Registered Report

Histomorphometric evaluation of skin wounds in rats submitted to biomodulatory therapies – research protocol

Avaliação histomorfométrica de feridas cutâneas de ratos submetidas a terapias biomoduladoras – protocolo de pesquisa

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ABSTRACT | INTRODUCTION: The use of biomodulatory therapies in order to help tissue repair has been increasingly common in different areas of health. **OBJECTIVE:** This study aims to comparatively evaluate the effects of 660 nm laser photobiomodulation, ozone therapy, and ozonized oil on repair through histomorphometric analysis in skin wounds in rats. Forty Wistar rats will be divided into 4 groups of 10 animals each, Control Group (CG), Laser Group (LG), Ozone Gas Group (OGG), and Ozonated Oil Group (OOG). **MATERIALS AND METHODS:** Standard skin wounds will be made on the back of the animals, and the different experimental groups will be treated with the biomodulatory therapies described for three consecutive days. Five and ten days after surgery, five rats from each group will be euthanized. Skin fragments, including the wound area, will be removed for histological processing and subsequent staining of histological sections with Hematoxylin-eosin and Sirius red. Micrographs of the histological sections will be obtained and ten standard images will be captured for quantitative evaluation of the variables collagen area, number of blood vessels, and epithelium thickness. The variables infiltrate of polymorphonuclear and monomorphonuclear inflammatory cells, as well as edema, will be analyzed semiquantitatively. Statistical analysis of the study variables will be performed, with a significance level of $p<0.05$. **CONCLUSION:** It is expected to verify which of the biomodulatory therapies used can favor the resolution of tissue repair, in particular, by promoting collagen biosynthesis.

KEYWORDS: Wound healing. Low-Level Light Therapy. Ozone. Wistar, Rats.

RESUMO | INTRODUÇÃO: A utilização de terapias biomoduladoras no intuito de auxiliar o reparo tecidual tem sido cada vez mais comum nas diversas áreas de saúde. **OBJETIVO:** Este estudo objetiva avaliar, comparativamente, os efeitos da fotobiomodulação laser de 660 nm, ozonioterapia e óleo ozonizado sobre o reparo através da análise histomorfométrica em feridas cutâneas de ratos. Serão utilizados 40 ratos Wistar distribuídos em 4 grupos de 10 animais cada, Grupo controle (GC), Laser (GL), Gás Ozônio (GGO) e Óleo Ozonizado (GOO). **MATERIAIS E MÉTODOS:** Serão realizados ferimentos cutâneos padronizados no dorso dos animais e os diferentes grupos experimentais serão tratados com as terapias biomoduladoras descritas, por três dias consecutivos. Cinco e 10 dias após a realização da cirurgia, 5 ratos de cada grupo serão eutanasiados. Serão removidos fragmentos de pele, incluindo a área da ferida, para processamento histológico e posterior coloração das secções histológicas com Hematoxilina-eosina e Sírius vermelho. Serão obtidas micrografias das secções histológicas e dez imagens padrão serão capturadas para avaliação quantitativa das variáveis: área de colágeno, número de vasos sanguíneos e espessura do epitélio. As variáveis infiltrado de células inflamatórias polimorfonucleares e monomorfonucleares, assim como edema, serão analisadas semiquantitativamente. Será realizada análise estatística das variáveis do estudo, com nível de significância $p<0,05$. **CONCLUSÃO:** Espera-se verificar qual das terapias biomoduladoras utilizadas pode favorecer a resolução do reparo tecidual, em especial, por promover a biossíntese do colágeno.

