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Original Article

A novel *in vitro* assay for electrophysiological research on human skin fibroblasts: Degenerate electrical waves downregulate collagen I expression in keloid fibroblasts

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Abstract: Electrical stimulation (ES) has been used for the treatment of wounds and has been shown to alter gene expression and protein synthesis in skin fibroblasts *in vitro*. Here, we have developed a new *in vitro* model system for testing the effects of precisely defined, different types of ES on the collagen expression of normal and keloid human skin fibroblasts. Keloid fibroblasts were studied because they show excessive collagen production. Both types of fibroblasts were electrically stimulated with alternating current (AC), direct current (DC) or degenerate waves (DW). Cells were subjected to 20, 75 and 150 mV/mm electric field strengths at 10 and 60 Hz frequencies. At lower electric fields, all types of ES upregulated collagen I in both cell types compared to controls. However, at higher electric field strength (150 mV/mm) and frequency (60 Hz), DW maximally downregulated collagen I in keloid fibroblasts, yet had significantly lower cytotoxic effects on normal fibroblasts than AC and DC. Compared to unstimulated cells, both normal skin and keloid fibroblasts showed a significant decrease in collagen I expression after 12 h of DW and AC stimulation. In contrast, increasing amplitude of DC upregulated collagen I and PAI-1 gene transcription in normal and keloid fibroblasts, along with increased cytotoxicity effects. Thus, our new preclinical assay system shows highly differential effects of specific types of ES on human fibroblast collagen expression and cytotoxicity and identifies DW of electrical current (DW) as a promising, novel therapeutic strategy for suppressing excessive collagen I formation in keloid disease.

Key words: collagen I – degenerate waves – differential gene expression – electrical stimulation – fibroblasts – *in vitro* electrical stimulation model – keloid scars and keloid disease

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Introduction

In both human and animal studies, electrical stimulation (ES) in its various forms has been shown to enhance wound healing by improving fibroproliferative responses (1–11). Alternating current (AC) voltages, which represent the periodic reversal of charge flow direction, show beneficial effect in the management of diabetic (12) and neuropathic foot ulcers (13), whilst direct current (DC) has been used for treating chronic skin ulcers of various aetiologies (14–16). Pulsed current, which consists of defined intermittent flow of current, has also been reported to promote healing of chronic wounds (17,18) and spinal cord injuries (19).

However, there is considerable variation in the ES protocols used in clinical studies, and these reports are usually based on small case series. In addition, the biological responses of cells to specific types of ES remain incompletely understood. Therefore, it is important to develop simple, highly standardized and reproducible *in vitro* assays that will enable better understanding of the effects of various types of ES on human skin fibroblasts.

In this context, it is interesting to compare the response of normal human skin fibroblasts to ES with that of keloid fibroblasts. Keloids are scars that grow beyond the boundaries of original wounds (20–22) and exhibit an elevated collagen type I mRNA expression (23,24) and fibronectin synthesis (23,25), along with increased plasminogen activator inhibitor (PAI-1) expression (26).

In the current study, we hypothesized that different waveforms of electrical current can differentially modulate gene and protein expression (27), such as collagen I expression (28) in keloids. In addition, our aim was to identify a form of ES that effectively downregulates collagen I production in keloid fibroblasts but is non-toxic to normal skin fibroblasts. As ES is a major determinant of cell migration in tissue healing (5–8), various types of currents such as AC, DC and degenerate wave (DW, degenerating sine waves) with different amplitudes and frequencies were investigated on keloid fibroblasts in comparison with normal human skin fibroblasts. During ES, voltage-gated ion channels are the key membrane proteins that mediate voltage-evoked cell signalling (29–32).

The unique assay system presented here allows for the assessment of differential effects of specific types of ES on human fibroblast

gene and protein expression as well as cytotoxicity. In additon, it identifies DW as a promising, novel therapeutic strategy for suppressing excessive collagen I formation evident in keloid disease fibroblasts.

