Two years of Functional Electrical Stimulation by large surface electrodes for denervated muscles improve skin epidermis in SCI

Giovanna Albertin (1), Helmut Kern (2,3), Christian Hofer (2), Diego Guidolin (1), Andrea Porzionato (1), Anna Rambaldo (1), Raffaele De Caro (1), Francesco Piccione (4), Andrea Marcante (4), Sandra Zampieri (3,5,6)

(1) Department of Neuroscience, Section of Human Anatomy, University of Padova, Italy; (2) Ludwig Boltzmann Institute of Electrical Stimulation and Physical Rehabilitation, Vienna, Austria; (3) Physiko- und Rheumatherapie, St. Poelten, Austria; (4) IRCCS Fondazione Ospedale San Camillo, Venice, Italy; (5) Interdepartmental Research Center of Myology, Department of Biomedical Science, University of Padova, Italy; (6) A&C M-C Foundation for Translational Myology, Padova, Italy

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Abstract

Our previous studies have shown that severely atrophic Quadriceps muscles of spinal cord injury (SCI) patients suffering with complete conus and cauda equina lesions, and thus with permanent denervation-induced atrophy and degeneration of muscle fibers, were almost completely rescued to normal size after two years of home-based Functional Electrical Stimulation (h-bFES). Since we used large surface electrodes to stimulate the thigh muscles, we wanted to know if the skin was affected by long-term treatment. Here we report preliminary data of morphometry of skin biopsies harvested from legs of 3 SCI patients before and after two years of h-bFES to determine the total area of epidermis in transverse skin sections. By this approach we support our recently published results obtained randomly measuring skin thickness in the same biopsies after H-E stain. The skin biopsies data of three subjects, taken together, present indeed a statistically significant 30% increase in the area of the epidermis after two years of h-bFES. In conclusion, we confirm a long term positive modulation of electrostimulated epidermis, that correlates with the impressive improvements of the FES-induced muscle strength and bulk, and of the size of the muscle fibers after 2-years of h-bFES.

Key Words: spinal cord injury, denervated-degenerating muscle, long-term effects, h-bFES, skin biopsy, morphometry of epidermis

Materials and Methods

Histologic morphometry of skin biopsies

The harvested skin biopsies were fixed in 10% buffered formalin and embedded in paraffin. Histological sections of 5 μm thickness were then collected and stained with immunohistochemical procedures to analyze in the skin biopsies some biomarkers (see below). Three sections per sample were considered for the analysis and from each measurements of the area of epidermis in cross sections of the skin harvested from both legs of 3 SCI patients before and after two years of h-bFES. Morphometric analyses of the area of epidermis determined in skin cross sections before and after two years of h-bFES support our recently published evidence of an increase of skin thickness randomly quantified in the biopsies.
transverse section 3-5 digital images were collected at a primary magnification of x10 using a light microscope (B-293 PLi, Optika Srl, Ponteranica, BG, Italy) mounting a HDMI camera (Optika 4083.13E HDMI Easy camera). Image of 1024x576 pixel size were then captured and stored as TIFF files on the workstation associated with the instrument. Care was taken to accurately align the epidermal layer parallel to the largest side of the frame before acquiring each image. Images were analyzed by using the ImageJ software (available at http://rsb.info.nih.gov/ij/).\textsuperscript{16} The boundary of the epidermal layer, from the outermost surface of the epidermis (but excluding the stratum corneum) to the dermo–epidermal junction, was interactively traced to estimate its area. One example is presented in figure 1.

