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In vitro Evaluation of a Surface Acoustic Wave Device on Epidermal Characteristics

Jonathan Rosenblum[1*], **David Friedman**[1], **Roslyn Rivkah Isseroff**[2] **and Farzam Gorouhi**[3]

[1]*Israel Amputation Prevention Center, Bet Shemesh, Israel*

[2]*Department of Dermatology, Dermatology Service Department of Veterans Affairs, Mather CA, University of California, Davis, USA*

[3]*CIRM Clinical Fellow at University of California, Davis, USA*

[*]**Corresponding author:** Jonathan Rosenblum, Israel Amputation Center, Jerusalem, Israel, Tel: 7207443222; E-mail: diabfootman@gmail.com

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Abstract

The authors evaluate a novel Surface Acoustic Wave (SAW) device as to its effect on epidermal health. The SAW device is indicated for use in phonophoresis as well as epidermal tissue growth in wound care. In order to understand the clinical effect the authors evaluate an *in vitro* skin model for SAW effect and examine the cellular and subcellular changes that occur with this device. CK17 proliferation, changes in GAG's (Glycosaminoglycans) and neoangiogenesis were positively affected by the SAW device.

Keywords: Ultrasound; Surface acoustic wave; Cell proliferation

Introduction

Ultrasound (US) is a sound wave with a frequency of greater than 20 KHz. It is generated by a transducer that delivers an alternating electrical current to a piezoelectric crystal. It can ultimately generate a wavelength that makes the molecules vibrate. It is primarily used as a diagnostic modality such as in prenatal medicine. It also has therapeutic indications such as destroying a kidney stone [1] or facilitating liposuction [2]. High frequency US can be used to treat tendon injuries [3]. Unfortunately, conventional US treatment at high intensity can cause skin and vascular damage, leading investigators to search for alternative settings US [4]. Hence, it was hypothesized that low intensity US can act as a slow action modality and will make less tissue damage and comparable tissue repair [5]. US with frequencies of less than 120 KHz and intensity of less than 1 W/cm^2 is considered "low intensity" US. It cannot penetrate deeper than the soft tissue underlying the skin [5]. Low intensity pulsed US can further decrease the heat formation and tissue destruction. Thus, low intensity pulsed US can be a safer modality for skin regeneration [6].

US have been used as a non-invasive facial rejuvenation modality [7]. The literature on low intensity US treatment is growing and needs a better understanding of its functions at cellular levels. *In vitro* and *in vivo* reports demonstrate partial or complete effect of this technique *via* enhancing collagen formation, cell proliferation and inflammatory response [8-12]. On the other hand, Lowe and co-workers failed to show any effect of low intensity US in the treatment of radiation induced wound [13]. Surface Acoustic Waves (SAW) is at the lowest end of the ultrasound spectrum with a frequency of 96 kHz and intensity of 0.4 W/in^2. Previously the limiting factor of devising a low intensity ultrasound unit was that the generator needed to be very large. NanoVibronix Inc. has developed a low intensity low frequency ultrasound, surface acoustic wave device that can be used in a patch form and the generator is small enough to fit in a pocket.

In this study, we explored the effects of such low intensity US on epidermal activity and proliferation in skin explants.

Materials and Methods

Skin explant preparation

Twenty four Viable human skin explants from same donor (cosmetic abdominoplsty from 36 year old Caucasian woman) to maintain homogeneity were obtained from a commercial source (Bio-EC; Clamart, France). The explanted skin was transported to the laboratory in a sterile container on ice within an hour post-operation, where it was cut into twenty four (1 cm X 1 cm) explants. Each explant was placed in a well of a 6 well culture plate and maintained in specific survival medium BEM (Bio-EC's explant medium) containing: Hank's buffered salt solution (HBSS).

US stimulation

The US box was fitted beneath each six well tissue culture plate with connections to the center of each well that contains a skin explants. The US box and the corresponding 6 well plate will form the NanoChambers (NanoVibronix inc. Elmsford, NY, USA).

Ultrasound stimulation was performed at 89 kHz ultrasound wave; pulse modulated with an output intensity of 0.4 mW/cm^2 on days 1, 2, 3, 6, 7, 8, and 9.

In the first experiment, skin explants were treated with either continuous or pulsed US therapy, and compared to positive control explants treated with Retinol (ROC company, France, daily) or left untreated (Table 1). In the second experiment, pulsed US therapy were compared with untreated control on days 0 and 10 (Table 1). The US treated explants were placed in Nano Chambers and were treated according to the protocol. At the end of the ten day period, samples were taken from the explants and were evaluated for the histology and immunostaining.