PALAVRAS-CHAVE: Cicatrização de feridas. Terapia de luz de baixo nível. Ozônio. Ratos Wistar

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Introduction

Currently, several therapeutic tools known as biomodulatory therapies have been used to optimize the healing process. Among them are acupuncture, essential oils, ozone therapy, photobiomodulation with laser and light-emitting diode (LED), and plasma jet. In general, such therapies can promote significant anti-inflammatory and analgesic effects, in addition to biomodulating different phases of tissue repair, without generating deleterious effects on the patient.[1,2]

Laser photobiomodulation, with a wavelength within the visible light spectrum, has been documented to positively affect the healing process[3] by increasing ATP biosynthesis, increasing microcirculation[4], and increasing collagen biosynthesis by fibroblasts.[5-7] Such effects are interdependent on the type of tissue treated, the energy density and power of the laser, and the application time and intervals outlined for the patient.[8]

Ozone is a type of gas abundant in nature whose molecule is composed of three oxygen atoms. It is an unstable gas that participates in the oxidation-reduction reactions that occur in cells.[9] The decomposition of ozone into O_2 and a free atom of oxygen is known as ozonolysis and is responsible for generating free radicals, also known as reactive oxygen species (ROS).[10,11] Ozone therapy has a documented therapeutic action, through its different routes of administration[12], on animal tissues[13] and human beings[14], based on the activation of protein synthesis mechanisms, resulting from the increase in the amount of ribosomes and the hydrogen potential of the mitochondrial respiratory chain. These changes at the cellular level explain the increased functional activity and the more significant potential for tissue and organ regeneration.[15,16]

Tissue repair is a highly dynamic and complex biological event characterized by the occurrence of primary phenomena of exudative nature resulting from changes in microcirculation in the wound area. The initial inflammatory phase is characterized by vasodilation and increased vascular permeability and may extend up to 48 hours after the onset of the injury. The first change is vasodilation, which enables the transcytosis of defense cells, such as neutrophils, responsible for being the first line of defense against infection.[17] As tissue repair advances, proliferative phenomena, such as neoangiogenesis, the deposition of new elements constituting the extracellular matrix, and the process of re-epithelialization, come to predominate in the lesion's microenvironment.

Studies comparing the tissue effects induced by biomodulatory therapies in the tissue repair process are scarce to date. Therefore, this research project aims to evaluate, through a histomorphometric study in rat skin, the polymorph and monomorphonuclear inflammatory infiltrate, the vascular density, the area of synthesized collagen, and the thickness of the newly formed epidermis at different stages of repair.

Background

Given the occurrence of complicating factors of tissue repair, such as bacterial contamination, excessive mechanical traction, immunodeficiencies, and metabolic diseases like diabetes mellitus, among others, the promotion of knowledge about alternative methods that can positively modulate the healing of affected tissues becomes relevant. Furthermore, checking the true efficacy of these therapies, which include laser photobiomodulation and the use of ozone, is necessary for their use to become justified in the clinical practice of health professionals, especially for patients who have difficulty healing wounds.

Although there are already some studies in the literature that address the potential action of the above-mentioned biomodulatory therapies, conducting an experimental, controlled, in vivo study will allow a better understanding of the tissue changes resulting from their use during the wound healing process.[18,19]

Objectives

General Objectives

- To evaluate the effects of laser photobiomodulation and ozone therapy on the tissue repair process of standardized surgical wounds in rat skin through histomorphometric analysis.

Specifics Objectives

- To compare the percentage of polymorphonuclear and monomorphonuclear inflammatory cells, vascular density, and the area of fibroplasia and epidermal thickness between the intervention groups.

- To compare semi and quantitatively the variables polymorphonuclear and monomorphonuclear inflammatory cells, vascular density, fibroplasia area, and epidermis thickness in the intervention groups at two different moments of tissue healing.

Materials and methods

Research Ethics Committee Approval

This study was submitted and approved by the Comissão de Ética no Uso de Animais - CEUA (Ethics Committee on Animal Use) of the Faculdade Adventista da Bahia - FADBA (Adventist College of Bahia), and it was registered under protocol number 67/2019. The approval opinion number by CEUA was CIAEP: 01.0039.2013. It began in December 2019, with the performance of all the procedures described below, except for the histomorphometric analysis, which will allow the performance of a correlation study between histological findings and thermographic recordings.

