EVIDENCE OF THE ACTION OF ILIB IN HEMORHEOLOGY THROUGH DARK FIELD MICROSCOPY: A RANDOMIZED CONTROLLED CLINICAL STUDY

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INTRODUCTION

Epidemiology of Hematologic Diseases and Main Aspects of the ILIB

In the scenario of hematological diseases, Intravenous laser irradiation of blood (ILIB) at a wavelength of 630-640 nm has been developed for the treatment of various diseases since 1988 [1]. The accumulation of clinical and experimental data made it possible to elucidate the mechanism of action of this type of treatment, defining the indications and contraindications for its use in clinical practice and revealing the myriad of effectively treatable diseases.

In this context, for more than 25 years, studies have shown that ILIB acts directly on the parameters of all blood cells, plasma status, and all structural components of the vascular wall. Furthermore, by acting on immune system cells, hormones, and exchange processes, ILIB can influence all other systems of an organism [2].

Therefore, there is an exponential growth of clinical applications of ILIB in various pathological processes, such as pain treatment, tissue repair, and post-endovascular restenosis [2]. The literature has also documented the morphological alteration of mitochondria as a result of the use of ILIB, as well as the activation of metabolic energy processes [3-5]. In this sense, the ILIB process seems to modulate redox signaling in the respiratory chain by stimulating mitochondrial components and the plasma membrane.

In this aspect, the production of adenosine triphosphate (ATP) and the reduction in the generation of free radicals would be increased through the improvement of the flow of electrons through the respiratory chain [4,5]. Mitochondria stand out from other cellular organelles by having their genome (mtDNA), distinct from nuclear DNA. In mammalian cells, mitochondrial DNA is a circular molecule of about 16,500 base pairs that encodes 13 proteins, 22 tRNAs, and 2 rRNAs. All 13 proteins encoded by mtDNA are components of 4 of the 5 complexes of the oxidative phosphorylation system. During electron transport through the respiratory chain of the inner mitochondrial membrane, molecular oxygen can be reduced monoelectronically, generating reactive oxygen species, or ROS. These species are highly reactive with biomolecules, and in the intracellular environment, they can attack nucleic acids, proteins, and lipids [6-8].

As mtDNA is located close to ROS generation sites, mtDNA accumulates high levels of oxidative modifications, such as oxidized bases and single-strand breaks. Therefore, the integrity of the mitochondrial genome is critical for the maintenance of cellular homeostasis [8-11]. Mitochondrial damage may be central to the impairment of cells in regulatory systems, such as the nervous, endocrine, and immune systems, and

to the communication between them [12-14]. Therefore, type 2 diabetes mellitus, obesity, hyperglycemia, hyperlipidemia, and atherosclerosis may be related to mitochondrial dysfunction [15].

In this context, ILIB can be used in peripheral nervous system injuries by stimulating microcirculation, paralyzing the precapillary sphincters, causing vasodilation of arterioles and capillaries, and vascular neoformation, thus leading to an increase in blood flow in the irradiated area. It is also used for healing various tissues, as it stimulates an increase in the production of cellular ATP, causing acceleration in cellular mitotic activity [15].

Also, concerning the formation of rouleaux, which is caused by an increase in cathodic proteins, such as immunoglobulins and fibrinogen, 4 or more red blood cells stack up. Red blood cell membranes have a negative charge that causes these cells to repel each other and thus establish the "Bulk" state (stable detachment) [16], with increased Zeta potential. Zeta potential is an indicator of the stability of a dispersion. Large Zeta potentials predict a more stable dispersion. Zeta potential is also known as electrokinetic potential, it is measured in millivolts (mV) [17].

In colloidal components such as blood, the zeta potential is the electrical potential difference across the ionic layer around a charged colloid ion. The higher the zeta potential, the more stable the colloid. Thus, p Zeta potential that is less negative than -15 mV usually represents the beginnings of red cell agglomeration. When the zeta potential is equal to zero, the colloid will precipitate into a solid [17].