Materials and methods

Electrical stimulation (ES) apparatus

A simple and precise apparatus for ES was designed for delivering specific electric current to a monolayer of cells. A shallow rectangular chamber was made in a glass petri dish (Duroplan, Germany), the sides of which were made up of glass and the chamber interior coated with silicone resin. The internal dimensions of the chamber were 44 mm × 13 mm × 11 mm. Two 10-mm-diameter holes were drilled in the glass cover lid of the petri chamber for agar bridges to enter the chamber.

Agilent function generator (33320A, Agilent, UK) was used as the source for generating the required amplitude and frequency, which was subsequently connected to an amplifier (Prism, UK). The power supply for the amplifier (Isotech IPS 1603D for AC and DW, TT*i* EX752M for DC) was set to constant current and the voltage adjusted until the chamber reached the desired field strength. The voltage to the chamber was continuously observed by an oscilloscope (Tektronix TDS 301213, UK). Voltage was checked for consistency at different points inside the chamber with silver strips (Advent, UK) attached to the end of the leads from the oscilloscope. The silver strips were dipped in the cell-growing medium in the chamber (electrical setting details are given in Supporting Information Data S1).

Apparatus set-up for AC and DW stimulations

The required electric current was conducted into the chamber through Ag–AgCl electrodes inserted in synthetic rubber – agar bridges filled with Steinberg's saline gelled with 1% agar (Fisher Scientific, UK). The ES apparatus is demonstrated in Fig. 1a. For complete apparatus set-up and detailed technical descriptions of DW, please refer to Supporting Information Data S1. A schematic representation of AC, DC and DW waves are shown in Fig. 1b, c and d, respectively.

Apparatus set-up for DC stimulation

The inverted 'U'-shaped agar bridges conducted the required current from silver electrode–dipped Steinberg's saline solution into the ES chamber. Further description about the apparatus is given in Supporting Information Data S1.

Cell culture

Primary normal and keloid fibroblasts were used in all the experiments (Table 1). Cells were seeded in DMEM (PAA, Austria) supplemented with 10% foetal calf serum (PAA, Austria), penicillin (100 U/ml), streptomycin (100 U/ml), non-essential amino acids (1X, PAA Sigma, UK) and L-Glutamine (2 mM, PAA Austria). Cell passages used in the experiments were restricted between 3 and 6.

Cell growth surface

Large microscopic cover slips (22 mm × 50 mm) (Scientific Laboratory Supplies, UK) were divided into four cover slips (11 mm × 25 mm) and were termed as 'slides'. They were sterilized, coated with collagen (BD Biosciences, UK) at a concentration of 5 $\mu g/cm^2$ and cells were seeded at a density of 10 000/cm^2.

