A DOSE-RESPONSE STUDY OF A NOVEL NON-THERMAL METHOD OF SELECTIVELY MODIFYING CELLULAR STRUCTURES IN SKIN WITH LOW ENERGY NANOSECOND ELECTRICAL STIMULATION

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BACKGROUND AND OBJECTIVES
Pre-clinical studies using Nano-Pulse Stimulation (NPS) technology have demonstrated the ability to stimulate a lasting immune response in animal models of tumor treatment, including melanoma. The objective of this first controlled human study is to evaluate the dose response effects of the NPS device on normal skin and subcutaneous structures and establish a safe dose range for use in future clinical applications.

STUDY DESIGN AND METHODS
Initially, five patients with healthy tissue planned for abdominoplasty excision were enrolled in a 60-day study to evaluate the effects of a unique method for modifying tissue using low energy, high voltage NPS. A total of 30 squares of 25mm² or less within the planned excision area were treated in 5 sets of 6 squares on the following days prior to surgery: 60 days, 30 days, 15 days, 5 days and 1 day. Six progressively higher NPS energy levels were applied and compared across all time points. Five different staining methods were used to assess tissue changes over time. Two patients were treated subsequently under a similar protocol with a longer follow-up (90 days) and additional histology analysis 2 hours and 4 hours after NPS treatment to further evaluate the cellular changes prior to 24 hours, and healing beyond 60 days.

RESULTS AND CONCLUSIONS
The majority of the original 150 test sites exhibited delayed epidermal loss and remarkably low levels of inflammation followed by re-epithelization by Day 15 and a normal course of healing by day 60. Histologic analysis identified a nucleolysis effect (“ghost cells”) of epidermal cells evident at 1 day post-treatment. Characteristic histologic and clinical effects, such as minimal long term changes in melanocyte counts, elastin density and collagen quality when compared with controls at day 60, were observed in the majority of evaluated tissue samples, with notable exceptions of 2 tested areas. These two areas, treated at the highest energy level, showed signs of collagen damage at 60 days. The selective effect of NPS on cellular structures in the epidermal and dermal layers suggests a non-thermal mechanism for targeting cellular structures that spares non-cellular dermal tissue within a range of energy levels.

Positive Caspase-3 staining at 2 and 4 hours post-NPS treatment precedes the appearance of ghost cells at 24 hours, thus confirming programmed cell death (PCD).

The histology results indicate that the lowest effective energy level for NPS was not established in this study. This observed specificity for cellular structures in the epidermal and dermal layers of the skin across all energy levels tested suggests a unique profile for targeting common benign and non-benign lesions with minimal damage to the dermis. The novel cellular mechanisms of NPS demonstrated in clinical and pre-clinical studies warrants further evaluation in a wide variety of skin conditions.

CAUTION: Nano-Pulse Stimulation (NPS) is an investigational use therapy.
Methods and Study Design

Subjects
5 Subjects were recruited and treated at 5 visits: 60, 30, 15, 5 and 1 day prior to their scheduled abdominoplasty. At each visit, 6 different energy levels (“doses”) were used on a defined skin location (TL1-TL6; Figure 1).

NPS Treatment Protocol
The skin was numbed locally with 2% Lidocaine. Pain was recorded on a standard visual analog scale of 0-10. Photographic documentation was acquired at each visit (Figure 2). Photographs were assessed by three qualified, blinded independent reviewers (blinded to time point and to treatment parameters). Assessments were made for erythema, flaking and crusting on a 5 point scale (0-4, 0 being low and 4 being high) for 150 treated areas across all subjects.

Histopathology
Harvested skin was stained with five different immunostaining methods: H&E, Trichrome, Caspase 3, MITF, and Elastin. Microscopic samples were evaluated by a qualified dermatopathologist to assess tissue changes.

Results

Clinical Assessment
The pain score across all treatment levels (TL) ranged from 0 to 7 in the 5 subjects, with a median pain score of 0 (0-10 Point Scale).
All wounds healed without exudate or discomfort to the patient. No signs of infection were present and no adverse events occurred.

Blinded Photographic Assessment
Low grade crusting and flaking were scored for 5 subjects across all time periods and treatment levels.
At 60 days post-treatment, slightly higher erythema scores for the 2 highest energy levels indicated incomplete healing.

