of scientific inquiry for promoting similar, beneficial gene responses in subjects with slow or poorly healing wounds. It also might be possible to measure expression in responders and nonresponders alike to predict treatment efficacy in different patient groups and to select ES treatment parameters that yield the best wound healing outcomes.

MATERIALS AND METHODS

A recent systematic review concluded that the ideal ES device needs to be noninvasive, portable, cost-effective, and cause minimal interference with patients' daily life.[3] In addition, a device that delivers a program of fixed parameters and duration is important in order to standardize findings and to make comparisons and draw conclusions from studies or clinical applications that use it.

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The device selected for this study (Accel-Heal; Synapse Electroceutical Ltd, Kent, United Kingdom) is a class IIA medical device (under medical directive 93/42/EEC, which has full Medical Devices Directorate approval under ISO 13485:2003) and satisfies the aforementioned qualities of an ideal ES device. The device delivers a proprietary frequency of pulsed electrical energy at the microcurrent level directly to the skin via 2 off-the-shelf treatment electrode pads placed directly onto intact skin for a duration of 48 hours (Figure 1A).

Study Design and Participants

The study was an investigator-initiated, double-blind, randomized, placebo-controlled trial undertaken at Bispebjerg Hospital in Copenhagen, Denmark. The study was reported to the Danish register (Datatilsynet) and was performed in accordance with Danish law (Lov om behandling af personoplysninger). This included an approval from the Regional Ethical Committee for the Hospital Region of Greater Copenhagen (Region H, H-3-2012-158).

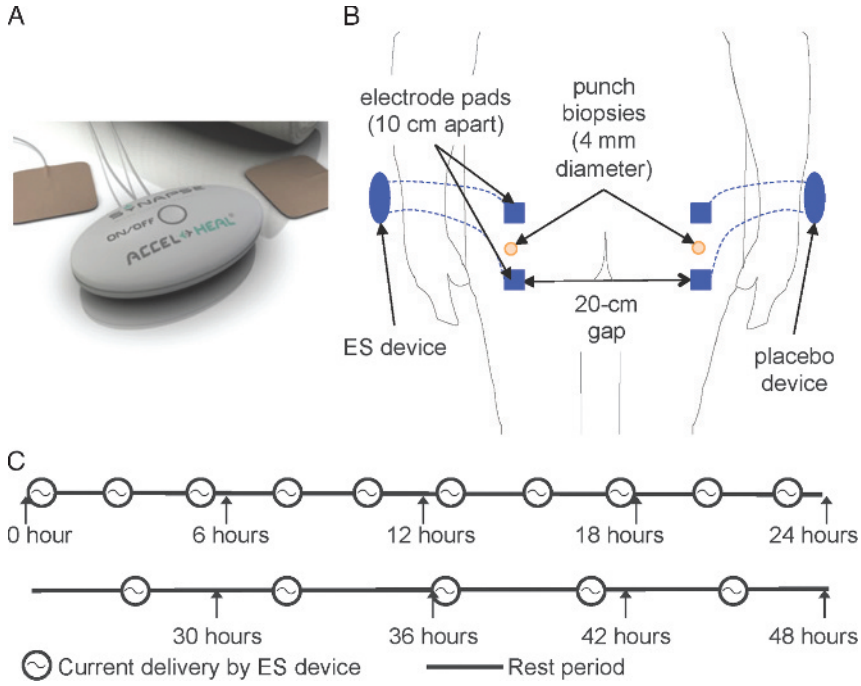
Only healthy, nonsmoking white men, 20 to 30 years old, with a body mass index between 19 and 25 kg/m^2 and no history of cancer, diabetes, or vascular disease, who undertook similar levels of moderate physical activity per week were recruited.