Masson's trichrome staining and epithelial thickness measurement

Formalin fixed samples were refixed in Bouins solution at room temperature and embedded in paraffin. Paraffin embedded samples were cut in 6 µm thick sections and stained according to Masson's trichrome staining protocol. [14] The thickness of the epidermis was measured using the measure module of the LEICA Image Manager (IM1000) software. For each treatment group, 6 to 7 images were analyzed and 4 different areas were measured on each image.

First Experiment (Figure 1)	I. Untreated, negative control, tissues were fixed on day 0 (UT0) II. Untreated, negative control, tissues were fixed on day 10 (UT1) III. Positive control of Roc® Retinol therapy for 10 days (R) IV. Continuous US therapy for 10 min daily for 10 days (US1) V. Pulsed US therapy for 6 min (3 two min) in a 12 min period with 2 min intervals daily for 10 days (US2)
Second Experiment (Figures 2-4)	I. Untreated, negative control, tissues were fixed on day 0 (UT0) II. Untreated, negative control, tissues were fixed on day 10 (UT1) V. Pulsed US therapy for 6 min (3 two minutes) in a 12 min period with 2 min intervals daily for 10 days (US2)

Table 1: Treatment groups.

Immunostaining

Immunostaining was done on 5 µm thick sections. Basal keratin 14 was visualized on frozen sections with an anti- keratin 14, monoclonal anti-body on mouse, with biotin/streptavidin amplifier system, revealed in FITC with nucleus post stained with propidium iodide. Ki67 positive cells were counted on frozen sections with an anti-Ki67, mouse monoclonal anti-body, with biotin/streptavidin amplifier system, revealed in VIP (alternative to DAB). The primary antibodies were omitted in controls. Microscopic observations were performed by optical microscopy with a Leica type DLMB microscope with a 40× magnification. Photos were taken using a CCD Sony DXC 390P camera. These images were stored and analyzed with Leica IM1000 image manager software.

Statistical analysis

P values less than 0.05 were considered as significant. Continuous variables were presented as the means ± SD and were tested for normality by Kolmogorov Smirnov test. Therefore, non-parametric test (Mann Whitney U test) was used in this regard.

Results

Epidermal thickness was increased significantly in skin explants that received pulsed low intensity US therapy (group V) as compared to the untreated controls (92.43 ± 16.08 µm *vs.* 52.46 ± 3.81 µm, $p<0.05$, Figure 1a-e). The treated and untreated groups showed 109.8% and 19% increase, respectively, in epidermal thickness as compared to the baseline. Keratin 14 positive basal cells were also significantly increased in the explants that received the pulsed US therapy (group V) (27.77 ± 6.35 *vs.* 15.52 ± 3.20, $p<0.05$, Figure 2a-d). Figure 3 represent the results of the number of cells in mitosis per centimeter of surface of epithelium between the treated group and the untreated control. Ki67 positive cells were not increased in the group V (data not shown). Acid GAGs (glycosaminoglycans) were decreased in US therapy and untreated control groups but neutral GAGs were only increased in US therapy group Figure 4a-c.

Discussion

The literature on low intensity US is not conclusive for therapeutic effects in bone repair, cartilage proliferation or wound healing. Cullum et al. [4] found 8 studies that compared ultrasound therapy with no ultrasound therapy for venous ulcers. All eight papers had significant biases, with different regimens of ultrasound. Only two of them applied low intensity ultrasound to the wounds. Based on this systematic review, there is no solid evidence that low intensity ultrasound improves healing of venous ulcers. The reviewers pointed out that all included trials were underpowered and had biased designs.

In our analysis of effects of US on explanted skin, we found significantly more keratin 14 positive cells in the pulsed US group when compared to the untreated controls. CK14 is expressed in basal epithelial cells [15] indicating precursors for cellular division. To the best of our knowledge, there is no similar study to investigate the number of CK14+ cells after US therapy.

In the present study, we found a decrease in KI67, a marker of cell proliferation, in the US treated explants. This is puzzling in view of the increase in epidermal thickness, and increase in basal keratinocytes in the treated explants. Indeed, the literature on the effects of US on cell proliferation is contradictory. In some systems (examples here) US has been found to decrease cell proliferation, while Monici et al. showed a stimulatory effect of low intensity short term US on Ki67 protein expression levels of osteoclasts that would ultimately increase the bone repair [16]. There are few more papers in support of low intensity US ability to trigger inflammation, and cellular proliferation in different settings. The extracellular regulated kinase (ERK) to c-Jun N-terminal kinase (JNK) ratio is significantly higher in fibroblasts that were treated with US [17]. Low intensity pulsed US is proven to be effective in fibroblast proliferation [17] *via* Rho/ROCK/ERK1/2 pathway [8]. Hill et al. studied the effects of low intensity pulsed US on epithelial cell monolayers *in vitro* [18]. They noted no difference in cell growth as well as cell migration and suggested that the US on wound healing could be indirect mechanism of action by modulating extracellular matrix composition and/or production of paracrine stromal agents.

