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# Immunohistochemistry Analysis of Animals with Post-Ischemic Stroke Treated with LED

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

**Objective:** To evaluate the effects of 630nm LED and 904nm LED in animals subjected to ischemic stroke, using the immunohistochemistry of these animals as analysis parameters.

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**Methodology:** The present work presents an experimental study with a controlled intervention that included 75 male Wistar rats. The animals were allocated into three groups: a control group consisting of 15 animals without treatment, a group treated with 630nm LED, and a group treated with0 904nm LED, composed of 30 animals each. All animals underwent stereotaxic surgery for electrode implantation and subsequent electrolyte injury. The results were compared on days 3, 7, and 21 of treatment after surgery.
**Results:** The immunohistochemical analysis showed an improvement in the inflammatory response and tissue repair of the animals of both groups treated.
**Conclusion:** The present study suggests that the treatments with 630nm LED and 904nm LED favored a significant response, which may be a promising technique because of its simplicity, low cost, and easy application.

*Keywords: 630nm LED; 904nm LED; neurogenesis; motor behavior.*

## ABBREVIATIONS

*21CG : 21-day control group*
*3CG : 3-day control group*
*7CG : 7-day control group*
*ATP : adenosine triphosphate*
*CD68 : Cluster of Differentiation 68*
*CG : Control Group*
*CNS : Central Nervous System*
*DAB : 3,30-diaminobenzidine*
*GPAF : Glial fibrillary acidic protein*
*IHC : Immunohistochemistry*
*PBS : Phosphate-buffered saline*
*PVC : Polyvinyl chloride*
*TG630 : Group treated with 630 nm LED*
*TG630-21 : Group treated with 630 nm LED for 21 days*
*TG630-3 : Group treated with 630 nm LED for 3 days*
*TG630-7 : Group treated with 630 nm LED for 7 days*
*TG904 : Group treated with 904 nm LED*
*TG904-21 : Group treated with 904 nm LED for 21 days*
*TG904-3 : Group treated with 904 nm LED for 3 days*
*TG904-7 : Group treated with 904 nm LED for 7 days*

## 1. INTRODUCTION

"Stroke is a condition that may result in neurological damage and lead to disability and death. Stroke is a vascular lesion that occurs in the brain region, causing damages through the interruption of blood support. This injury becomes increasingly challenging, as, over the last 40 years, it has remained one of the leading causes of death and severe long-term disability, triggering great economic and social impact worldwide" [1,2].

According to the World Health Organization [3] "stroke is the second leading cause of death globally". "Neurological injury is the second leading cause of death, according to the Ministry of Health (2013) data, reaching 400 thousand cases per year and more than 100 thousand deaths. Every 5 minutes, there is a death by stroke" [4].

"Stroke is characterized as a temporary or definitive deficit caused by an alteration in blood circulation in the brain, which can damage one or more parts of this organ. The damage may be of two kinds, ischemic or hemorrhagic, and compromises neurological function. Several risk factors are associated with stroke development; however, the incidence is increased among the elderly, the most vulnerable population to this condition" [5].

"The LED has shown promising results when used as a treatment for several diseases,

benefiting the pathological state of numerous neurological disorders, including stroke. In addition, to increase adenosine triphosphate (ATP) production, the LED treatment may modulate reactive oxygen species, activate replication of mitochondrial DNA, increase early response genes, increase growth factor expression, induce synapses, and stimulate cell proliferation. Moreover, in animal studies investigating neurogenesis, the LED treatment showed satisfactory results" [6,7,8].

"The FBM therapy was introduced as an innovative modality for stimulating neural activity to improve brain function. This therapy is based on a light that involves exposing neural tissue to a low fluence through various light delivery methods capable of absorbing several wavelengths" [9,10].

"The FBM has been investigated as an alternative treatment for stroke and showed neuroprotective effect while regulating several other biological processes" [11,12]. "The light is able to penetrate many tissues, including the scalp and skull, and reach the brain. Several clinical and preclinical studies showed that this process could improve stroke recovery" [13]. "These positive results may be due to the increase in local blood flow, especially in brain tissue, enhancing protein expression through signaling mediators and transcription activation, promoting neurogenesis and synaptogenesis" [14, 15,16].

"Neurogenesis is the biological process that involves coordinated proliferation, differentiation, and migration of cells to form neural tissue in the brain and may be involved in targeting and repairing tissue damaged by an ischemic stroke. Animal studies using LED as a treatment for neural injuries showed increased neurogenesis and tissue repair" [17,18,19].

The objective of the present study was to evaluate the effects of 904nm LED and 630nm LED as a method of treatment in animals submitted to ischemic stroke, using immunohistochemistry as the parameters for analysis [20].

## 2. METHODOLOGY

### 2.1 Sample

The sample consisted of 75 male Wistar rats, acquired from the bioterium of the State University of Londrina – UEL, of approximately 2 months of age and weighing approximately 200 g. The animals were separated into groups, and five rats were allocated per cage.

