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Stereological and molecular studies on the combined effects of photobiomodulation and human bone marrow mesenchymal stem cell conditioned medium on wound healing in diabetic rats

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ABSTRACT

We investigated the effects of conditioned medium (CM) from human bone marrow mesenchymal stem cells (h BMMSC)s and pulse wave photobiomodulation (PW PBM), applied alone or in combination, on the stereological parameters and gene expression of some growth factors, during wound healing in a streptozotocin (STZ)-induced rat model of type one diabetes mellitus (T1DM).

T1DM was induced in 72 rats and two incisions were made in each animal. The rats were assigned to one of four groups: a control (placebo) group, a Laser group (890 nm, 80 Hz, 0.2 J/cm^2); a CM group, and a combined CM + Laser group. On post-surgical days 4, 7, and 15, skin samples were extracted for stereology and reverse transcription PCR (RT-PCR) analyses of gene expression of basic fibroblast growth factor (bFGF), hypoxia-inducible factor (HIF-1α), and stromal cell-derived factor-1α (SDF-1α).

The stereological examinations of the proximal and distal wounds revealed significantly enhanced healing in all the treated groups, compared to the control group. The extent of healing was significantly greater in the CM + Laser group than in the other treatment groups. The RT-PCR results also indicated greater gene expression in the CM + Laser and Laser groups than in the CM and control groups.

Application of CM and PW PBM, alone or in combination accelerated the process of wound healing in T1DM rats. The results of combined application of CM and PW PBM, indicated a synergistic effect, and the combination treatment was statistically more effective than single applications of CM or PW PBM.

1. Introduction

Diabetes mellitus (DM) is known to impair the healing of skin wounds [1]. Estimates made in 2104 in the United States indicated that approximately 29.1 million individuals were suffering from DM [2]. Of these patients, 15–20% are predicted to experience diabetic foot ulcers (DFUs), resulting in 73,000 amputations per year [2,3]. Under non-diabetic conditions, wound healing takes place as a cellular defense against further harm and consists of precise cellular activation of specific cell elements such as neutrophils, macrophages and fibroblasts [4] and numerous cytokines and growth factors that coordinate [4] and promote healing. This wound healing process is dysregulated in the

Abbreviations: Adipose-derived stem cells conditioned medium, (ASC-CM); Analysis of variance, (ANOVA); Basic fibroblast growth factor, (b-FGF); Colony-forming unit, (CFU); Conditioned medium, (CM); Diabetes mellitus, (DM); Diabetic foot ulcer, (DFU); Endothelial progenitor cell, (EPC); Federal Drug Administration, (FDA); Fluorescence isothiocyanate, (FITC); High glucose, (HG); Human bone marrow mesenchymal stem cell, (hBMMSC); Human dermal fibroblast, (HDF); Human epidermal growth factor, (hEGF); Hypoxia-inducible factor, (HIF-1α); Interleukin, (IL); Least significant difference, (LSD); Monocyte chemoattractant protein-1, (MCP-1); Monocyte chemoattractant protein-1, (MCP-1); Nitric oxide synthase, (NOS); Numerical density, (Nv); Phosphate-buffered saline, (PBS); Photobiomodulation, (PBM); Pulse wave, (PW); Recombinant platelet-derived growth factor, (rPDGF); Reverse transcription-polymerase chain reaction, (RT – PCR); Standard deviation, (SD); *Staphylococcus aureus*, (*S. aureus*); Stromal cell-derived factor-1α, (SDF-1 α); Total volume, (TV); Type one DM, (T1DM); Vascular endothelial growth factor, (VEGF)

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DFUs of patients with DM [4], as these wounds are largely unable to mount an adequate inflammatory response [4]. The DFU then becomes a preferred location for the colonization of *Staphylococcus aureus*, and the resulting infection can necessitate amputation of limb [4].

The development of *S. aureus* infection creates innumerable problems for patients with DM and delays treatment [5], making DM and DFUs a pivotal challenge for physicians [3]. However, photobiomodulation (PBM) has been introduced as a promising new healing agent in recent years. Recent scientific data have revealed that PBM applied, using frequency mode or pulse wave (PW), at frequencies of 80 Hz [6–12], 700 Hz (13), 2336 Hz, 3000 Hz [14,15], and 5000 Hz [16], wavelengths of 660 nm [22], 780 nm (19), 820 nm [20], and 890 nm [12–18,21,22] and intensities of 0.2 J/cm^2 [6], 0.324 J/cm^2 [10], 0.396 J/cm^2 [11], 924 J/cm^2 [12] 2 J/cm^2 [17], 8 J/cm^2 [13], 11.7 J/cm^2 [14,15], and 12 J/cm^2 [16], promote the healing processes in healthy (nondiabetic) skin wounds [17], deep second degree burns [14,19], and third degree burns [15]. The use of PW PBM, in particular, was shown to heal wounds in an experimental model of type one DM (T1 DM) [6,7,9], as well as in an animal model of type two DM (T2DM) [10]. PW PBM has also been shown to promote superficial wound healing in humans [13], and to cause a continued depletion of chronic venous ulcerations in patients [16]. PW PBM can reduce the inflammatory response and enhance the proliferative phase of normal wound healing by increasing angiogenesis, numbers of fibroblasts, and basic fibroblast growth factor (b-FGF) gene expression [7,17]. Wound healing is accelerated by PW PBM, in streptozotocin (STZ)-induced T1DM.