In this context, and as a corollary, the increase in immunoglobulins and fibrinogen decrease the density of negative charges on the surface of red blood cells, allowing them to attract each other through electrostatic interactions, Van Der Waals interactions, and even covalent bonding (base formation of Shiff). In this way, ILIB can break these binding forces between red blood cells, allowing hemorrhoids and consequent blood tissue homeostasis [16].

In this scenario, in the blood, its elements such as plasma proteins, some electrolytes, erythrocytes, leukocytes, and platelets are negatively charged. As they are of similar charge, they move away, that is, the solid elements remain close to the center of the vessel. This charge is measurable and is called Specific Conductivity (SC), which in human blood is, on average, 12,000 SC. This is the state of dispersion of the formed elements of the blood that ensures optimal vascular function [17].

In addition, human blood contains 19 electrolytes, 8 of which are essential (must be acquired from food) and 11 non-essential. Of the eight essential electrolytes, four are cationic and four are anionic. Of the eleven non-essential, also called trace minerals, eight are cationic and three are anionic. The main anions in blood plasma are chlorides (Cl-), carbonates (HCO3-), phosphates (HPO4-), and sulfates (S3O4-). They are primarily responsible for maintaining the forces of dispersion of the elements in the blood. The main cations are sodium (Na+), potassium (K+), calcium (Ca++) and magnesium (Mg++) [17].

The total dissolved electrolytes in plasma are equal to 9 g per liter of plasma (about 1 tablespoon), including essential and non-essential, so the average does not exceed 12,000 SC. Sodium, calcium, potassium, and magnesium are all cationic, totaling about 3.5 grams. Chlorides, carbonates, phosphates, and sulfates are anionic and amount to 5.5 g in an ideal combination [18].

Also, normal blood pH balances between 7.35 and 7.40. Above or below normal, amino acids play an important role in maintaining balance, as they can be transformed into anions or cations, as needed. If the medium is alkaline, anions are produced, if in an acidic medium, cations are released. We call this mechanism the Alkaline Reserve. Liquids or fluids are divided into three compartments: intravascular (plasma), extracellular (interstitial), and intracellular space. Plasma and interstitial can rapidly exchange ions, whereas intracellular ions are not easily exchanged [18].

If the ionic concentration of the plasma increases, half of the cations migrate to the extracellular medium, where they are stored to balance the medium. When the plasma concentration returns to normal, the electrolytes return to the plasma to be later eliminated by the kidneys. However, if the ionic concentration remains high, cation migration will continue until the extracellular fluid itself becomes hypertonic as well.

At this point, the body will have to produce water in an attempt to dilute the high concentration at the site. Edema, for example, is the accumulation of interstitial fluid resulting from high ionic concentration [18]. For this filtration to occur properly, the blood must be in constant motion. Therefore, it is essential to preserve its fluidity, which is only possible if there is no clotting [18]. In this aspect, ILIB can restore blood fluidity, as the greater the number of ions present, the greater the chance of decreasing the Zeta Potential, reducing dispersion forces, and increasing the possibility of clotting.

Chemical structure of erythrocytes and their physicochemical antigen-antibody mechanisms

The composition of the erythrocyte plasma membrane contains 39.5% proteins, 35.1% lipids, and 5.8% carbohydrates which are present on the extracellular side of the lipid bilayer. Glycolipids, which represent 10% of plasma membrane lipids, are sugar-containing lipids. These molecules are found exclusively in the extracellular layer (outer layer) of the plasma membrane. They have the function of allowing the cell to interact with the extracellular environment [19].

Glycophorins A is integral membrane proteins that contain a sialic acid residue [20]. Sialic acid residues are abundant in the plasma membrane of the erythrocyte, and 60% of the negative charge present in the erythrocyte membrane comes from the presence of sialic acid. The maintenance of the negative charge on erythrocytes is important in erythrocyte-erythrocyte and erythrocyte-blood cell interactions [21].

