Official Title of the study

Application of photobiomodulation therapy by LED using a combined red and nearinfrared light spectrum improves the vascular function of the radial artery *in vitro*.

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Authors: André Timóteo Sapalo, PhD[1,2,5]; Carlos Henrique Miranda, PhD[2]; Rinaldo Roberto de Jesus Guirro PhD[1]; Elaine Caldeira de Oliveira Guirro PhD[3]; Carlos Eduardo Girasol PhD[2]; Gisele Moceline Junkoski PhD[3]

[1]HCRP-USP Echocardiography Laboratory, Department of Clinical Medicine, FMRPUSP, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto -SP, Brazi

[2]Division of Emergency Medicine, Department of Internal Medicine, Vascular Biology Laboratory, School of Medicine, Ribeirão Preto, University of São Paulo, Brazil.

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[1] Postgraduate Program in Rehabilitation and Functional Performance, Department of Health Science, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto – SP, Brazil.

[2] Postgraduate Program in Physics Applied to Medicine and Biology, University of São Paulo (USP), Ribeirão Preto, Brazil.

[3] Department of Cardiac Surgery, Hospital das Clínicas, University of São Paulo.

Abstract

LED photobiomodulation therapy (PBMT) is widely recognized for its beneficial effects in several clinical conditions; however, its potential in modulating vascular function is not yet fully established. **Objective**: This study aimed to evaluate the application of PBMT by LED (light-emittion diode) using a combined red and near-infrared light spectrum, with a focus on improving the vascular function of the radial artery *in vitro*. **Methodology**: A total of 30 radial artery segments were studied and divided into three groups of 10 segments: the PBMT LED group combined with red and infrared light (R + IR), the red light (R) group, and the infrared light (IR) group. Prior to light irradiation at wavelengths of 650 nm and 940 nm, vascular function in these segments was pharmacologically inhibited using L-NAME. The drugs phenylephrine, acetylcholine, and sodium nitroprusside were used to determine if irradiation could restore the inhibited vascular functions. Superoxide anion levels, an important reactive oxygen specie (ROS), were measured using a chemiluminescence assay with lucigenin. The bioavailability of nitric oxide was evaluated using a Griess Reagent Kit assay, while nitric oxide production was assessed through the DAF fluorescence assay. The vascular inflammation was determined by the Intercellular Adhesion Molecule 1 (ICAM-1) expression.

Key words: Photobiomodulation; light-emitting diode; vascular function; radial artery

Introduction

LED photobiomodulation therapy (PBMT) is widely recognized for its beneficial effects in various clinical conditions. This technique is used as a therapeutic resource for a variety of pathologies in various fields, including musculoskeletal disorders[1], dermatological conditions[2], dental issues[3], and even mood disorders[4], with increasingly promising results.

When applied with appropriate parameters, PBMT has the potential to induce fundamental biological changes in tissue via the absorption of light photons, stimulating or inhibiting various processes as needed[5]. To achieve these outcomes, PBMT can be administered through different methods, either directly in contact with the target area or systematically via the vascular system, which may involve transdermal or intravascular application[6].

PBMT has found extensive application, particularly in the treatment of

cardiovascular diseases[7]. Initially, the technique involved the application of laser light through an intravenous catheter, typically inserted into the radial artery. This approach, known as intravascular laser irradiation of blood (ILIB)[8], was first reported by Russian researchers (PMID: 3191241), who attributed the therapeutic effects to the normalization of blood lipid levels, as well as the reduction of platelet aggregation and clot formation. Furthermore, it is suggested that the technique may activate the immune system, involving dendritic cells, macrophages, and lymphocytes[9].

For a long time, the evolution of this technique stagnated due to the limitations of its intravenous application. However, in recent years, modifications have been made to facilitate its use, and systemic PBMT has begun to be applied transdermally, such as on the radial artery. This approach, known as vascular photobiomodulation (VPBM), has been indicated for clinical use[10]. The ease of application has led to an expansion of the technology's use in clinical settings; however, the scientific validation of its effects remains unclear in light of available clinical reports.

In recent years, photobiomodulation mediated by light-emitting diodes (LEDs) has gained significant prominence and clinical focus as a more viable and cost-effective therapeutic option. Compared with laser devices, LEDs offer several advantages, including smaller size, lighter weight, lower cost, and ease operation[11,12]. Although some studies have demonstrated that LED irradiation can promote improvements in microcirculation and enhance the healing of diabetic ulcers in clinical settings[13,14], the dose-response relationship and underlying mechanisms of LED-mediated PBMT remain unclear. Several parameters are crucial for PBMT, including wavelength, light irradiance, energy, treatment duration, and frequency. Red or near-infrared light (600–1000 nm) is generally considered the optimal range for enhancing vascular function[15]. The present study aimed to assess the effects of LED photobiomodulation therapy on vascular function *in vitro* using human radial arteries harvested during coronary artery bypass grafting.