Furthermore, low intensity pulsed US increased the thickness of the skin explants in our study. This could be another indication of increased proliferative response. Collagen synthesis and proliferation is increased in fibroblasts after treating the cells with 1 MHz (0.1-0.4 W/cm^2). Additionally, US had stimulatory effect on production of many angiogenetic and/or growth factors such as VEGF, and FGFβ in osteoblasts [19]. The same study note increased cell proliferation in cells treated with lower US intensities as opposed to higher intensities (47% *vs.* 37% increase in fibroblast proliferation with 0.7 W/cm^2 and 1 W/cm^2, respectively [19]. Lower intensity of US (0.1 W/cm^2, with 0.75 MHz and 3 MHz frequencies) is associated with improved early wound healing in an *in vivo* model, ascribed to an concomitant increase in the number of fibroblasts in the wound at day 5 [9]. Lipoperoxidation and collagen synthesis were investigated in a rat wound healing model [11].

Hydroxyproline levels as a predictor of collagen production were increased and levels of thiobarbituric acid-reactive substances as an index of lipid peroxidation were decreased with pulsed low intensity US (0.4, 0.6 and 0.8 W/cm^2, 1 MHz, 3 min) [11].

Our study noted an increase in the number of cells expressing neutral GAGs only in US group. GAGs are one of the responsible molecules for skin turgor and elasticity and its maintained or increased levels can prevent wrinkle formation in skin. Vogel et al. [20] mentioned that the viscosity of skin is correlated with the content of glycosaminoglycans. Increased neutral GAG levels is an indication of stronger cell attachment in basal layer. There is no similar study in the literature to support or contradict our finding in this regard.

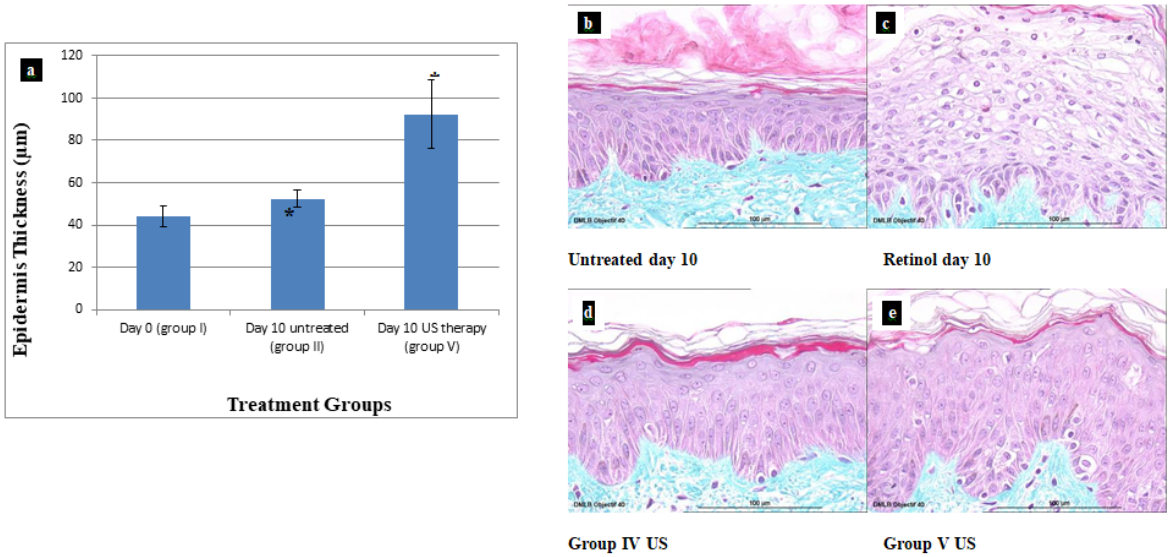

Figure 1: a. Epidermal thickness of skin explants were measured by using LEICA 1000 software on day 0, and day 10 in untreated and US treated groups. Panels b, c, d, and e demonstrate Masson's trichrome staining of samples from untreated, retinol, continuous US and pulsed US groups, respectively.