In this sense, the immunoglobulin molecules, or antibodies, present differences in the sequence of amino acids in the Fab portions, in regions called complementarity determining regions (CDRs, from the English complementary determining region). These regions form a surface complementary to the epitope (antigen-antibody binding site or site). In the antigen, the epitope determines the specificity of the antibody, conferring specific activity in the binding domains. The diversity at these antigen binding sites ensures that there is an almost limitless repertoire of antibody specificities [22].

In addition, CDRs determine the conformation of antigen-antibody binding sites. Antigens can bind to the antibody in different ways. Variation in the sequences of the antibody's variable chain domains determines antigen specificity. The variable chain regions of an antibody are different for each antibody molecule, and this variation is concentrated in a few locations. The regions located in the hypervariable sequence form the antigen binding site [23].

Furthermore, the antigen-antibody binding is done reversibly and can be understood as an interaction of macromolecules with their ligands in general. The antigen-antibody complex exhibits a high degree of chemical and structural complementarity, with the interaction of its surfaces [24].

Thus, the basic principle of thermodynamics in antigen-antibody interaction is the same as that of a reversible ligand reaction. The antigen-antibody reaction obeys the principle of the law of mass action. The equilibrium constant (Keq) measures the intrinsic affinity of the antibody for the antigen. Keq is defined as the concentration of [ac-ag] binding over the concentration of [ag] and [ac] [25]. This is the equation for the equilibrium constant:

$$\mathbf{Keq = k1/k2 = [ac - ag]/[ac] - [ag]}$$

Antibodies bind to antigens by contact in CDRs with amino acids, but the details of binding depend on the size and shape of the antigen. The light and heavy chains of CDRs create an antigen binding site. The sequences of the CDRs differ among antibodies, as do the forms created by these CDRs.

In this context, the binding forces involved in the specific interactions between antigens and antibodies do not present covalent bonds of a physicochemical nature. These specific interactions involve a variety of forces and can be undone by ILIB, high salt concentrations, extreme pH, temperature, detergent, and sometimes competition with high concentrations of the pure epitope itself. The forces involved in these conditions interfere with the antigen-antibody interaction, causing its disruption [26].

Electrostatic forces (ionic bonding) can be repulsive or attractive, depending on whether they act on equal charges or charges of opposite signs. Electrostatic interactions between antigen and antibody are a result

of the presence of one or more ionized epitope sites. These sites are typically formed by COO– and NH2+ or NH3+ groups of amino acids from antigen or antibody molecules, or similarly, altering structures of carbohydrates or other non-protein antigens. A hydrogen atom shared between electronegative atoms (F, N, O) leads to the formation of hydrogen bonds [26].

In this scenario, van der Waals forces, or electrodynamic forces, are fluctuations in the electron clouds around molecules oppositely polarizing neighboring atoms. Hydrophobic forces are hydrophobic groups interacting unfavorably with water that tend to cluster together to the exclusion of water molecules. The attraction also involves van der Waals forces. These interacting forces contribute to antigen-antibody binding. The distance between antigen and antibody molecules can change the forces involved in specific binding [27].

Electrostatic interactions occur between charged amino acid side chains. In hydrogen bonds and shorter-range van der Waals forces, interactions between electric dipoles can also occur. High salt concentrations and extreme pH weaken electrostatic interactions and/or hydrogen bonds, breaking the antigen-antibody bond [25].

In this context, electrostatic interactions can occur in the form of ion-ion, ion-dipole, and dipole-dipole, Van der Waals interactions, π-electrons and charge transfer complexes, forming ionic, hydrogen, and covalent bonds between the components polymers, as well as negative Gibbs free energy ($\Delta G<0$) [27,28].

This mixture of polycations and polyanions may have led to spontaneous aggregation and release of counter-ions, increasing entropy ($\Delta S>0$) [27]. Thus, the presence of counter-ions, as well as the presence of cations and anions of the individual polymeric chains, presents cellular and tissue reactivity, causing several diseases. Thus, it is necessary to use ILIB in an attempt to neutralize these harmful reactions in the blood colloidal system through laser photobiomodulation.