### 2.2 Experimental Groups

The animals selected for the study were allocated into 3 groups: Control Group (CG), with 15 animals equally allocated into three subgroups (there was no intervention of LED irradiation in the animals belonging to this group). The subgroups were labeled: 3-day control group (3CG), in which animals were euthanized on the fourth day, 7-day control group (7CG), with euthanasia on the eighth day, and 21-day control group (21CG), with euthanasia on the twenty-second day.

Group treated with 630 nm LED (TG630), with 30 animals equally allocated into three subgroups: group treated with 630 nm LED for 3 days (TG630-3), with euthanasia on the fourth day, group treated with 630 nm LED for 7 days (TG630-7), with euthanasia on the eighth day and group treated with 630 nm LED for 21 days (TG630-21), with euthanasia on the twenty-second day.

Group treated with 904 nm LED (TG904), with 30 animals equally allocated into three subgroups: group treated with 904 nm LED for 3 days (TG904-3), with euthanasia on the fourth day, the group treated with 904 nm LED for 7 days (TG904-7), with euthanasia on the eighth day, and the group treated with 904 nm LED for 21 days (TG904-21), with euthanasia on the twenty-second day.

### 2.3 Surgical Procedure

The animals were anesthetized with a solution containing 80mg/kg of Ketamine Hydrochloride (10mL bottle) to 15mg/kg Xylazine Hydrochloride (10mL bottle) using an intra-abdominal application. After anesthetized, the animals were located on a stereotaxic apparatus (David Kopf model, USA), where their heads were fixed by the external auditory meatus and upper incisors.

Part of the tissue overlying the skull was cut, leaving the calvaria exposed and cleaned with 2% hydrogen peroxide. Thus, the bregma was located as a reference point, and the skull was

drilled with a 2mm dental drill to implant the electrode in the calvaria and reach the internal capsule.

The implant location was determined from the stereotaxic coordinates obtained with the atlas of Paxinos and Watson [6]. The stereotaxic coordinates used were AP= -1.72 mm, ML= -3.4 mm, and DV= 4.4 mm, respectively, using the bregma as a reference, with the lambdoid and bregmatic sutures in the same horizontal plane. After implantation, the electrodes were fixed to the calvaria by a self-curing acrylic prosthesis, using the Self-curing Acrylic Resin Kit VIPIFLASH®.

## 2.4 LED Treatments

The LEDs used in the present study were manufactured of Polyvinyl chloride (PVC) material. The device consisted of seven LEDs, each with a 5mm diameter encapsulation, geometrically positioned 1mm apart from each other so that they all focused the light on a single area. Therefore, the device's intensity value is compatible with the value of the clinical application using the "photobiomodulation" technique from a height of one centimeter away from the application region. The equipment consisted of LEDs that emitted red light with λ = 630nm, in which the power measured by the equipment (brand Thor Labs model: PM100D) was 70 mW. The LED model used was RL5-R12008. The LED used in this study was produced by Superbright LEDs company [7,21].

The application was punctual, with contact, optimizing the LED biophysical properties. The application, was performed in the animal's brain region for 104 seconds, once a day, starting after 12 hours of stroke induction, for the amount of time required for each experimental group. The dose used in this study was 7 J/cm² in the e]. experimental groups, totaling an energy density of 21 J/cm² in the group treated for 3 days; 49 J/cm² in the group treated for 7 days; and 147 J/cm² in the group for 21 days [21].

With the λ= 904nm LED, the application method was similar; however, the application duration was 63 seconds, with a dose of 7 J/cm2, obtaining 110 mW in the treated groups, to obtain useful irradiation intensities for clinical application in phototherapy [17].

## 2.5 Euthanasia

The animals were carefully separated in order to avoid any stress. First, the animal was immobilized by a scientist; then, a second scientist infused the anesthetic via intraperitoneal with 80 mg/kg of Ketamine and 15 mg/kg of Xylazine. After checking the anesthetic status, the animal received 175mg/kg of intraperitoneal thiopental [21].

## 2.6 Immunohistochemistry

For the Immunohistochemistry (IHC) technique, slides were made as described for histological analysis, containing two sections per slide, per experimental group. First, the thermal deparaffinization was performed for 16 hours in an incubator at 60°C. Subsequently, the chemical deparaffinization was performed, immersing the slides in a xylene bath twice for 10 min, and immersing the slides in alcohol at 70 °C for 10 extra minutes. After staying in the water bath, the slides were immersed in a sodium citrate solution in a closed container and placed in a water bath for 30 min. for antigen recovery.

The field marking was performed as described by Panis, 2011. First, the sections were delimited with a Dako Pen® hydrophobic pen, and the endogenous peroxidases were blocked with a 10% hydrogen peroxide solution for 30 minutes, followed by the blocking of nonspecific binding by incubation in 0.1% fetal serum for 1 hour.

Subsequently, the sections were incubated with primary antibodies from the Santa Cruz Biotech brand, CD68 (Cluster of Differentiation 68), and GPAF (glial fibrillary acidic protein) (1:300) in a humidity chamber at 4°C for 2 hours. After incubation, the slides were subjected to three baths (5 minutes) in Phosphate-buffered saline (PBS) and then incubated with the secondary antibody for 15 minutes. Next, the sections were jet-washed with PBS, followed by three extra washes with PBS drops.