Materials and Methods Ethical aspects

This study was approved by the Human Research Ethics Committee of the Hospital das Clínicas, FMRP/USP (**protocol no. 4.829.712, approved on April 22, 2023**). The research was conducted in the Vascular Physiology Laboratory, Department

of Physiology, University of São Paulo. Surgical patients who consented to donate a segment of their radial artery were fully informed about the study objectives and the intended use of the donated tissue. Written informed consent was obtained from all participants.

Removal of radial arteries

Radial arteries (RAs) were harvested during coronary artery bypass grafting (CABG) by surgeons at Hospital das Clínicas – FMRP/USP – Brazil. Harvesting the radial artery is a standard procedure and is the most commonly used artery for coronary artery bypass grafting due to its favorable characteristics. All surgeries were performed by the same surgical team using cardiopulmonary bypass and cardioplegic arrest. The radial artery was primarily used to provide secondary target vessels, mainly the obtuse marginal branches or posterior descending arteries, in 30 patients. The radial artery was selected for this purpose due to its ability to yield a sufficiently long segment, making it suitable for use as a coronary graft, with the remaining tissue being used for the present study.

Before radial artery extraction, all patients underwent Doppler vascular assessments to evaluate arterial function. An ultrasound system equipped with a 13-6 MHz linear transducer (HFL38x/13-6 MHz Transducer, FUJIFILM Sonosite, Inc., Bothell, WA, USA) was used, which is designed for small-part imaging. Vessel compression was avoided, and oblique images of the vessel were captured using conductive gel, in accordance with recommendations from studies such as the one referenced[16], due to the varying degrees of vascular impairment among patients. RA was always harvested from the non-dominant arm, and bilateral RA harvesting was not performed. As there was no control group, baseline assessments to evaluate normal vascular function are provided in the supplementary files, along with patient demographics. Although these patients already had vascular impairment, the graft arteries were chosen to be as healthy as possible to minimize complications after surgery and avoid shortening the graft's lifespan, thus preventing the need for another surgery within a short period. Data are available in the supplementary files. For the study, three groups were considered: mixed red and infrared LED (R + IR, n = 10), red LED (R, n = 10), and infrared LED (IR, n = 10).

LED Irradiation in vitro on Radial Arteries

After the arterial segments were collected for the study, they were transferred to HTK solution (commercially known as Custodiol® Contatti, manufactured by São Geraldo, Porto Alegre, Brazil), an intracellular crystalloid cardioplegia solution used in the surgical setting at: Clinical Hospital of Ribeirão Preto, University of São Paulo (USP), Brazil. This solution is used for myocardial protection in complex cardiac surgeries and organ preservation during transplant procedures. It aids in maintaining the integrity of vascular function after the removal of arterial segments, allowing for their safe transfer to the vascular biology laboratory.

The arterial segments were positioned on a plate placed beneath an LED device. The distance between the LED panel and the bottom of the plate containing the arterial segments was approximately 5 mm. The total irradiation times were 94 seconds for the infrared LED blanket, 73 seconds for the mixed blanket (red + infrared), and 83 seconds for the red LED blanket.

To ensure direct light exposure to all arterial segments, a pre-test was conducted using a light sensor (model E18-D80NK) to verify the effectiveness of the light delivery, given the 5 mm distance between the LED panel and the arterial segments. The LED blankets were procured from PlatinumLED Therapy Lights: BIOMAX 900 Series. Prior to use, all LEDs were tested in the Photobiophysics Laboratory at the Ribeirão Preto School of Philosophy, Sciences, and Letters, University of São Paulo, where the wavelengths, radiation angles, power, and power density of each LED were measured

Vascular Function

Vascular function was assessed using a wire myograph after the arterial segments were exposed to a preservation solution (costodiol) and subsequently immersed in a saline solution containing L-NAME (0.5 mg/ml). The L-NAME solution was used to induce vascular dysfunction for one minute, after which the segments were transferred to a modified Krebs-Henseleit solution at 4°C, with the following composition (in mM): NaCl, 130; KCl, 4.7; $NaHCO_3$, 14.9; KH_2PO_4, 1.18; $MgSO_4$, 1.17; glucose, 5.5; $CaCl_2 \cdot 2H_2O$, 1.56; EDTA, 0.026.