Laser Photobiomodulation

The absorption of laser light by tissues can occur through photochemical, photothermal, photomechanical, and photoelectric processes. In the group of photochemical effects, we can include biomodulation, which is the effect of laser light on molecular and biochemical processes that normally occur in tissues [29].

In this sense, it has been demonstrated in several studies, in vitro, and in vivo, that laser photobiomodulation at the cellular level stimulates the cytochrome-C-oxidase photoreceptor, resulting in increased metabolism and energy production, consequently increasing mitochondrial oxidative metabolism and initiating a cascade of cellular reactions that modulate biological behavior [29].

Thus, the low-intensity laser has the property of stimulating the plasma membrane and mitochondrial membranes, inducing the cell to biomodulation, that is, stimulating the normalization state of the affected region. When laser therapy is used in the visible electromagnetic spectrum, there is an initial photobiostimulation in the mitochondria, which activates a chain of biological events [30].

When irradiation occurs in the infrared spectrum, plasma membrane channels are stimulated, resulting in changes in membrane permeability, temperature, and pressure gradient. Both visible and infrared light can be absorbed by different components of the cellular respiratory chain, such as chromophores in cytochrome-C-oxidase or porphyrins, resulting in the production of reactive oxygen species or superoxide radicals [31].

The radiation emitted by low-power lasers has demonstrated analgesic, anti-inflammatory, and healing effects, and is therefore widely used in the tissue repair process. The therapeutic effects of morph differentiation and cell proliferation, tissue neoformation, revascularization, reduction of edema, greater cell regeneration, increased local microcirculation and vascular permeability are observed [31].

Therefore, laser therapy presents itself as an alternative for processes that present an inflammatory reaction, pain, and the need for tissue regeneration. The repair process constitutes a dynamic tissue reaction, which encompasses the following phenomena: inflammation, cell proliferation, and synthesis of constituent elements of the extracellular matrix, including collagen, elastic and reticular fibers. The molecular absorption of laser light allows an increase in cellular metabolism, characterized by the stimulation of photoreceptors in the

mitochondrial respiratory chain, changes in cellular ATP levels, the release of growth factors, and collagen synthesis. Acceleration of microcirculation results in changes in capillary hydrostatic pressure, with edema resorption and elimination of the accumulation of intermediate metabolites.

Main Clinical Findings of the ILIB – Literary Support

Low-level laser therapy, or photobiomodulation, is capable of inducing a photobiological response within cells; activating the production of adenosine triphosphate (ATP), NO, and reactive oxygen species; and alteration of sodium-potassium pumps and calcium channels in cell membranes, in addition to proving to be an efficient, non-invasive, low-cost and safe tool [32].

Among the different photobiomodulation methods, ILIB has been shown to provoke systemic effects. ILIB has been studied since 1981 by Soviet scientists; was developed for the treatment of cardiovascular diseases with evidence of improved blood rheological properties and microcirculation, as well as reduced infarction area, cardiac arrhythmias, and sudden death [32].

One study investigated the clinical effects of intravascular laser irradiation blood therapy (ILIB) on oxidative stress and mitochondrial dysfunction in individuals with chronic spinal cord injury (CCI) resulting from trauma. Twenty-four subjects with CCI (assigned to a sham group and a study group) and 12 normal subjects were recruited (sham). The study group underwent 1 hour of ILIB daily for 15 days for 3 weeks. The sham group underwent ILIB without laser power. Baseline measurements established greater oxidative stress and mitochondrial dysfunction in subjects with CCI than in normal subjects. On day 15 of therapy, the study group revealed significantly higher mitochondrial DNA (mtDNA) copy number, white blood cell adenosine triphosphate synthesis (WBC ATP), and total antioxidant capacity (CAT) with significantly reduced malondialdehyde (MDA), than the sham group. Intragroup comparison of the study group revealed significantly increased mtDNA copy numbers, WBC ATP, and CAT synthesis, with significantly reduced MDA compared to their baseline measurements. Intragroup comparisons of the sham group showed no statistical differences. Low-density lipoprotein (LDL) in the study group was significantly reduced on days 10 and 15, with high-density lipoprotein (HDL) significantly higher on day 45. Therefore, this study showed the efficacy of ILIB in relieving oxidative stress and mitochondrial dysfunction in CML patients [15].