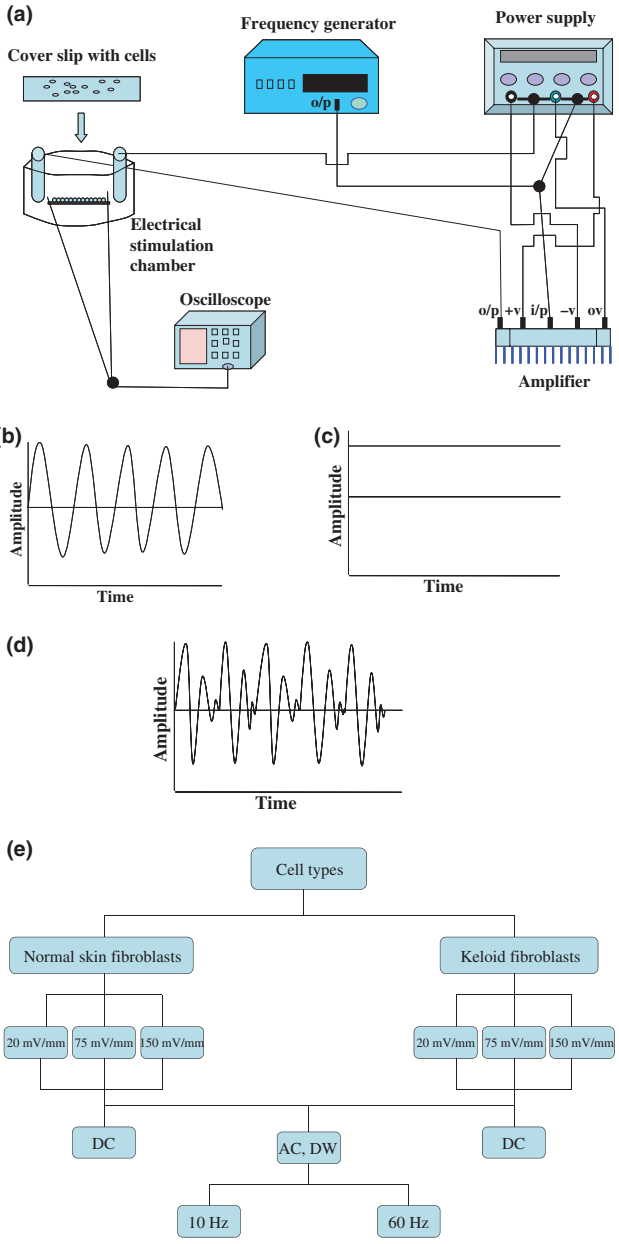

Figure 1. General electrical stimulation (ES) apparatus. (a) shows a schematic representation of the ES apparatus. Different types of waveforms applied are represented in (b) alternating current (AC), (c) direct current (DC) and (d) degenerate waves (DW), and (e) the mode of ES applied to different cell types.

Electrical stimulation (ES)

For the purpose of ES, six slides were placed in the chamber for a particular time point. We had 0, 1, 4, 8 and 12 h time points, of which the 0-h time point was before any ES. Before ES, 2.5 ml of DMEM medium containing 25 mM HEPES buffer (Lonza, UK) was added into the chamber. The agar bridges were introduced into the chamber and electrical connections were immediately set up for the customized current parameters (Fig. 1e).

Table 1. Primary cell sources

Primary cells	Patient	Age	Sex	Biopsy site	Duration (years)	Ethnicity	Treatment
Keloid fibroblasts	KS68	62	Male	Sternal	1	White	No
Normal fibroblasts	NS34	43	Female	Left cheek	–	White	Surgery

LDH (Lactate Dehydrogenase) cytotoxicity assay

One millilitre of cell growth medium from the ES chamber was collected at different time points (0, 1, 4, 8 and 12 h) for the assay. This assay was performed following the manufacturer's instructions (Cytotoxicity Detection kit, Roche Mannheim, Germany). The endpoint absorbance of the samples was measured at 490 nm using an ELISA reader.

RNA extraction, complementary DNA synthesis and qRT-PCR

Cells from three slides were extracted with 0.2 ml Trizol (Invitrogen, UK) and were further processed with RNeasy kit (Qiagen, UK) according the manufacturer's instructions. qScript™ cDNA SuperMix (Quanta Biosciences, MD, USA) was used for complementary DNA (cDNA) synthesis using manufacturer's protocol. Quantitative polymerase chain reactions (qPCR) were performed in real-time using the LightCycler®480 II platform (Roche Diagnostics GmBh, Germany), and corresponding LightCycler® (Roche Diagnostics, UK) 480 software release 1.5.0 (version 1.5.0 SP3, Roche Diagnostics) was used to amplify target genes for which 5 ng cDNA was used along with PCR primers (Supporting Information Fig. S2).

Enzyme-linked immunosorbent assay (ELISA)

Cell growth medium was collected at 0, 1, 4, 8 and 12 h for collagen I detected by ELISA. It is presumed that at 0 h, there is no secretion of collagen because this was a time point when the slides were just moved into the cell-growing ES chamber. Therefore, complete medium with HEPES was validated for collagen production at this time point. For all the other time points, 500 μl of medium was collected from the cell growth chamber for analysis.