Growth factors and cytokines responsible for wound healing are released by mesenchymal stem cells (MSCs), also referred to as secrotomes [18]. Application of secrotomes to wounds could be achieved, using conditioned medium (CM) from cultured MSCs [18]. A combination of growth factors and cytokines has been shown previously to increase healing in diabetic wound [19]. For chronic wounds, CM from MSCs represents a desirable treatment for reducing the period of wound contraction [20]. At present, positive outcomes have been reported for the use of CM, in non-diabetic and T1DM animals [19–21].

Previous studies have shown that bFGF, hypoxia-inducible factor (HIF-1α), and stromal cell-derived factor-1α (SDF-1α) are involved in wound healing [7,22–26].

Recently Pouriran et al. [6] reported positive effects of a combined administration of CM from human bone marrow mesenchymal stem cells (hBM-MSC) and PW PBM (890 nm, 80 Hz, 0.2 J/cm^2) on the tensiometric parameters of the healing wounds in an animal model for T1DM. Pouriran et al. concluded that PWLLLT and hBM-MSC-CM, alone or in combination, significantly increased biomechanical parameters, within the healing wounds. However, PWLLLT was statistically more effective, compared with the hBM-MSC-CM (CM) [6].

Understanding the mechanisms underlying the beneficial effects of PW PBM requires studying the changes in tissue histology and growth factors that occur with this treatment [7].

The new concept introduced in this paper is the investigation of the effects of combining the administration of the PW PBM and CM, on the stereological parameters and on the expression of SDF-1α, bFGF, and HIF-1α in a repairing wound, in a STZ-induced rat model of T1DM. The goal of the present research was to bring about awareness, among clinicians that this method could be an innovation for the treatment of patients with DM. This combined treatment may ease their pain and suffering, by accelerating the healing process and preventing the need for amputation and its devastating emotional effects on these patients.

2. Materials and Methods

2.1. Study Design and Animals

This study was approved by the Institutional Medical Ethics Commission (protocol no: 5137). We used 72 *Wistar* male adult rats, weighing approximately 260 g. The animals were caged individually in standard cages and provided with food and water *ad libitum*. T1DM was induced in all animals. The animals were randomly assigned to four groups, each containing 18 animals: a control group (placebo), a Laser group that underwent PW PBM, a group treated with CM, and a CM + Laser group that received both CM and PW PBM. On days 4, 7, and 15, six animals of each group for each day were euthanized, and skin samples were extracted for stereology and for the analyses of gene expression of bFGF, HIF-1 α, and SDF-1 α by reverse transcription-polymerase chain reaction (RT – PCR).

2.2. Isolation and Expansion of hBM-MSCs

The hBM-MSCs were isolated and expanded, as reported previously [6]. Briefly, the BM sample was obtained from a healthy adult female donor. The aspirated BM was diluted and mononuclear cells were cultured and isolated. Non-adherent mononuclear cells were eliminated, during the initial medium changes. Four cell passages were implemented by detaching the attached fibroblast-like cells [6].

2.3. Immunophenotyping of Cultured hBM-MSCs by Flow Cytometry

The BM-derived MSCs were isolated and cultured as previously reported [6]. Briefly, the MSC surface antigens were detected by incubating the obtained samples with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human antibodies: CD34-FITC, CD105-PE, CD45-PE, CD73-PE, and CD90-PE. Mouse Isotype antibodies were applied, as negative controls. Each antibody in the study was evaluated by flow cytometry, using WinMDI software [6].

2.4. Preparation of CM

The CM was prepared by culturing 1×10^6 hBM-MSCs at passage 4, in a TP75 culture flask, as previously reported [6]. The cells were grown to approximately 80% confluence, and CM was collected and concentrated roughly 20-fold by freeze-drying, according to the instructions from the instrument manufacturer [6].

2.5. Induction of Type I DM

The T1DM model was induced by an intraperitoneal injection of STZ (40 mg/kg body weight) to cause the development of DM [6]. The DM was defined, as a blood glucose level > 250 mg/dl seven days, following the injection of STZ [27]. To ensure that DM was established in the animals, the rats were kept for 30 days under standard conditions in the animal house, before any further procedures.

2.6. Surgery

The animals were placed on their backs. Under general anesthesia and sterile conditions, two 12 mm-longitudinal full-thickness incisions were made with a No. 15 blade in the upper thoracic and lumbar regions (Fig. 10, in Supporting information) [6].

2.7. PBM and CM Administrations

An infrared laser [MUSTANG 2000, LO7 pen, Technica Co., Moscow, Russia] was used to irradiate the proximal incisions, in the third and fourth groups. The followings are the characteristics of the laser:

- Wavelength: 890 nm
- Average power: 1.08 mW
- Peak power output: 75 W
- Pulse frequency: 80 Hz
- Pulsed duration: 180 ns