Also, one study evaluated the effects of ILIB on blood metabolites in type 2 diabetic patients using metabolomics. Blood samples from nine type 2 diabetic patients were compared using metabolomics before and after ILIB. Results showed a significant decrease in glucose, glucose-6-phosphate, dehydroascorbic acid, R-3-hydroxybutyric acid, L-histidine, and L-alanine and a significant increase in blood L-arginine level ($p<0.05$). These findings support the therapeutic potential of ILIB in diabetic patients [33]. Furthermore, the verification of the effects of systemic photobiomodulation in the control of blood pressure (BP) in humans is a current, relevant and promising area of study, as it has contributed to the reduction and control of BP [34].

Another study investigated the clinical effects of ILIB on crossed cerebellar diaschisis (CCD) and evaluated the therapeutic effect in the subacute post-stroke phase. The 77-year-old man with cerebral infarction in the territory of the right anterior cerebral artery only underwent conservative treatment including hydration and aspirin in the acute post-stroke phase. Once the patient was in a stable condition, he underwent a daily one-hour ILIB (He-Ne laser) for ten consecutive days during the subacute post-stroke stage. Single-photon emission computed tomography (SPECT) was used before and after intravascular laser irradiation to detect changes in cerebral and cerebellar perfusion. Then, the two images were compared. DCC was detected using the first SPECT. After ILIB intervention, the second SPECT showed greater perfusion in the affected cerebellar hemisphere. Stroke patients can therefore benefit greatly from ILIB [35].

Besides, a study clarified the specific features of immunological disorders in patients with chronic endometritis and corrected them with the help of the application of ILIB in combination with standard treatment. The study included 30 women of reproductive age with a proven diagnosis of chronic endometritis in partial remission. Patients were divided into two groups. Patients in group 1 (control) were treated with pharmacotherapy alone, while those in the main group (group 2) received standard therapy supplemented by

intravascular laser blood irradiation in the form of daily 25-min sessions over 7 days with the use of the ≪Mulatto≫ device with an output power of 2 MW at a wavelength of 0.63 microns. In addition, a third comparison group was formed, to which healthy women of the same age were recruited. The levels of cytokines, complement components, and immunoglobulins were determined in blood plasma. More reliable correction of immunological disorders was achieved with the use of low-level laser blood irradiation compared to drug therapy alone [36].

An integrative literature review study that included non-randomized and randomized controlled trials that specifically evaluated the therapeutic effect of ILIB in chronic systemic diseases. After applying the inclusion and exclusion criteria, 13 articles were selected, mainly randomized controlled studies. Despite the varied parameters and protocols for the use of this type of therapy, all studies have shown satisfactory results in the clinical picture of patients. ILIB proved to be effective in all organ systems, showing some positive results. However, studies on the effect of this therapy on various diseases are still scarce in the literature, and there is a need for better-designed clinical trials to better understand the role of ILIB in various systemic diseases [37].

OBJECTIVE

Primary

To analyze, employing dark field microscopy, the effect of ILIB irradiation on slides containing blood samples from 20 participants of this study who will receive radiation for a time of one-hundredth of a second, as well as to compare these samples with the slides that were obtained later by in vitro irradiation. live through the carotid for 12 seconds to assimilate the radiation.