Two ES devices identical in appearance (1 active and 1 placebo), attached to pairs of electrodes identical in appearance, were placed on intact healthy skin on the buttocks of 12 healthy male subjects. This site was selected because it would be possible to place the devices out of sight of the subject to secure the blinded element; further, the skin would be covered all of the time in all of the subjects. The electrode pads of each device were placed 10 cm apart, and there was a 20-cm gap between the electrode pads of the different devices (Figure 1B). This arrangement was used because it most accurately reflected use in wound management, where wounds are often covered by a 10-cm square dressing. Only a third independent person had knowledge of which device was active, ensuring the study was double-blind. After 48 hours, full-thickness 4-mm skin biopsies were excised from a region midway between the 2 electrodes.

Comparative Microarray

Subjects' RNA was isolated from skin biopsies and cells using Invitrogen TRIzol reagent (ThermoFisher Scientific, Carlsbad,

Figure 1.
ELECTRICAL STIMULATION (ES) APPLICATION TO SKIN OF HEALTHY PARTICIPANTS



A, The ES device. B, Two ES devices were applied to the skin overlying the buttocks (1 on each side). The electrode pads of each device were 10 cm apart, and there was a 20-cm gap between the electrode pads of the different devices. C, Program specification of the ES device.

California) following manufacturer protocols.[10] The RNA concentrations and integrity were measured using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California). The RNA was amplified and hybridized to Human Genome U133 2.0 GeneChip arrays (Affymetrix, Santa Clara, California). Microarray data are available in the ArrayExpress database under accession number E-MTAB-3935. Technical quality control and outlier analysis was performed with dChip (V2005)[11] using the default settings. Background correction, quantile normalization, and gene expression analysis were performed using RMA in Bioconductor (Buffalo, New York).[12]

To assess whether ES altered gene expression in the skin, an analysis of variance linear model was used to evaluate where the variation within the data set originated (Partek Genomics Solution version 6.5; Partek Inc, St Charles, Missouri). Differential expression analysis was performed for subjects who were exposed to ES using a paired model in Limma (Bioconductor) using the functions lmFit and eBayes.[13] A gene list of differentially expressed genes was formed from probe sets that had a greater than 1.5-fold change ($P < .05$; see Supplemental Digital Content 1, http://links.lww.com/NSW/A12).

Data Analysis

Gene ontology analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resources, version 6.8) as well as Ingenuity Pathway Analysis (Qiagen, Hilden, Germany) showed that ES-stimulated genes were enriched with receptor for advanced glycation end products (RAGE)–binding proteins: S100A7, S100A8, and S100A9. A comparison was performed with wound-regulated genes in a microarray data set generated by Cooper et al[14] under the ArrayExpress accession number E-MTAB-3836(14) from genes differentially regulated in venous leg ulcer wound edges.[15]

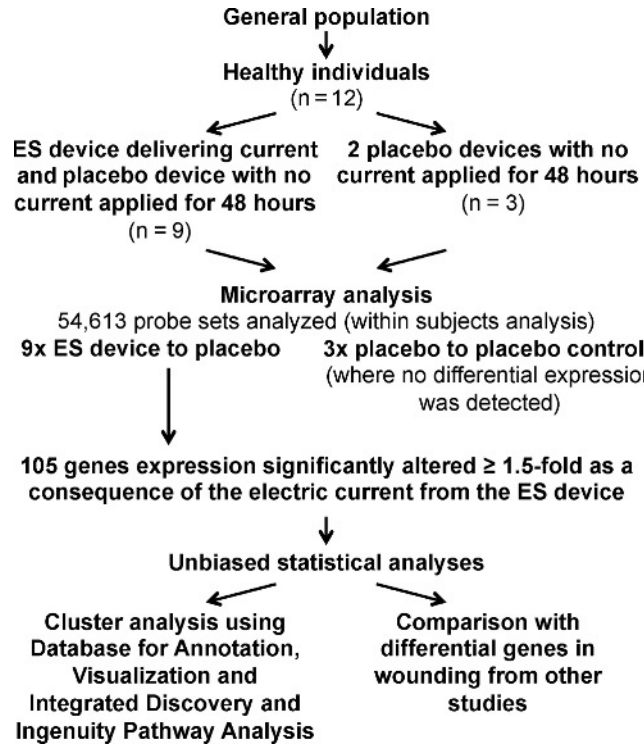
RESULTS

The study included 12 participants, 9 of whom received 1 activated and 1 placebo ES device and 3 of whom received only placebo devices (Figure 2). The activated device delivered a series of preset programs of varying amplitude (40–500 μA), frequency (10–900 Hz), and polarity (alternating every 0.1 second). The electrical pulses were delivered in repeated 240-minute treatment sessions with a 2-hour resting period between each in the first 24 hours and a 4-hour resting period between each in the second 24 hours (Figure 1C).