Radial artery rings (2 mm) from the R + IR, R, and IR groups were mounted in a myograph (model 620M; Danish Myo Technology – DMT, Copenhagen, Denmark)

containing Krebs-Henseleit solution, gassed with 5% CO_2/95% O_2 to maintain a pH of 7.4 for recording isometric tension. The rings were subjected to a basal passive force of 5 mN, as defined by a voltage-response curve with rings subjected to tension between 0.5 and 10 mN and stimulated with 10^{-7} M phenylephrine. A stabilization period of 45 minutes was allowed.

Following the stabilization period, potassium chloride (KCl, 120 mM) was used to test arterial contractility. Cumulative concentration-response curves were then constructed for phenylephrine (PE, 10^{-10} - 10^{-4} M), acetylcholine (ACh, 10^{-10} - 10^{-4} M), and sodium nitroprusside (SNP, 10^{-10} - 10^{-4} M).

Lucigenin Chemiluminescence

The superoxide anion ($O_2^{\bullet-}$), an important reactive oxygen species (ROS), generation in arteries from the R + IR, R, and IR groups was measured using a chemiluminescence assay with lucigenin as a nicotinamide electron acceptor and nicotinamide adenine dinucleotide phosphate (NADPH) as the substrate. Following stimulation, the arteries were washed and collected in a selective lysis buffer containing 2×10^{-2} M KH_2PO_4, 10^{-3} M EGTA, and protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 10^{-3} M phenylmethylsulfonyl fluoride [PMSF]). The samples were then transferred to glass tubes containing 990 µL of assay release solution [5×10^{-2} M KH_2PO_4, 10^{-3} M EGTA, 1.5×10^{-3} M sucrose, pH 7.4] and 5×10^{-6} M lucigenin (98% purity, Sigma) for baseline measurement.

NADPH (98% purity, 10^{-4} M; Sigma) was added to each sample, and the luminescence signal was measured for 30 cycles, each lasting 18 seconds, in a luminometer (Lumistar Galaxy, BMG Lab Technologies, Germany). A blank control was included, and baseline and blank readings were subtracted from the respective sample readings. The results are expressed as relative light units (RLU) per µg protein, as quantified by the BCA assay (Thermo Fisher, 23225).

Nitrite Assay

Nitrate/nitrite concentrations were measured using a Griess reagent kit (Thermo Fisher Scientific, catalog G7921) and nitrate reductase (Sigma-Aldrich, catalog N7265), following the manufacturer's instructions. Briefly, arterial segments were homogenized in 1x phosphate-buffered saline (PBS) and then centrifuged at 10,000 × g for 10 minutes at 4°C. Following centrifugation, 50 µL of the supernatant was transferred to a 96-well

plate, and 50 µL of Griess reagent was added (composed of 5% H_3PO_4, 0.2% N(1Naphthyl) ethylenediamine, and 2% sulfanilamide). After a 10-minute incubation, the absorbance was measured at 540 nm using a FlexStation 3 microplate reader (Molecular Devices, San Jose, USA). Nitrite concentrations were determined from a standard curve prepared with nitrite solutions at final concentrations of 0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µmol/L.

In Situ Measurement of Nitric Oxide (NO)

Arteries were homogenized in 1x phosphate-buffered saline (PBS), and a 5,6diaminofluorescein diacetate (DAF-2 DA, 5 µM, Sigma-Aldrich, catalog #50277) probe was added to the homogenate for a 30-minute incubation at 37°C. Nitric oxide (NO) levels were analyzed by fluorimetry using a FlexStation 3 microplate reader (Molecular Devices, San Jose, USA), with excitation and emission wavelengths set at 485 nm and 538 nm, respectively. The results are expressed as relative light units (RLU) per milligram of protein.

Immunoblotting

The Intercellular Adhesion Molecule 1 (ICAM-1) expressionwas assessed by homogenizing samples in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml pepstatin A, 1 µg/ml leupeptin, and 1 µg/ml aprotinin. The proteins were then collected. For immunoblotting, 30 µg of protein was separated by electrophoresis on 10% polyacrylamide gels and transferred to 0.22 µm nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 hour. Primary antibodies were incubated overnight at 4°C: anti-ICAM-1 (1:500 dilution; Santa Cruz, catalog sc-8439) and antiGAPDH (1:20,000 dilution; Sigma-Aldrich, catalog G9545).

Statistical Analysis

Continuous variables are presented as means ± standard deviation, and categorical variables are presented as frequencies and percentages. The normality of the variables was assessed using the Shapiro-Wilk test. Vascular changes following photobiomodulation were analyzed using Two-way ANOVA, followed by Tukey's post

hoc test. A p-value < 0.05 (two-tailed) was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 9.1.2 (GraphPad Software, San Diego, CA, USA).

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