Secondary

- Try to understand through laboratory tests the possible reduction in inflammatory and immunological processes in the blood colloidal system through the use of ILIB;
- To seek to know the possible physicochemical mechanisms of laser photobiomodulation, both at the level of blood sampling and the level of the organism.
- Analyze and compare the effects that the laser causes on the cells of stationary tissue, such as skin, bone, etc., with dynamic blood.

Hypothesis

It is hypothesized that photobiostimulation provides the photochemical stabilization of the cytoplasmic membrane of said cells, allowing the maintenance of the predominance of negative charges and the increased Zeta potential, with a decrease in entropy ($\Delta H<0$), thus allowing the dissolution of the rouleaux and consequent reduction inflammatory processes and comorbid processes.

JUSTIFICATION

The process of light absorption by biological tissue is the central point of laser-tissue interaction and involves the transfer of photon energy (or quantum of light radiation) from the light beam to the absorbing molecular species present in the tissue, called chromophores. This process is present in a series of applications such as fluorescence diagnostic techniques, therapeutics (Low-Level Laser Therapy), currently called Photobiomodulation (Photobiomodulation) and Photodynamic Therapy-Photodynamic Therapy) and surgical (photophotothermal and photomechanical coagulation and ablation) involving coherent luminous electromagnetic radiation, namely the Laser [38-40].

The absorption of light by a specific biological tissue depends on the optical properties of the tissue (absorption coefficient, μA (cm^{-1}). of the wavelength (type of laser) to be used in the procedure in question. The absorption rates of photons from a laser by the tissue are directly related to the power density (W/cm^{-2}) or in the present case the linear energy density (J/cm), absorption coefficient μA (cm^{-1}), and the exposure time t(s) of the tissue to the Laser jointly determine the predominant type of Laser-tissue interaction, 1) photochemical 2) photothermal, 3) photomechanical, with always a predominant mechanism and effect [39].

In this context, as a justification for the present study, before the blood passes through the laser beam, it will be in the dark, and after passing it, it returns to the dark. Therefore, within the possible actions of the laser in such a short exposure (hundredth of a second), there may be the possibility of dissolution of the rouleaux. This facilitates the rate of red blood cell sedimentation, a phenomenon that can be seen on a peripheral smear. When a rouleaux formation is present, it is caused by an increase in cathodic proteins such as immunoglobulins and fibrinogen. Rouleaux formation refers to the stacking of 4 or more red blood cells. Red blood cell membranes have a negative charge (zeta potential) that causes red blood cells to repel each other. In the presence of increased positively charged plasma proteins, such as fibrinogen or immunoglobulins, the negative charge on the surface of the red blood cells decreases, allowing the red blood cells to bind through electrostatic interactions. Although myeloma and macroglobulinemias are considered first by hematologists, other causes occur more frequently, such as acute and chronic infections, connective tissue diseases, and chronic liver disease.

Therefore, any other known effect of ILIB such as fibrinolytic action on fibrinogen, SOD, serum-plasmin, catalase-peroxidase, and poikilocytosis may be part of the secondary rather than primary effects.

METHODS

Study Design

This study is a prospective and randomized clinical trial with 20 participants who will be properly selected based on the inclusion and exclusion criteria. The rules of the CONSORT Platform (The clinical research, available at: http://www.consort-statement.org/) will be followed.

Sample Calculation (Sample Power)

The determination number of patients needed will be calculated using the Minitab software. The calculation was performed using ANOVA for repeated measures, with a significance of 5% and a test power of 95%. According to sample calculation, 20 participants will be divided into 2 groups. The number of participants per group will depend on the randomization process.