**Results**

**Histopathology**

- At 1 day post-treatment (Post-Tx, Figures 3 & 4), efficacy in achieving non-viable epidermis with evidence of unique “ghost cells” (intact cell membrane with absence of stained nuclei) along with nearly complete loss of melanocytes and minimal inflammation was seen despite complete epidermal destruction.
- At study end, the epidermis, collagen, elastin fiber density and orientation all appeared normal and similar to controls with exception of TL6 samples (TL6 determined to be near upper threshold for some patients).
- Impressive recovery of normal melanocytes (at or approaching control levels) by study end (Figure 5).

*Figure 3: Photomicrographs (H&E) capturing epidermal and dermal wound progression, TL4*

<table>
<thead>
<tr>
<th>Control</th>
<th>1 day Post-Treatment</th>
<th>7 days Post-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Control</td>
<td>“Ghost cells”</td>
<td>Original necrotic epidermis separating</td>
</tr>
<tr>
<td>Epidermal cells with dark nuclei</td>
<td>Non-viable epidermis</td>
<td>New epidermal layer, healthy nuclei</td>
</tr>
<tr>
<td></td>
<td>Minimal inflammation</td>
<td>Minimal inflammation</td>
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*Figure 4: Epidermal Integrity (H&E)*

Example of TL3: Day 1 Post Treatment shows non-viability of most epidermal cells. Day 15 post treatment almost completely normal epidermis seen.

*Figure 5: Melanocyte Density (MITF)*

Loss of melanocytes for all treatment levels (T11-TL6). By 60 days post treatment, the number of melanocytes return to normal density, comparable to controls.

Evidence of Programmed Cell Death (PCD)

- Two additional subjects were evaluated at earlier timepoints (2 and 4 hours) for Caspase-3 presence across a range of energy settings.
- Active Caspase-3, a marker for PCD, is detected at both 2 hours and 4 hours post-NPS treatment, and dissipates by 24 hours post-treatment.
- This is the first evidence of NPS-activated Caspase-3 in humans, confirming prior results observed in vitro.
- PCD is postulated as a mechanism for subsequent non-viable “ghost cells” seen at 24 hours.
- Prior observations in pre-clinical models of lasting immune response after NPS treatment of malignant tumors may be triggered by PCD.

**Figure 6: Caspase Staining**

Presence of active Caspase-3 (brown stain), two hours and four hours post-treatment. Active caspase dissipates by 24 hours post-treatment.

<table>
<thead>
<tr>
<th>2 Hours Post Treatment</th>
<th>4 Hours Post Treatment</th>
<th>24 Hours Post Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 = Clear Presence (&gt;50% treated area)</td>
<td>1 = Faint Presence (10-50% treated area)</td>
<td>0 = No Presence (0% treated area)</td>
</tr>
</tbody>
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- Viable epidermal cells with dark nuclei
- “Ghost cells” (Non-viable epidermal cells)
Summary of Findings

- NPS demonstrates selective activity on cellular structures in skin and subcutaneous tissue at all tested energy levels.
- Detection of active Caspase-3 within hours of NPS treatment is evidence of PCD mechanism.
- The appearance of “ghost cells” in the epidermis is present for all tested energy levels 1 day post-treatment, indicating the achievement of non-viable epidermal cells (Figure 8).
- New and “normal” epidermis is evident after 1 week beneath the non-viable crusted layer of the originally treated epidermis.
- There is a loss of melanocytes for all treatment levels 1 day post-treatment. By 60 days post-treatment, the number of melanocytes return to normal density, comparable to controls (Figure 9).
- Low dermal inflammation scores and lack of fibroplasia suggest dermal sparing property of NPS.
- By study end, histology analysis shows normal epidermis, collagen, elastin fiber density and orientation for the majority of treated areas.
- The increased tissue response scores correlate with longer healing time at higher settings (Figure 7).
- These findings are basis for “dose” selection in future clinical trials for benign and non benign lesion treatment.

NPS Mechanism of Action
NPS stimulates delayed cell destruction, characterized by “ghost cells” (nucleolysis effect) indicating non-viable epidermal cells, very low grade inflammation and sparing of the non-cellular dermal structures.

Prior Publications using NPS technology