EFFECT OF LASER BIOSTIMULATION ON CELL PROLIFERATION IN THE HEALING OF CUTANEOUS SURGICAL WOUNDS IN PIGS

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Abstract

The objective of this study was to investigate the effect of low-level laser therapy (LLLT) on the proliferation of epithelial and connective tissue cells in the healing of incised cutaneous wounds in pigs. The experiment was conducted on 12 young pigs divided into four groups. Group I (undamaged skin) and group III (damaged skin) served as control. Group II - pigs with undamaged skin and group IV - pigs with incised wounds in the dorsal area were subjected to laser irradiation. Laser biostimulation was carried out using a CTL 1106 MX semiconductor laser in the continuous wave mode of operation at a wavelength of 810 nm and a maximum output of 100 mW. Following three weeks of observation, skin specimens were collected for histopathological analysis (HE), immunohistochemical detection of PCNA, and determination of apoptosis (TUNEL) and presence of mast cells (toluidine blue staining). Laser irradiation administered at E=8 J/cm for 1 min over a period of three weeks accelerated the proliferation of stratum basale cells, stimulated fibroblast proliferation, increased the number of mast cells in the wound area, and inhibited apoptosis in cells participating in the skin regeneration process.

Key words: pig, surgical wounds, laser therapy, PCNA, apoptosis.

Laser irradiation was first used in medicine when a pioneer laser apparatus was built by T. Maiman in 1960. The first low-level laser for tissue biostimulation was applied by E. Mester in 1969. Despite initial doubts concerning its efficiency, low-level laser therapy (LLLT) has been used for 30 years, and it occupies a prominent place in contemporary medicine. The range of LLLT’s medical applications in tissue stimulation continues to increase as new devices are constructed.

Continued research into tissue biostimulation revealed that LLLT has a beneficial effect on living organisms. The use of laser irradiation for biostimulation broadens our knowledge of the mechanisms responsible for polarised light effects on living tissue. Nevertheless, there is still a scarcity of published data on the type of processes taking place in healthy and damaged skin after laser therapy.

The objective of this study was to investigate the effect of LLLT on cell proliferation in the process of skin regeneration and to determine the adaptive response of cells to polarised light. This study is a continuation of the authors’ previous research and clinical observations (1-3) investigating the effects of laser irradiation as a factor modifying tissue regeneration, based on the results of histological and histochemical analyses.

Material and Methods

The experiment was conducted on 12 immature Polish Large White female pigs weighing approximately 20 kg. The animals were kept under standard laboratory conditions, with free access to feed and water. All surgery and treatment procedures were carried out with the consent of the Local Ethics Committee, in particular in view of the guidelines concerning the minimisation of stress during and after the procedure. Anaesthetics were applied to ensure that the performed procedures are painless. The animals were immobilised with special emphasis on stress reduction, and they were premedicated through intramuscular injection to lower anxiety, minimise pain, and induce muscle relaxation (azaperone, xylazine, and ketamine) before general anaesthesia. The animals were anaesthetised by
intravenous infusion of thiopental in fractionated doses. After surgical anaesthesia has been induced, the procedure involved a skin incision in the loin area, at level L1 - L2, on the left, parallel to spinous processes, followed by the closure of the surgical wound.

The animals were divided into four equal groups. Group I was a control group. Group II comprised pigs whose undamaged skin was subjected to laser irradiation in the dorsal area. Pigs from group III with incised wounds on the dorsal area were subjected to a routine procedure of surgical stitching and antibiotic treatment. Group IV pigs with incised wounds on the dorsal area, closed surgically, were biostimulated by laser irradiation. Laser biostimulation was done with the use of a CTL 1106 MX semiconductor laser in the continuous wave mode of operation at a wavelength of 810 nm and a maximum output of 100 mW. The transmission depth of radiation emitted by the applied laser was around 7 cm. Laser beam irradiation was applied at P=50 mW and E=8 J/cm for 1 min. Prior to laser treatment, skin was shaved and degreased with 70% ethyl alcohol. This procedure was carried out daily for 21 d. Since laser light initially causes blood vessels to contract, thus to reduce skin reactivity, non-contact irradiation was administered for approximately 2-3 min at the beginning of every procedure, i.e. the laser head was positioned at a certain distance from the animal's skin. In the proper part of the biostimulation procedure, the laser head was set perpendicularly to the skin. Contact point-irradiation was applied by pressing the wound area with the laser head.