Sample

For the sample of the present study, it was established as inclusion criteria regarding the animals: male Wistar rats from the animal house of the FADBA weighing between 150-300 g; and, as exclusion criteria: animals presenting any alteration, wound, or skin disease. Forty animals will be randomly divided into four groups of 10 rats each, which will be sacrificed on the 5th (5 animals) and 10th (5 animals) days after the proposed treatments. Group 1 is the Control Group and will receive no treatment; Group 2, called Laser Group (LG), will be submitted to conventional laser photobiomodulation; Group 3, the Ozone Gas Group (OGG), will receive gaseous ozone therapy, through insufflation at the edge of the lesion; and Group 4, Ozonated Oil Group (OOG), will be treated with ozonated oil on the surface of the lesion.

The rats will be kept in an animal facility, isolated in cages with identification, containing one animal each. The temperature (average of 23 ºC), humidity (50 to 52%), as well as the light conditions of the facility (12 hours with artificial light and 12 hours in darkness), will be strictly controlled. The specimens will be fed daily with a Nuvilab® (Suprilab) ration specific for the species and water at will. All care regarding cleanliness will be taken, and the environment will be protected from auditory stimuli to avoid stress.[20] All animals will come from the FADBA laboratory animal facility. Before the beginning of the research, the animals will be submitted to one week of adaptation in the cage.

Surgical procedures and use of biomodulatory therapies

According to the specific weight established for each animal, the rats will be anesthetized with a combination of 10% ketamine hydrochloride (Dopalen®, São Paulo, Brazil) 75 mg/ml (2 mg/kg) and 2% xylazine hydrochloride (Anazedan, São Paulo, Brazil) 5 mg/ml (3 mg/kg). After sedation, hair will be removed from the dorsal region, close to the fat pad, followed by antisepsis with povidone-iodine (Rioquímica, São Paulo, Brazil). Then, a standardized wound will be made on the animals' skin with a 6 mm diameter circular scalpel (Biopsy Punch, Stiefel, Germany). Only one calibrated operator will perform the wound induction. The animals from the first experimental group, the Control Group (CG), will be treated with a simulation of the proposed therapies.

The Laser Group (LG) animals will be treated with a semiconductor diode laser (Laser VR-KC-610-Dentoflex, Brazil) ALGaAs, 9 mW, 670 nm, 0.031 W/cm^2, with continuous emission and active tip area of 0.28 cm^2. In each diametrical vertex of the circular wound, 1 J/cm^2 will be applied, corresponding to a total dosimetry of 4 J/cm^2 per daily application. The animals will be treated for three consecutive days, and the total fluence provided to the tissue will be 12 J/cm^2.

In the third group (OGG) of the experiment, represented by the ozone gas, the daily application of ozone for three consecutive days will be performed with a 5 ml syringe, and approximately 1 ml of the mixture of ozone with oxygen will be insufflated in the diametrical vertices of the circular wound, at the external edges of the lesion. The ozone generation will be produced through the Philozon generator® (Philozon - Indústria e comércio de geradores de ozônio - LTDA, Santa Catarina, Brazil). By sharing the cylinder with medical oxygen, a concentration of 13 µg/ml of ozone and a constant flow of 1 L/min will be adopted. As for the fourth experimental group (OOG), which will be treated with ozonated oil, the rats will undergo the wounds following the same patterns of the Control Group (CG) plus instillation in the center of the lesion of 1 drop of 100% ozonated sunflower oil (Philozon - Indústria e comércio de geradores de ozônio - LTDA, Santa Catarina, Brazil), also for three days.

At the end of the 5th and 10th days of the experiment, five animals belonging to each experimental group will be sacrificed. The euthanasia will be performed using an overdose of the anesthetic solution already specified, and after verification of deep sedation, the specimens will be placed in a CO_2 chamber with a concentration of 5 liters per minute.