The sandwich ELISA utilized rabbit anti-human polyclonal antibody (Abcam, UK), which was added to the surface coating buffer (AbD Serotec, UK). Additionally, a mouse anti-human monoclonal antibody (Abcam, UK) capture antibody was used against collagen I. TMB substrate (Thermo Scientific, USA) was used for developing the plates and the endpoint absorbance was measured at 450 nm.

Immunocytochemistry (ICC)

Immunocytochemistry for type I collagen was performed in 24-well plates with customized slides. The cells were incubated with rabbit anti-human polyclonal antibody (Abcam, UK) followed by Alexa 488 conjugated goat anti-rabbit secondary antibody (Invitrogen, UK). Rhodamine-phalloidin staining (Sigma-Aldrich, UK) for F-Actin and DAPI (Invitrogen, UK) staining for nucleus were followed.

Statistical analysis

Statistical significance was calculated using Student's *t*-test for distributed data groups. Potential differences amongst various groups were considered significantly different from others for $P < 0.05$.

Results

In this study, we have compared the effects of different types of ES (DC, AC, DW) on cultured normal human skin and keloid fibroblasts over time and at distinct electrical current amplitudes and frequencies with respect to:

(a) cytotoxicity, (b) collagen I gene and protein expression as well as (c) the transcription of genes involved in (i) the regulation of collagen production/deposition (ii) apoptosis and (iii) cellular activity during ES.

ES waveforms differentially affect keloid and normal fibroblast cytotoxicity and apoptosis

First, we asked whether ES has any deleterious effect on skin cells. This was studied by LDH cytotoxicity assay and API5 gene transcription. Apoptosis was increased by DC stimulation at higher and lower electric fields (Supporting Information Fig. S3) when compared to all other wave forms. At 150 mV/mm and 60 Hz, keloid fibroblasts subjected to DW stimulation underwent higher level of apoptosis than by AC stimulation (Supporting Information Fig. S3d2). The cytotoxic effects of high electric field stimulations by AC and DW on keloid fibroblasts were significantly higher than for normal cells. DW was demonstrated to be the least cytotoxic to normal fibroblasts when compared to all other stimulations.

Collagen I and PAI-1 gene expression are downregulated in keloid fibroblasts by AC and DW, with opposite effects for lower and higher electric field intensity and frequency

Amongst all forms of ES, collagen I gene expression in keloid fibroblasts was shown to be the highest by AC treatment at 20 and 150 mV/mm at 10 Hz (Fig. 2a1, c1). PAI-1 pathway was also upregulated by AC stimulation at 20 mV/mm and 10 Hz (Fig. 2a2). At 75 mV/mm and 10 Hz electric field, collagen I gene was increasingly expressed with time by DW and AC (Supporting Information Fig. S4c1). But when the frequency was increased to 60 Hz, the expression of collagen I gene in keloid fibroblasts was significantly downregulated, and this appeared more by AC than DW (Supporting Information Fig. S4d1).

During AC stimulation at 150 mV/mm and 10 Hz electric field, keloid fibroblasts exhibited the same collagen I gene expression pattern as in 20 mV/mm and 10 Hz. But collagen I gene was downregulated and apoptosis for keloid fibroblasts was increased