Eligibility

Inclusion criteria

- Individuals with comorbidities, but with normal daily activities.**Exclusion Criteria**
- Individuals hospitalized or with compromised daily activities.
- Patients over 60 years of age, of both genders, using anticoagulants, with or without a history of cancer, diagnosed with psychiatric disorders, anemia, or specific blood disorders.**Participants and Samples**According to the sample calculation, 20 participants will be selected according to the eligibility criteria. These participants will be divided into two groups after the randomization process. Only participants who will receive ILIB irradiation via the carotid artery will be randomized. Each participant will provide a total of 3 blood samples (in triplicate each sample), with a total of 9 slides, 3 slides will be irradiated in a dark field microscope (positive control), and 3 slides will not be directly irradiated (from the blood later irradiated from the participant) and 3 slides will not be irradiated (negative control), as shown below:

- **Slide 1:** Blood samples that will receive direct irradiation from the ILIB (positive control from the same participant);
- **Slide 2:** Blood samples from ILIB irradiation via the carotid in the participant. This subgroup of participants will be randomized.
- **Slide 3:** Blood samples without ILIB irradiation (negative control from the same participant).

As 20 participants will be compared, the total number of samples on slides will be one hundred and eighty (n=60). Blood samples irradiated by ILIB on slides will be analyzed under a dark field microscope. The total number of participants in each group will be known after the randomization process.

Groups

Each participant in each group will provide a total of 3 blood samples (in triplicate). The groups will be divided into:

Group I - 3 slides will be irradiated under a darkfield microscope (positive control);

Group II - 3 blood samples from ILIB irradiation through the carotid in the participant;

Group III - 3 slides will not be irradiated (negative control);

Recruitment and Randomization

Recruitment will be carried out in compliance with Resolution 466/2012, which regulates research with human beings in Brazil. Recruited patients will have their medical records analyzed for demographic and descriptive characterization of the sample. The nursing staff will conduct an initial interview to complete the clinical record and perform hemodynamic and anthropometric measurements, as well as blood collection and ILIB interventions. For the application of photobiomodulation. The ILIB will be positioned for 12 seconds in the carotid artery.

The randomization mechanism provided by Sealed Envelope Ltd will be used. (London, UK) [Sealed Envelope Ltd. 2017: Create a blocked randomization list. [Online]. Available at: https://www.sealedenvelope.com/simple-randomiser/v1/lists. Participants will be randomized into two groups, Group 1 to perform direct irradiation of the ILIB via the carotid artery, and Group 2 to perform direct irradiation of the ILIB in blood slides. Participants will be identified by sequential numbers, according to the order of recruitment. Only the participants who will receive the irradiations via the carotid will know the group they were directed to. Data analysis will be performed by a third person who will also not know the group allocations.

A data collection instrument will be developed to contain the information necessary to achieve the proposed objectives. The variables of comorbidities such as diabetes, cardiovascular diseases, dyslipidemia, metabolic syndrome, anthropometric data, arterial hypertension, heart rate, biochemical markers before and after the use of the ILIB, as well as an approach to the use of medications, diet, and complementary information will be recorded.

Location and Equipment

The procedures will be performed at Instituto Ricardo Trajano, R. Alm. Noronha, 558 - Jardim São Paulo, São Paulo - SP, 02043-060.

The laser equipment to be used in the present study has the Easy-Laser model. Registered with Anvisa and certified by Inmetro, the RIWT laser is a laser therapy device designed by Ricardo Trajano, Scientific Director of RIWT (Ricardo Trajano Institute) and Founding President of the Brazilian Laser Society. Launched in 2009, the laser therapy device has advanced technology to meet demands in all medical, paramedical, general health, aesthetics, and well-being specialties. Available at: https://www.ricardotrajano.com.br/aparelho-laserterapia.php. Accessed December 22, 2020.

The dark field microscope to be used in this study is the H 600 LL 100 HP dark field

microscope, manufacturer: Helmut Hund GmbH©, 2013). Specifications were available at https://www.hund.de/images/Microscopy_Pres.pdf. Accessed December 22, 2020.

Procedures and Interventions

According to the absorption spectra of blood and hemoglobin, a photon binding energy formula is established using physical methods and the effects of low power laser at different wavelengths are analyzed. Results show that lasers with peak wavelengths of 200 240, 275, and 342 nm on the whole blood absorption spectrum curve are easy to destroy protein molecules and then lead to loss of biological activity. of hemoglobin. Although lasers with wavelengths greater than 800 nm reduce the oxygen-carrying capacity of the blood, only lasers with wavelengths between 630 and 670 nm have the best effectiveness [41].