Histological processing

After death confirmation, a portion of tissue will be removed from the back of the rats, which will comprise the surgical wound. The surgical specimen will be fixed for a minimum period of 18 hours in 10% buffered formalin solution.

The tissue will be processed for staining with hematoxylin-eosin and Syrius red.

Histological and morphometric evaluation

The images of tissue sections submitted to the stains described above will be captured using the software Motic Images Advanced 3.0® (Motic China Group Co. Ltd.) in theLaboratório de Bioquímica Oral do Instituto de Ciências da Saúde da Universidade Federal da Bahia. A standard area will be established to analyze all cases, namely, 13107.200000 pixels. Ten standard images, corresponding to each case, will be captured with the established size, in which the number of vessels, the collagen area, and the thickness of the newly formed epidermis will be measured. Each area will be captured at 40 x magnification and saved in JPEG format.

The slides will be coded, and two double-blind calibrated examiners will do all the analyses. The degree of inflammation in the tissue will be assessed as described by Sampaio et al.[21] A semi-quantitative study of the sections will be done, analyzing the variables of the inflammatory process as polymorphonuclear infiltrate, monomorphonuclear infiltrate, and edema, adopting the criteria of absent (0), mild (+), moderate (++), and severe (+++). In order to define these histological grades, the following criteria will be adopted: when the alteration is present in a percentage equal to or greater than 50% in the section analyzed, the degree will be considered severe; for 25 to 50% of the tissue, moderate degree; and less than or equal to 25%, mild.

Statistical Analysis

The sample size calculation for the present study will follow the principles of the 3Rs of Russell and Brush[22], which advocate replacement (replace, if possible, animal experiments for invertebrates or in vitro); refined (the technique should be refined so that there is no suffering, and it should be done with anesthesia and analgesia); and reduce (the minimum number of animals should be used to achieve the scientific objectives). In this aspect, according to studies by Eckelman et al.[23], to determine the number of animals needed to achieve a significance of $p<0.05$, with a difference in percentage between the Control Group and the treatment/disease group of 30% and with a coefficient of variation (CV) of 20% for Wistar rats, the number of 5 rats per group will be established (Table 1).[24] For this, the collagen area will be chosen as the primary endpoint, and, according to the literature, laser therapies can increase the collagen increment by 30%.[25]

Table 1. Sample size calculation

Animals needed for a statistically significant result			
Percent difference between control and treatment group averages	Percent CV due to biological variability	Number of animals	Significance ($P<.05$)
20	20	2–7	Not significant
20	20	8	Significant
20	15	5	Significant
25	20	5	Nearly significant
30	20	5	Significant
25	15	5	Significant

Source: Eckelman, Kilbourn, Joyal, Labiris, Valliant.[23]

Three quantitative variables will be evaluated: collagen area, number of vessels, and epithelium thickness; and three semi-quantitative variables: polymorphonuclear cells, monomorphonuclear cells, and edema.

A database will be created in Microsoft® Excel® 2010 (version 14.0.7132.5000), Microsoft® Office Professional Plus 2010, USA, and analyzed in R software (version 3.1.1). The distribution of the data for normality will be tested. ANOVA test will be used, followed by the post hoc Bonferroni test. The significance level will be $p<0.05$.

Expected results

It is expected to verify the efficacy of the biomodulatory therapies previously described concerning the variables of tissue repair and to determine which of the three therapeutic resources will have a more beneficial effect on the skin healing process.

Authors' contributions

Lima FQ participated in the literature survey and discussion of the research project. Marchionni AM and Medrado AP elaborated on the project's initial conception and participated in its methodological development. Medrado AP performed the critical review of the project.

Conflicts of interest

No financial, legal, or political conflicts involving third parties (government, private companies and foundations, etc.) have been declared for any aspect of the submitted study (including but not limited to grants and funding, advisory board participation, study design, manuscript preparation, statistical analysis, etc.).

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