In groups III and IV, the skin in the wound area was protected against potential bacterial infections by spraying with atomised aluminium. Antibiotic protection was applied in group III pigs, which were administered two injections of amoxicillin of prolonged activity. After the animals had woken up from the anaesthesia, they were transferred to the post-op room with free access to water and feed. To eliminate unnecessary pain, animals of groups III and IV were administered two noraminophenazone injections every 8 h after the treatment.

After three weeks, the animals were sacrificed through the administration of sodium pentobarbital. Skin sections were collected from all animals for laboratory analysis. The sections were rinsed with a buffered saline solution (0.1 M, pH 7.4, at 4°C) and transferred to 18% phosphate-buffered saccharose where they were stored until dissection. The collected skin samples were fixed with 4% neutral formalin (pH 7.4), embedded in paraffin, and microtome sections were stained with hematoxylin and eosin or were placed on silanised slides and subjected to immunohistochemical analysis.

**Immunohistochemical analysis.** Proliferating cell nuclear antigen (PCNA) expression was determined in cells of the epidermis and dermis. Monoclonal mouse antibodies (DAKO), designed for paraffin-embedded sections, were used for immunohistochemical staining. PCNA antibodies (DAKO) of clone PC-10 (Ig G2 K) were diluted 1:200. Dewaxed and rehydrated sections were placed in a citrate buffer (pH 6.0) and microwaved twice for 3 min each at 650 W to expose the antigens. 3,3'-diaminobenzidine (DAB) was used as the chromogen to obtain a stained reaction product. Negative control was performed with the use of IgG K and IgG1 K antibodies, and positive control involved DAKO sections. The percentage of cells showing a positive response to the antigen was determined.

**Determination of apoptosis.** Apoptotic cells were defined by the TUNEL method (TdT- mediated deoxyxuridine triphosphate- biotin/digoxigenin nick end-labelling) with the use of an R&D Systems labelling kit. Paraffin-embedded sections were deparaffinised with xylene and hydrated with decreasing concentrations of ethyl alcohol. Then the sections were rinsed in double distilled water and microwaved twice in a 650 W oven for 3 min each. The sections were transferred to PBS buffer for 10 min, dried, treated with proteinase K diluted 1:50, and incubated for 15 min at room temperature. Afterwards, the sections were rinsed twice in deionised water, and 3% hydrogen peroxide solution was applied. The excess of hydrogen peroxide was removed, and the specimens were placed in the TdT Labelling Buffer for 5 min. At the next stage, the specimens were incubated in the Labelling Reaction Mix containing labelled nucleotides TdT-dNTP, TdT Enzyme, and cation (cobalt), diluted 1:50, for 60 min in a humidity chamber at 37°C. Then the sections were placed in the Stop Buffer for 5 min, rinsed with the PBS buffer, and treated with Streptavidin-HRP antibodies, diluted 1:50 and labelled with peroxidase for anti-digoxigenin and anti-biotin antibodies, for 5 min. Peroxidase-labelled antibodies were identified with 3,3'-diaminobenzidine (DAB) as the chromogen by incubating the sections in a commercially available solution for 2-5 min. At the last stage of the reaction, the specimens were stained with haematoxylin, rinsed in tap water, dehydrated in increasing concentrations of alcohol, cleared with xylene, and mounted in Gurr® DPX mounting medium. The nuclei of apoptotic cells containing a high number of free 3'-OH DNA ends were stained brown, and the nuclei of unmodified cells – dark blue. Positive control was carried out using commercially available R&D systems sections. The percentage of apoptotic cells was determined in the specimens.

**Determination of mast cells.** The sections were stained with toluidine blue and the number of mast cells in the field of view was determined with the use of a 60x Olympus objective.