**Immunohistochemistry**

Immunological characterization was performed by Dako Autostainer/Autostainer Plus (Dako, Milan, Italy) with the following antibodies diluted in EnVision™ FLEX: S100 diluted 1:4000 (Z0311 anti-S100 Dako), CD1a 1:100 (M3571 anti-human CD1a Dako) and TUBB3 1:5000 (poly18020 BioLegend). Every section was incubated with peroxidase-blocking serum (EnVision™ FLEX Peroxidase-Blocking Reagent; Dako) for 5 min to remove unspecific bindings and for 30 min with the primary antibody. After, it was used a labeled polymer (EnVision™ FLEX /HRP, Dako) for 20 min and 3,3'-diaminobenzidine (EnVision™ FLEX Substrate buffer, + DAB + Chromogen; Dako) in order to show the positivity of the reactions, that is display the primary antibody binding. All the sections were finally counterstained with hematoxylin (EnVision™ FLEX Hematoxylin, Dako) for 5 min to reveal the presence of nuclei, dehydrated with a increasing scale of alcohol solutions (70%, 95%, 99%), cleared with xylene and mounted. These steps were performed at RT.\textsuperscript{17}

**Statistical analyses**

Statistical analyses were performed by using GraphPad Prism 5.0 software (GraphPad software, La Jolla, CA, USA). Area values from each biopsy were first averaged to obtain a representative value for each leg and the values before h-bFES and those observed (on the same legs) after two years of h-bFES were then compared by paired Student’s t-test. Unpaired Student’s t-test comparing leg-by-leg the set of measurements before h-bFES with the one obtained after h-bFES was also applied. Results are reported as absolute area in µm$^2$, and as $\Delta$%, i.e., the percent difference comparing before and after 2-years of h-b FES. The limit for statistical significance was always considered p<0.05 (Table 1).

**Results and Discussion**

The twelve skin biopsies here analyzed were harvested from both the right and left thighs of 3 SCI patients before and after approximately two years of h-bFES. All the twelve biopsies presented with a regular stratified squamous epithelium rich in cells. The epidermis, consisting of several layers of cells, was distinguishable from the underlying dermis and exhibited a regular and consistent basal membrane. The H-E staining revealed
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Table 1. Area of epidermis (µm²) in transverse sections of thigh skin biopsies. Skin biopsies were harvested from denervated SCI-patients before and after 2 years of home-based functional electrical stimulation (h-bFES, 5 days per week) using large surface electrodes and a purpose-developed electrical stimulator. The column of “Years from SCI” shows the number of years from SCI to the beginning of h-bFES.

<table>
<thead>
<tr>
<th>Years from SCI</th>
<th>Before FES</th>
<th>After FES</th>
<th>Δ %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subjects</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>39558±6074</td>
<td>54953±14034</td>
<td>+39</td>
</tr>
<tr>
<td>1</td>
<td>Right</td>
<td>37143±8044</td>
<td>49154±10821</td>
<td>+32</td>
</tr>
<tr>
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<td>Left</td>
<td>39683±2682</td>
<td>66070±8530</td>
<td>+66</td>
</tr>
<tr>
<td>3</td>
<td>Right</td>
<td>42780±2512</td>
<td>54139±4544</td>
<td>+26</td>
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<tr>
<td></td>
<td>Right</td>
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<tr>
<td></td>
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<td>42940±14239</td>
<td>42010±9780</td>
<td>-2</td>
</tr>
<tr>
<td></td>
<td>ALL-After</td>
<td>53477±10378</td>
<td>41062±7404</td>
<td>+30</td>
</tr>
</tbody>
</table>

Results are reported as absolute area in µm², and as Δ%, i.e., the percent difference comparing before and after 2-years of h-b FES. The limit for statistical significance was always considered p<0.05. The data in bold are significant or highly significant.