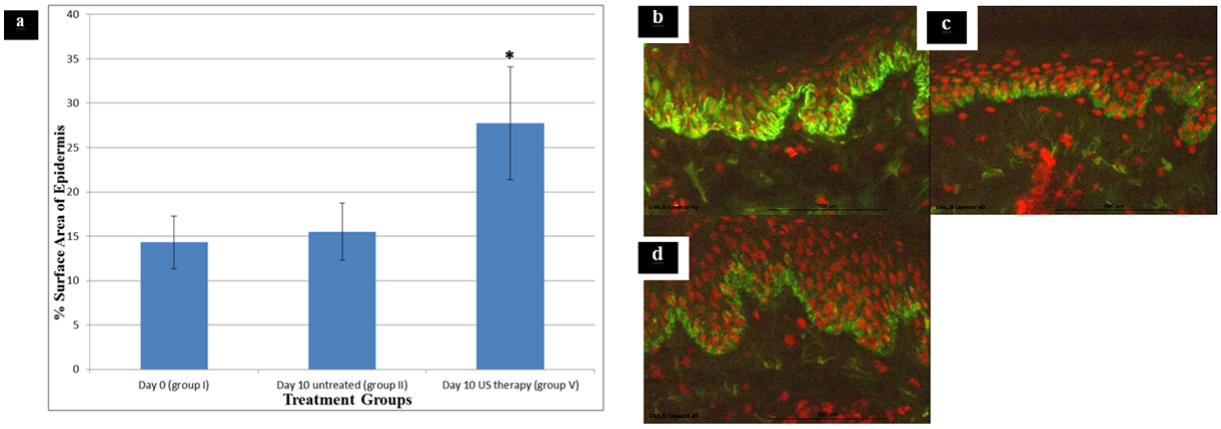

Figure 2: The percentage of surface area that was stained for CK14 per field averaged in 6 different fields per group (a), Immunostaining of skin explants with anti-CK14 antibody at baseline (b), untreated group after 10 days (c) and US group after 10 days (d).

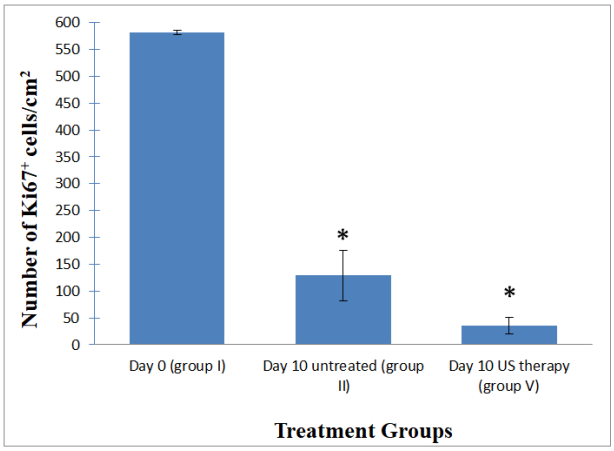

Figure 3: Number of Ki67 positive cells per cm^2 (All three groups were significantly different from each other, $p<0.05$).

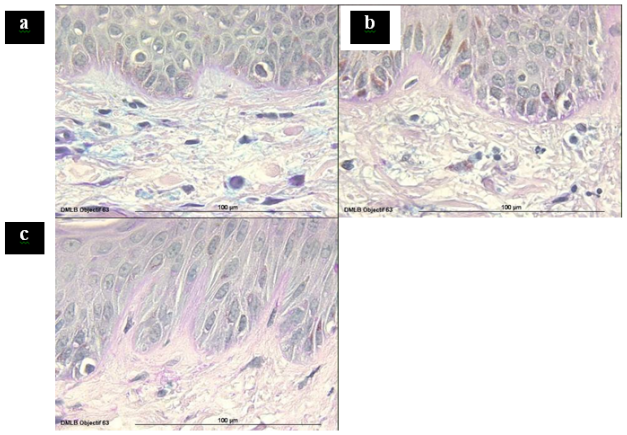

Figure 4: Glycosaminoglycan immunostaining at baseline (a), untreated group after 10 days (b) and US group after 10 days (c).

In an informative study, 167 genes were identified to be mainly upregulated in response to low intensity US. This includes integrins and cytoskeleton genes, TGF-β family genes, IGF family genes, MAPK and AKT genes and apoptosis related genes [21]. These findings warrant more comprehensive experiments including proteomics methods to reveal more details of US molecular mechanisms.

The present study shows an increased activity in the basal layer of the epidermis as demonstrated by increased number of CK14+ and GAG+ cells in this layer. Moreover, pulsed US treatment increased the epidermal thickness in the explants.

These findings need to be confirmed with thorough *in vivo* dose-response models in addition to well-designed phase II and III randomized controlled trials.

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