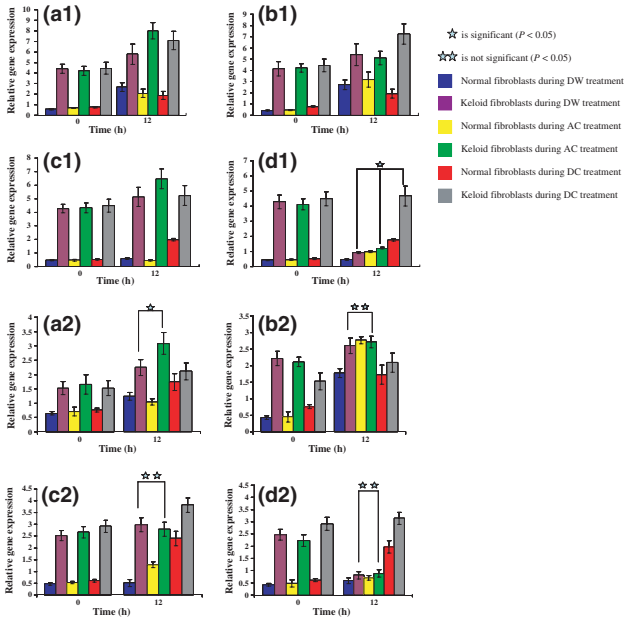

Figure 2. Collagen I gene expression by normal and keloid fibroblasts at 20 mV/mm and 10 Hz (a1), 20 mV/mm and 60 Hz (b1), 150 mV/mm and 10 Hz (c1) and 150 mV/mm and 60 Hz (d1), PAI-1 gene expression by normal and keloid fibroblasts at 20 mV/mm and 10 Hz (a2), 20 mV/mm and 60 Hz (b2), 150 mV/mm and 10 Hz (c2) and 150 mV/mm and 60 Hz (d2). At 20 mV/mm and 10 Hz electric field, collagen I gene was expressed more in keloid fibroblasts than in normal fibroblasts at 12 h by alternating current (AC) stimulation when compared to degenerate waves (DW) stimulation (Fig. 3a1). At higher frequency (60 Hz), both the normal and keloid fibroblasts exhibited the same level of collagen expression by AC and DW treatments (Fig. 3b1). At 150 mV/mm and 10 Hz electric field, the collagen gene in keloid fibroblasts was upregulated by DW and AC (Fig. 3c1) when compared with control keloid fibroblasts. Collagen I gene was downregulated and apoptosis for keloid fibroblasts was increased after 12 h upon AC and DW stimulations at 150 mV/mm and 60 Hz (Fig. 3d1).

when the frequency was increased to 60 Hz. This was also observed for DW (Fig. 3d1). Collagen I and PAI-1 syntheses were increased at 150 mV/mm by DC stimulation more than AC and DW for both normal and keloid fibroblasts. The activity of voltage-gated calcium ion channel gene showed a statistically significant difference ($P < 0.05$) when measured in keloid and compared to normal skin fibroblasts (Supporting Information Fig. S2a1). The gene expression increased with time ($P < 0.05$) for lower amplitude ES (Supporting Information Fig. S4a2, b2), whilst it was highly dependent on the frequency for higher amplitudes (Supporting Information Fig. S4c2–f2).

Keloid fibroblasts subjected to DW and AC stimulations at higher electric fields produce reduced levels of collagen I

Our ELISA results showed that at lower electric field of 20 mV/mm, collagen I expression by both cell types increased with electric field exposure time. However, there was higher synthesis of collagen I by keloid fibroblasts when compared to normal skin fibroblasts ($P < 0.05$) throughout ES as shown in Fig. 3a1, a2. Keloid fibroblasts had a decreased expression ($P < 0.05$) of collagen I at 75 mV/mm and 60 Hz by DW and AC stimulations, (Supporting Information Fig. S5a, b), when compared to stimulations at 10 Hz. This emphasizes the variation in gene expression at different frequencies, even at the same electric field strength. Normal and keloid fibroblasts increased the level of collagen production by all stimulations at 150 mV/mm and 10 Hz, when compared to cells without ES. But the effect was decreased when subjected to ES at 150 mV/mm and 60 Hz. Therefore, it appears that type I collagen synthesis is highly dependent on the applied frequency of ES.

Decreased synthesis of collagen I by keloid fibroblasts at higher electric fields using AC and DW was also confirmed by immunocytochemistry (ICC)

ICC was performed on ES samples at 150 mV/mm and 60 Hz because these samples varied significantly in the expression of collagen I during the ES period for 12 h. Collagen I expression in keloid fibroblasts was reduced by DW (Fig. 3b2) and AC application (Supporting Information Fig. S6a1) after 12 h when compared with normal skin fibroblasts (Fig. 3b1). Collagen I expression in normal fibroblasts and keloid fibroblasts increased after treatment with DC for 12 h (Fig. S6a2). This finding corroborates data from qRT-PCR experiments (Supporting Information Fig. S2).