the various layers of keratinocytes with small, nucleated basal cells (see Figure 1. in Albertin et al. 2018). The surface layer is the stratum corneum which sloughs away naturally and is evenly colored, thus it was not included in the measured cross area of the epidermis. Although some samples shown the skin organization as having ridges and papillae, in most samples the epidermis appears thinner and flattened, in particular in the skin biopsies harvested before h-bFES, that is two years after SCI. We performed the skin morphometry, measuring the total area of epidermis in transverse sections of skin biopsies (Figure1), by acquiring ImageJ pictures. Each skin biopsy taken before h-bFES was evaluated versus its corresponding post h-bFES biopsy. Despite the heterogeneity of gender and time from SCI of these three cases, the results in Table 1 show that the two SCI persons have a significantly increased epidermal area after two years of electrical stimulation. More important, the grouped statistical analysis of the epidermal area in the three subjects show a significant 30 % increase after h-bFES (Table 1). The skin is a sensory organ that protect the internal body from chemical, physical and biological insults and several disorders may affect epidermis and dermis due to physical, chemical, metabolic and genetic factors. Different studies have shown that electrical stimulation has positive effects, specifically on wound closure. Based upon this evidence we were encouraged to study the effects of functional electrical stimulation on the skin of patients with spinal cord injury (SCI), specifically in persons suffering with permanent denervation of the leg muscles as the result of conus and cauda equina complete lesion. Earlier, we demonstrated by sound functional, structural, and cellular approaches that 2-years of home-based functional electrical stimulation (h-bFES) produced significant improvements in muscle size and assisted function. We have indeed shown that denervated, degenerating muscles (DDM), of patients suffering with permanent disconnection of muscle fibers from the nervous system were rescued by two years of h-bFES, using very large skin-contact electrodes and a new electrical stimulator designed in Vienna, Austria. The use of those equipments and the related protocols of stimulation of DDM has been validated by the successful EU Program: RISE [Use of electrical stimulation to restore standing in paraplegics with long-term DDMs (QLG5-CT-2001-02191)]. Thanks to the generous efforts of the Schuhfried Company based in Vienna, Austria the needed stimulators and large electrodes that recover DDM muscles are now commercially available. We have previously demonstrated that the positive effects of h-bFES are extended to the epidermal layer of the skin. The aim of the present short report was to describe a different approach to hopefully confirm the improvement of epidermis after two years of h-bFES. The previously study evaluated the histological structure of skin and the extent of changes that occurred in the thickness of the epidermis of three human SCI subjects before and after...
two years of continuous treatments of thigh muscles with h-bFES by surface stimulation revealing that there was a significant 28% overall increase in the thickness of the epidermis in response to h-bFES. Here with the evaluation of the epidermis area in cross sections of the skin and an unpaired statistical analysis (Table 1) the results are that: i) in two subjects (1 and 2) the skin biopsies from both the right and left legs show a significant increase in cross area of epidermis; ii) in the third subject there is a non statistically significant increase in epidermis area in the right leg, while there are no changes in the left leg; iii) the analysis of the three grouped subjects shows a significant 30% increase after h-bFES. Whether those differential behaviors are related to the time from SCI, as reported in table 1, will be tested analyzing more subjects (research in progress). If confirmed by further analyses in the whole series of skin biopsies of the RISE Project, the results will provide conclusive evidence that electrical stimulation could also be of foremost importance in prevention/management of decubitus ulcers in SCI, metabolic diseases like diabetes, age-related sarcopenia and in wound healing.20-29 Furthermore, the evidence of a FES-induced modulations of the skin suggests to extend similar studies to adaptation of the skin to other physical and pharmacological therapies, based on rehabilitation managements applied to the skin, specifically in decubitus ulcer recovery and pain relief.

List of acronyms
DDM - denervated, degenerating muscles
FES - Functional Electrical Stimulation
h-b FES - home-based FES
RISE Project - EU Program: RISE [Use of electrical stimulation to restore standing in paraplegics with long-term DDMs (QLG5-CT-2001-02191)]
SCI - Spinal Cord Injury

Author’s contributions
Authors equally contributed to the manuscript.

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Conflict of Interest
The authors report no conflicts of interests.

Ethical Publication Statement
We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Corresponding Author
Giovanna Albertin, Department of Neuroscience, Section of Human Anatomy, University of Padova, Italy.
E-mail: giovanna.albertin@unipd.it

E-mails of co-authors
Helmut Kern: helmut@kern-reha.at
Christian Hofer: stefan.loefler@kern-reha.at
Diego Guidolin: diego.guidolin@unipd.it
Andrea Porzionato: andrea.porzionato@unipd.it
Anna Rambaldo: anna.rambaldo@unipd.it
Raffaele De Caro: raffaele.decaro@unipd.it
Francesco Piccione: Francesco.piccione@ospedalesancamillo.net
Andrea Marcante: andrea.marcante@ospedalesancamillo.net
Sandra Zampieri: sanzamp@unipd.it

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