Unbiased statistical analyses showed that 1 of the 24 RNA samples was an outlier and was excluded from subsequent analyses (Figure 3A). Both principal component analysis and linear model evaluation suggested that the largest source of variation in global gene expression changes was from differences among individuals (Figure 3B). No significant differential expression

Figure 2.

FLOW CHART OF STUDY TO IDENTIFY GENES IN SKIN THAT ARE REGULATED BY ELECTRICAL STIMULATION



was detected between biopsies from the placebo group in any of the 3 control participants. A within-subject analysis of ES-treated versus placebo-treated skin produced a significant variation score of 1.47, as illustrated by the 15 most differentially regulated genes (Figure 3C). As a result, alterations in levels of gene expression could be attributed to the 48-hour ES treatment.

To accommodate the large individual variation, a paired test was used, resulting in 105 annotated genes with a significant differential regulation (at least 1.5-fold [$P < .05$]) in ES-treated skin compared with placebo treatment (see Supplemental Digital Content 1, http://links.lww.com/NSW/A12). A volcano plot of the microarray data shows that the majority of these differentially regulated genes were reduced in expression, with the exception of 2 genes (Figure 4A).

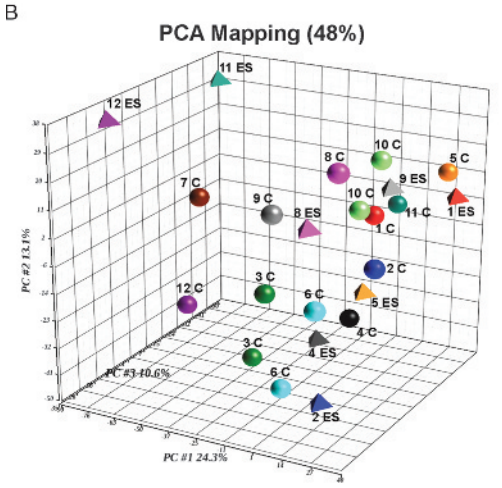
Study authors subjected this list of ES-regulated genes to unbiased functional annotation analyses using DAVID and Ingenuity Pathway Analysis. Functional gene ontology analysis showed that there was an overrepresentation of cellular compartment genes, including focal adhesion (ANXA6, PDIA3, TPM4, LPP), protein-DNA complex (CTNNB1, HNRNPK, JUP), and endoplasmic reticulum–Golgi intermediate compartment