Thus, based on this study, the present study will use the ILIB with continuous emission mode, device power of 100mw, 4J, and 660 nm, in the time of one-hundredth of a second and 12 seconds.

Δετερμινατιον οφ τηε ζετα ποτεντιαλ (ζ) οφ τηε βλοοδ σαμπλε

To determine the zeta potential of blood samples, 50 µL of each sample will be diluted in 1.5 mL of NaCl solution (0.001 M) prepared with Milli-Q ultrapure water, to ensure adequate signal intensity by the equipment. Measurements will be made by measuring electrophoretic mobility by laser Doppler anemometry, in triplicate, at a temperature of 25 °C and pH 7.0, using the Zetasizer 3000 HS equipment.

Viability of blood cells with trypan blue

10.0 uL of cell solution will be placed in the Neubauer chamber for the quantification of live and dead cells, with replicates of n = 3.0. After placing a sample of the cell suspension in this chamber so that it is filled, but there is no extravasation, the viable (unstained) cells are counted under the microscope. The chamber has four quadrants (Q1, Q2, Q3, and Q4) with an area of 0.1 mm2 each. After determining the n (total cells in all four squares), Equations 1 and 2 below indicate the concentration of cells in the suspension (number of cells per mL):

$$Cells / mL = \frac{\sum Q1Q2Q3Q4}{4} * FD * 1 \times 10^4 / mL$$

Cell viability calculation: CV (%) = number of live cells / number of total cells (live + dead) (Equation 02).

Ethical Aspects

This research project will be submitted to an Ethics and Research Committee. The data will be kept confidential in accordance with the ethical principles that are in resolution 466/12 of the National Health Council. Patients will provide a free and informed consent form, with the possibility of withdrawing from the registration at any time, free of charge for their follow-up.

Clinical Trials Record

The protocol of this study will be registered in the Brazilian Registry of Clinical Trials - ReBEC (http://www.ensaiosclinicos.gov.br), as well as registered in the International Platform of Nature and Scientific American.

Statistical Analysis

Data were collected using a table previously built in Excel, containing the dates of collection, the variables that were collected and the medical record number. The variables were presented in percentage, mean and standard deviation format. Depending on the Gaussian distribution (Test of normality), the comparisons of the variables were performed using the Person Test and One-Way ANOVA Test (Tukey) between the

variables of the present study, considering $p<0.05$ with statistical significance, in the 95% CI. A logistic regression analysis was also performed to analyze the association between the diameter of the gastrojejunal anastomosis and complications, considering $p<0.05$ with a statistically significant influence, in the 95% CI. Statistical analysis was performed using the Minitab 18® program (version 18, Minitab, LLC, State College, Pennsylvania, USA) [42].

OUTCOMES

Primary Outcome

It is expected to observe the effectiveness and efficiency of ILIB in the dissolution of reouloux from the blood, both directly on the slides and on the subject of this study.

Secondary Outcome

It is expected to observe an improvement in the comorbidities presented by the participants of the group that will receive irradiation in the carotid artery.

RISKS AND BENEFITS

Risks

The risks that participants will be exposed to will be minimal and will be related to common procedures for obtaining blood samples by nurses, such as contamination and bruising in the blood collection area. There are no facts yet documented with scientific evidence of possible risks that ILIB may cause.

BENEFITS

The benefits will outweigh the risks, given that this study will provide valuable information about the photobiomodulatory effect of ILIB on the blood colloidal system, dissolving agglomerations of red blood cells, restoring blood circulation homeostasis, reducing inflammatory processes, activating balanced mitochondrial functions and reduction of important comorbidities such as diabetes and arterial hypertension.

FUNDING

Own funding.

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