**Results**

The obtained results indicate that biostimulation with the use of laser treatment enhanced the proliferation of stratum basale cells and fibroblasts in the area of the surgical wound. Table 1 shows the type and localisation of histopathological changes in the epidermis and dermis in pigs of each examined group.
Table 1
Results of histopathological and immunohistochemical analysis of the epidermis and dermis of pigs

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>epidermis</td>
<td>dermis</td>
<td>epidermis</td>
<td>dermis</td>
</tr>
<tr>
<td>PCNA*</td>
<td>3.67 ±0.11</td>
<td>0.02 ±0.04</td>
<td>12.84 ±2.45</td>
<td>1.45 ±0.04</td>
</tr>
<tr>
<td>Apoptosis**</td>
<td>0.1 ±0.92</td>
<td>0.01 ±1.43</td>
<td>0.0 ±0.07</td>
<td>0.0 ±0.01</td>
</tr>
<tr>
<td>Number of mast cells in microscopic field</td>
<td>0-2-3</td>
<td>1.0-4-9</td>
<td>1-2</td>
<td>4-35</td>
</tr>
</tbody>
</table>

* Percentage of cells showing a positive reaction for PCNA,
** Percentage of apoptising cells.

Discussion

LLLT accelerates the regeneration of damaged tissue, which has profound implications for the healing of cutaneous wounds (7). The results of this experiment indicate that laser therapy has a strong biostimulating effect at the level of cutaneous tissues and cells, which renders laser stimulation a suitable technique for treating surgical wounds. LLLT has the following mechanism of action on living organisms: radiant energy is absorbed at the level of the electron transport chain, and that way the cellular metabolism is stimulated through increased supply of bound energy in ATP form. During laser irradiation, electromagnetic radiation is directly absorbed by the mitochondria. According to many authors, this transfer of electrons to higher energy levels corresponds to plant photosynthesis, which stimulates metabolism and ATP production (4).

Laser therapy contributes to the preservation of the energy balance of the body and cells through the direct absorption of photons, ADP phosphorylation, activation of cell enzymes, free radical reduction, and electromagnetic effects (5). Laser irradiation also affects the quantity of DNA in cells, stimulating its synthesis. The above leads to higher cell proliferation rates and enhances protein synthesis – vital processes in cell regeneration (8).

LLLT's immunomodulative effect on tissues has not been fully investigated yet. One of the cited studies (10) evaluated the effect of laser irradiation on the expression of TGF, an immunosuppressive cytokine. The results of the above study, which analysed the presence of apoptotic cells in the epidermis and connective tissue, demonstrated that laser irradiation has an inductive effect on apoptosis in the process of skin regeneration. Apoptosis affects very few healthy cells of the epidermis and the dermis. The findings of this study indicate that the number of apoptotic cells increases significantly during skin regeneration. Laser irradiation inhibits apoptosis. This is an important observation since the excess of cells produced in the granulation process have to necrotise, therefore, a prolonged irradiation could have an adverse effect. Irradiation is beneficial at the first stage of the healing process because it stimulates the formation of connective tissue.

The benefits of laser irradiation on the healing of cutaneous wounds have also been recognised by other authors (9, 11). In a study on rats, the authors observed higher number of vessels and greater quantities of mature collagen in animals subjected to laser therapy. The observations of stratum basale cell cultures also point to the beneficial effects of laser irradiation. Laser therapy increases cell mobility, stimulates the proliferation of wound keratinocytes, and thus significantly accelerates wound healing (6).
Fig. 1. Group I. A small number of stratum basale cells showing a PCNA-positive reaction. 120x

Fig. 2. Group II. An increase in the number of PCNA(+) cells after laser irradiation. 480x

Fig. 3. Group IV. A small number of apoptotic cells (brown nuclei) in the connective tissue after laser irradiation. TUNEL, 60x

Fig. 4. Group IV. Absence of apoptotic cells in the newly formed granulation tissue covering the wound area after laser irradiation. TUNEL, 120x

Fig. 5. Group IV. An increase in the number of mast cells under the epidermis and in the connective tissue after laser irradiation. Toluidine blue, 120x

Fig. 6. Group IV. Cellular infiltration in the dermis. HE, 120x
An increase in the number of mast cells was observed in the presented study. This could have various implications, including positive – in addition to secreting pro-inflammatory mediators, mast cells also significantly affect the immunological process, thus accelerating the healing process. Higher number of mast cell could contribute to the immune capacity of the connective tissue formed during the healing of surgical wounds. The presence of infiltrating PNM cells in the wound area supports the above observations.

References