Figure 3.
VARIATION AMONG INDIVIDUALS

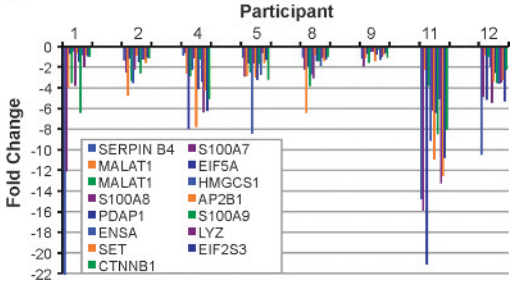
A

Sample	Key	Median Intensity (unnormalized)	P call %	% Array outlier	% Single outlier
1 control	●	120	61.4	0.219	0.07
1 electrical stimulation	▲	98	59.1	0.519	0.129
2 control	●	136	61.5	0.04	0.01
2 electrical stimulation	▲	102	59.1	0.176	0.023
3 control_1	●	119	60.1	0.051	0.011
3 control_2	●	141	62.9	0.035	0.009
4 control	●	121	61.6	0.126	0.025
4 electrical stimulation	▲	124	59.9	0.155	0.028
5 control	●	112	60.3	0.302	0.078
5 electrical stimulation	▲	99	59.1	0.388	0.238
6 control_1	●	126	62.3	0.06	0.011
6 control_2	●	128	60.5	0.298	0.037
7 control	●	108	60.9	0.296	0.026
7 electrical stimulation		87	53.2	7.29*	0.2
8 control	●	103	61.8	0.252	0.021
8 electrical stimulation	▲	107	61.5	0.134	0.018
9 control	●	106	62.1	1.205	0.164
9 electrical stimulation	▲	103	61.1	0.201	0.046
10 control_1	●	125	62.3	0.077	0.011
10 control_2	●	131	61.6	0.059	0.009
11 control	●	108	60.6	0.101	0.025
11 electrical stimulation	▲	113	59	0.658	0.03
12 control	●	113	59.6	0.293	0.046
12 electrical stimulation	▲	97	57.4	2.065	0.104

B



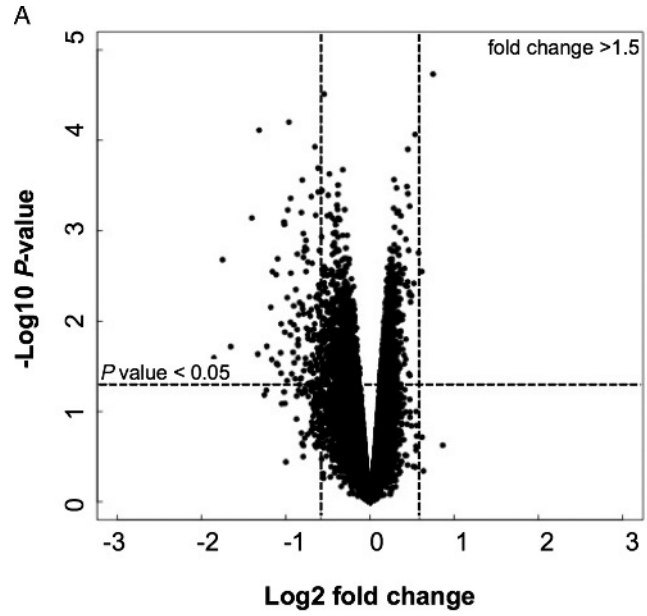
C



A, dChip analysis of samples obtained from the 12 participants of the study. Subject 8's ES treatment was an array outlier and was subsequently excluded from further analyses. B, Principal component analysis was used to provide a statistical summary of the array samples, and the first 3 principal components are shown. Abbreviations: C, control; MC, microcurrent. C, The top 15 differentially regulated genes (as ranked by fold change) of the 8 participants who were treated with a placebo and the ES device. Note that SERPINB4 expression in subject 1 is not included in the graph, but it showed a fold change of over -90 with treatment.

Figure 4.
ELECTRICAL STIMULATION TREATMENT TO HEALTHY SKIN LEADS TO DOWN-REGULATION OF GENE EXPRESSION

A



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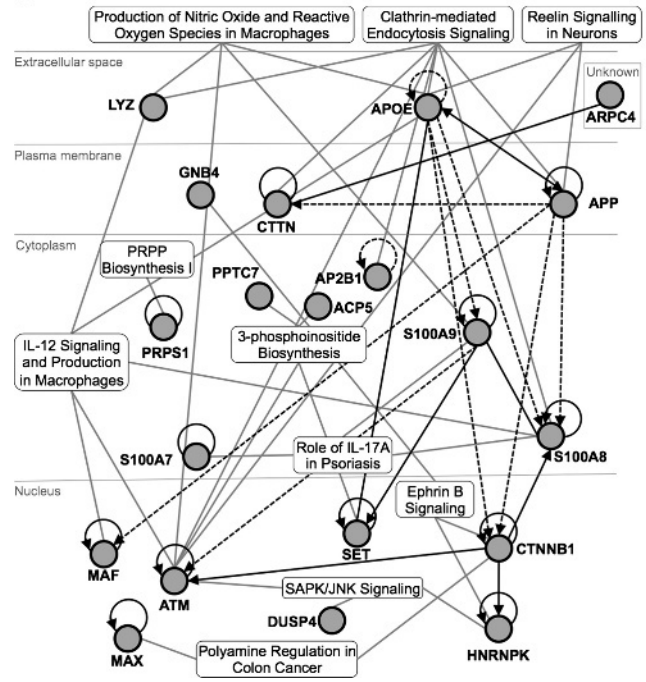