Discussion

This study presents a novel way to establsish a simple, highly standardized *in vitro* assay that allows objective assessment of the effects of defined types of ES on human skin fibroblasts. We demonstarte that our unique assay meets these criteria and show that collagen 1, API5, PAI1 and voltage-gated calcium ion channel genes provide sensitive, biologically meaningful and quantifiable read-out parameters. Specifically, our new preclinical assay system can be employed to systematically characterize the differential effects of DC, AC and DW on human fibroblast gene and protein expression as well as on fibroblast cytotoxicity. Moreover, we demonstrate that normal human skin and keloid fibroblasts differ markedly in their responses to defined types of ES. Namely, we identify DW as a promising, novel therapeutic strategy for suppressing excessive collagen I formation in keloid disease.

We have shown that DW offer a form of ES that effectively downregulates collagen I production in keloid fibroblasts *in vitro*, without being toxic to normal human dermal fibroblasts. Furthermore, we demonstrate that collagen I gene and protein expression are dependent on the amplitude and frequency of specific applied currents within the normal electrical field range used for cutaneous wound healing (0–150 mV/mm).

During wound healing, cell migration is initiated by higher wound currents as the current of injury is higher immediately after wounding and slowly subsides as it heals (33). As cell migration occurs during the wound-healing process, a sustained DW or AC stimulation will be better than DC to keep the migrating cells viable. In our study, the keloid fibroblasts showed reduced collagen I expression after treatment with AC ($P < 0.05$) and DW ($P < 0.05$) stimulation at 150 mV/mm and 60 Hz than with DC stimulation at 150 mV/mm. LDH assay at this particular ES parameter proved cytotoxic for both cell types stimulated by AC and DW but was significantly cytotoxic to keloid cells than normal fibroblasts ($P < 0.05$) with only DW treatment (Fig. 2d). This finding demonstrated that DW was better than AC at higher electric fields.