A, Volcano box plot to illustrate the expression changes of all genes. The 105 genes whose expression was significantly altered more than 1.5-fold ($P < .05$) are shown in the top left and top right boxes. B, Top 10 canonical pathways identified in ES-regulated genes in skin by Ingenuity Pathway Analysis.

membrane (TMED9, RAB2A, ERGIC1), and in genes encoding ribonucleoprotein (TEP1, HNRNPH1, MRPS16, MRPL30), gene activators (SUPT4H1, SMARCA2, MAX, RORC, EBF1), and RAGE-binding proteins (S100A7, S100A8, S100A9; see Supplemental Digital Content 2, http://links.lww.com/NSW/A13). Pathway analysis showed that these ES-regulated genes were implicated in canonical pathways, including regulation of macrophage production of interleukin 12, nitric oxide and reactive oxygen species, and interleukin 17A in psoriasis (Figure 4B).

Next, study authors compared the list of ES-regulated genes to 2 publicly available array data sets that identified genes regulated by wounding. First, the new data set was compared with more than 2600 probe sets differentially regulated 24 hours after wounding in vivo identified in a mouse skin injury model.[14] In common were 25 genes that were up-regulated in wounding but down-regulated with ES; 1 gene (RAD23B) was similarly down-regulated in both microarray studies (Table). Next, the new data set was compared with the top 50 most up-regulated and top 50 most down-regulated genes identified in the wound edge of venous leg ulcers.[15] All 3 S100 genes and SERPINB4 were up-regulated in the venous leg ulcer wound edge but were down-regulated in ES-treated skin (Table).

Table.

GENES COMMON WITH THOSE DIFFERENTIALLY REGULATED IN SKIN 24 HOURS AFTER WOUNDING AND IN VENOUS LEG ULCER WOUND EDGE

Gene Symbol	Therapy vs Placebo (Fold Change)	Acute Wound vs Control (Fold Change)	Ulcer Wound Edge vs Control (Fold Change)[15]
SERPINB4	−3.62	—	94.96
S100A7	−3.37	—	105.80
EIF5A	−2.52	1.62	—
S100A8	−2.26	90.42	95.11
A02B1	−2.24	1.47	—
PDAP1	−2.17	1.52	—
S100A9	−2.16	97.95	119.00
LYZ	−2.14	1.24	—
SET	−2.09	1.48	—
VCAN	−1.98	8.59	—
CPR124	−1.93	2.72	—
CD47	−1.92	1.60	—
RAD23B	−1.92	−1.26	—
SPP1	−1.90	7.52	—
CALD1	−1.88	1.52	—
ARPC4	−1.87	1.77	—
LCN2	−1.81	44.12	—
PDIA3	−1.67	1.56	—
ERGIC1	−1.67	1.70	—
RNF125	−1.66	1.27	—
KRT17	−1.64	2.72	—
KRT6B	−1.63	146.32	37.66
APP	−1.62	—	−9.17
TPM4	−1.55	1.7	—
RBM3	−1.53	1.53	—
SOX7	−1.53	1.23	—
GNB4	−1.52	1.51	—
AQP3	−1.52	2.80	—
GNG12	−1.50	1.36	—

DISCUSSION

The primary finding of this study is that ES delivered with a preset, fixed program and duration to healthy human skin reduces the expression of a specific set of genes that are up-regulated in skin inflammation. This study used healthy volunteers instead of patients with chronic wounds primarily because of the risk that biopsy could have adverse effects on wound healing. It is unclear from the results of this study, but possible to hypothesize, that the same ES device applied in the same manner would have the same effects on healthy wound edge skin adjacent to a chronic wound. However, many patients with chronic and nonhealing wounds commonly present with comorbidities so this hypothesis would have to be fully tested in a variety of patients and the results compared with this study to draw a safe conclusion.