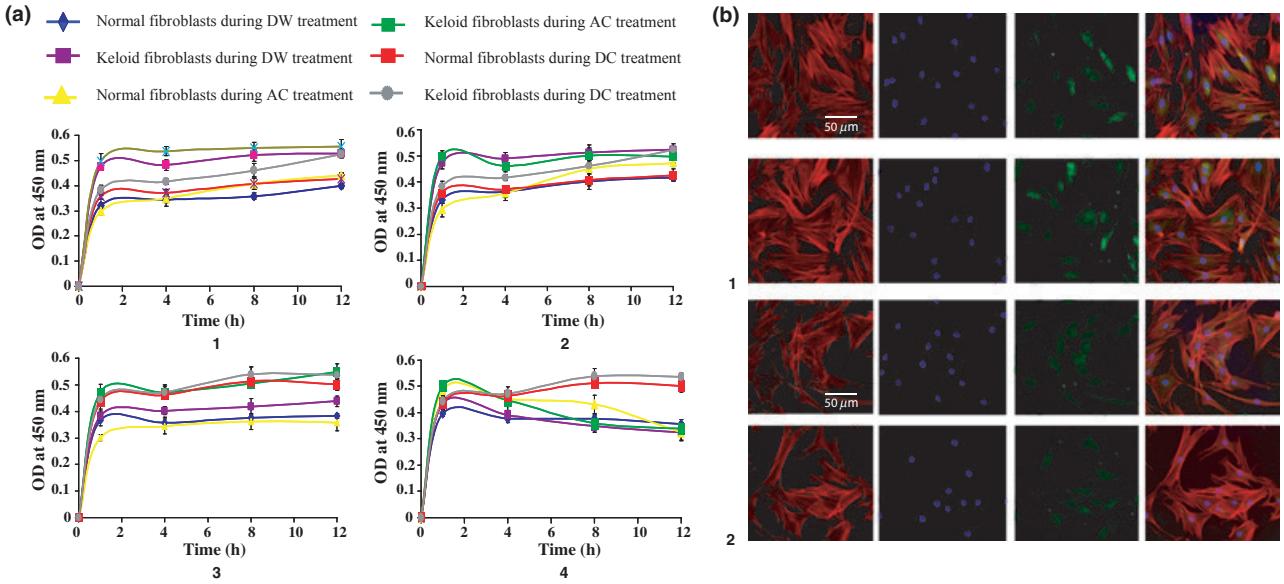

Figure 3. Collagen IA expression by normal and keloid fibroblasts at 20 mV/mm and 10 Hz (3a1), 20 mV/mm and 60 Hz (3a2), 150 mV/mm and 10 Hz (3A3) and 150 mV/mm and 60 Hz (3a4) by degenerate waves (DW), alternating current (AC) and direct current (DC) stimulations. Collagen I production was decreased by AC stimulation at 20 mV/mm and 60 Hz when compared with 10 Hz, whilst during DW stimulation there was no significant change. Collagen I production after 1 h was significantly higher for AC when compared to DW ($P < 0.05$) at 150 mV/mm and 60 Hz. At 12 h, there was a significant decrease in collagen I production by keloid fibroblasts when compared with normal fibroblasts by DW ($P < 0.05$) than by AC stimulation ($P < 0.05$). Immunocytochemistry of normal and keloid fibroblasts exposed to 150 mV/mm and 60 Hz for 12 h is shown in Fig. 3b. Red fluorescence is F-actin staining by rhodamine-phalloidin, green fluorescence is collagen-1 staining by Alexa 488 and blue fluorescence is DAPI staining of the nucleus. (3B1) shows normal (top) and keloid (bottom) fibroblasts without any ES (untreated). (3B2) shows normal (top) and keloid (bottom) fibroblasts exposed to DW waves. When compared to untreated keloid fibroblasts, the green fluorescence of keloid fibroblasts after DW has been significantly reduced. Immunocytochemistry (ICC) with collagen I antibody showed the overexpression of collagen I in keloid than in normal fibroblasts under normal conditions (Fig. 3b1).

There are different upstream proteins that guide the production of collagen I in wound healing. Plasminogen activator (PA) pathway is one amongst them, by which plasmin activates pro-collagenase into collagenase that results in the production of collagen I (34). Our qRT-PCR experiments showed that PAI-1 expression in keloid fibroblasts is four- to fivefold higher than normal fibroblasts and the application of customized AC and DW can bring down the expression of PAI-1. The expression profile of PAI-1 was analysed at mRNA level for keloid and normal fibroblasts, which showed its direct correlation with the qRT-PCR results and ELISA for collagen I (Figs 2a1-2d1 and 3a).

The verified gene expression changes in this report could be indicative of migratory activity of fibroblasts (35,36), although migration was not analysed here. The gradual elongation of cells in DC could be an indication of cell migration by ES. Future studies could include performing gene expression microarray profiling on relevant wound-healing genes subjected to ES with specific currents. Different genes involved in wound healing could be identified by microarrays even though there are limitations regarding data interpretation of the biological subsets. The pathway for collagen I synthesis in keloid fibroblasts could be further elucidated with the *in vitro* model, with providing further insight into the downregulation of specific proteins. As keloid tissue is different from a monolayer of keloid cells, in the absence of animal models of keloid; further optimization is needed with keloid organotypic cultures for demonstrating the effect of DW on collagen production.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Apparatus set-up for AC and DW stimulations.

Figure S1. General electrical stimulation (ES) apparatus.

Figure S2. Primers used for qRT-PCR.

Figure S3. LDH cytotoxicity assay for normal and keloid fibroblasts.

Figure S4. Collagen 1, Voltage gated ion channel, PAI-1 and API5 gene expression by normal and Keloid fibroblasts.

Figure S5. Collagen IA expression by normal and keloid fibroblasts.

Figure S6. Immunocytochemistry of normal and keloid fibroblasts.

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