The 48-hour duration of treatment was chosen because it constitutes 1 application of the device's full delivery cycle. Because the device was applied over the buttocks, study authors determined that 48 hours was an acceptable length of time for participants to withhold from showering, bathing, and activities that could cause excessive sweating.

Analysis of the sources of variation reinforced the fact that there is extensive variation among participants but did indicate that ES significantly affected the expression of specific genes. While some variables could be controlled (eg, age, smoking status), other variations in the participants' lifestyle (eg, activity and diet) or underlying medical conditions were unknown. However, the participants were all healthy young individuals without any signs of abnormal conditions or diseases. The participant profiles of the most regulated genes varied among participants, suggesting that some of the participants could be viewed as either responders or nonresponders to the ES treatment.

Functional annotation analysis of the 105 ES-regulated genes showed that there is an enrichment of RAGE-binding proteins: S100A7, S100A8, and S100A9. Study authors could compare these findings with only 2 other publicly available array data sets produced from wound-related studies[14,15]; however, the comparisons confirmed that S100 genes are up-regulated in both acute and chronic wounds. These proteins are expressed at low levels in keratinocytes in healthy skin but are up-regulated upon wounding. The expression of S100 genes is crucial for re-epithelialization of the wound,[16] recruitment of inflammatory cells,[17,18] regeneration of the hair follicle,[19] and regulation of keratinocyte growth and differentiation.[20] Controversially, S100 proteins are also readily

detected in wound exudates[21,22] and up-regulated in chronic wounds[15,23] and psoriasis,[24,25] which suggests that their expression is a biomarker for nonhealing wounds. Overexpression of S100 genes in HaCat keratinocytes impaired collective migration in the cell sheet immediately behind the migrating front in an in vitro scratch wound assay, and staining of β-catenin showed cells adjacent to gaps in the cell sheet had no positive staining in the periphery but accumulated cell junction proteins in the cytosol when S100 genes were overexpressed (data not shown).

It is thought that a prolonged inflammatory phase and the increased expression of proteolytic enzymes prevent wounds from progressing into the proliferative phase and are major factors that contribute to nonhealing wounds.[26] Pathway analysis indicated that the ES application dampened the release of proinflammatory cytokine interleukin 12, nitric oxide, and reactive oxygen species by macrophages responsible for the breakdown of connective tissues.[27] Reduction of S100A7, S100A8, and S100A8 and activation of macrophages by ES could potentially improve wound healing by dampening these pathways in chronic wounds.

In summary, study authors hypothesize that ES-mediated healing of chronic wounds can be achieved via down-regulation of certain gene pathways that are known to compromise wound healing, enabling an improved rate of repair.

Limitations

This study was performed on a small number of participants, limiting the generalizability of results. Further, it cannot be inferred from these results that the same ES device applied in the same manner to the periwound skin of a poorly healing wound would have the same effect as on the skin of healthy volunteers. Findings may be different in different areas of the body where skin thickness varies.

There are no known or anticipated adverse reactions from this electroceutical application. A single type of ES device was used for this investigation, delivering a fixed pulse sequence and duration of ES. Therefore, it is impossible to know whether the same changes in gene expression reported here would be seen if a different set of ES parameters were used.

CONCLUSIONS

This study is a first step toward a mechanistic understanding of the potential benefits of ES for improving skin healing in pathologic conditions. The identification of these changes in gene expression, albeit in healthy skin, provides potential novel therapeutic targets for individuals who present with delayed skin healing. Identifying higher levels of these proteins may help to identify those who are predisposed to poor healing. However, further investigation is required to see if the same changes in gene expression result from a different set of ES parameters. ●

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