The marking was revealed by incubation with 3,30-diaminobenzidine (DAB) for 15 minutes, followed by two washes with PBS, the first using a jet and the second, a drop. In the last stage, the sections were lightly counterstained with Harry's Hematoxylin (Merck) for 30 seconds and then rinsed under running water.

The sections were incubated in alcohol at 70°C for 5 min. in an immersion bath. The material was then incubated in alcohol at 95°C for 5 min. in an immersion bath, followed by incubation in xylene for 5 min. and another incubation in xylene for 10 min. After draining all the liquid, the slides were mounted with the Canadian balm and a coverslip.

## 3. RESULTS AND DISCUSSION

In the immunohistochemical analysis, the presence of glial fibrillary acidic protein (GFAP) we evaluated. According to Nardin 2016 and Grangeiro 2016, central nervous system (CNS) disorders can cause changes in glial cells and trigger reactive astrogliosis, which can lead to synaptic loss and neuronal death, being observed by the increased expression of GFAP.

Fig. 1 shows intense astrocytic expression (GFAP) in CG in the first two moments (3 and 7 days), suggesting reactive astrogliosis and consequent neuronal impairment. In the groups treated by both 630nm LED and 904nm LED, a mild response was noted, in which astrocytes with normal structures were observed, without thickening of the extensions, as observed in the CG 3 and 7 days, possibly indicating that the inflammatory response was more controlled in these groups.

Fig. 2 shows the immunohistochemical sections labeled with CD68, a lysosomal membrane marker that stains microglia in a state of active phagocytization. After a stroke, harmful agents are released and trigger pro-inflammatory responses that cause local tissue damage and activate the microglia present (Rolim et al, 2020).

Intense marking was observed in 3CG, 7CG, and 21CG with rounded aspects, indicating the microglia's alteration, constituting macrophages with phagocytic capacity. In the TG630-3 and TG630-7 groups, a moderate change in microglia was observed, while in TG630-21, TG904-3, TG904-7, and TG904-21 groups, the changes in microglia were milder.

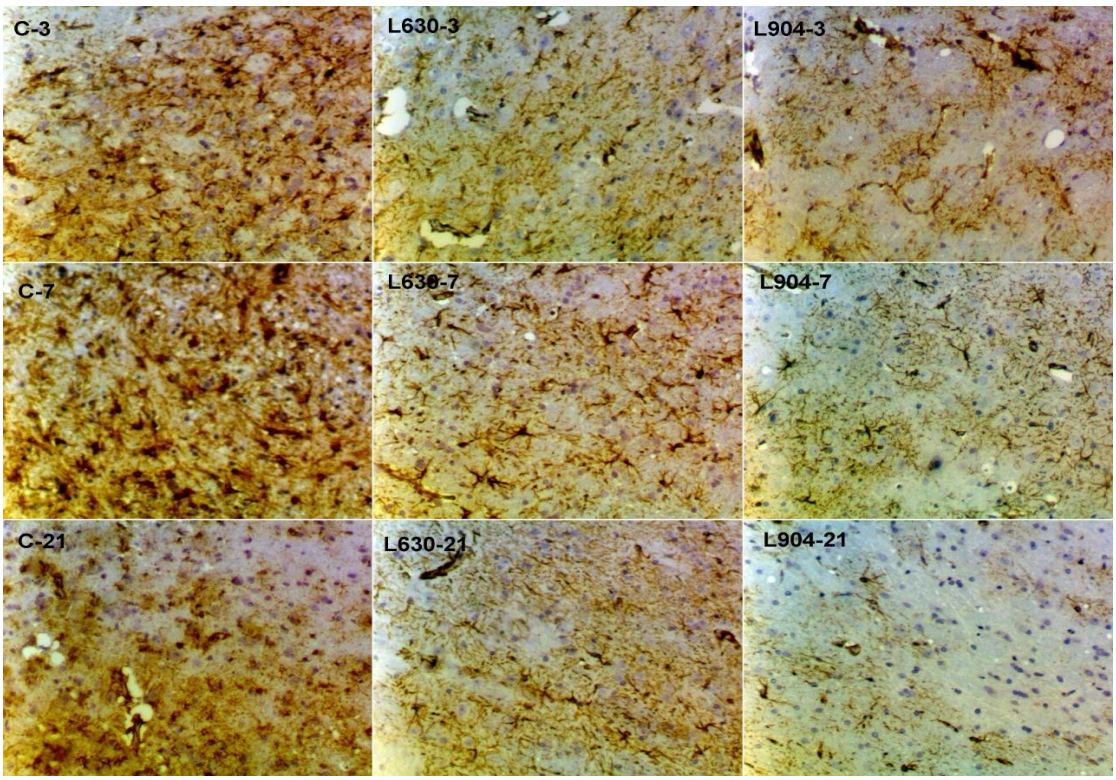


**Fig. 1. Photomicrograph of the immunohistochemical sections of the CG, 630nm LED, and 904nm LED groups at 3, 7, and 21 days**

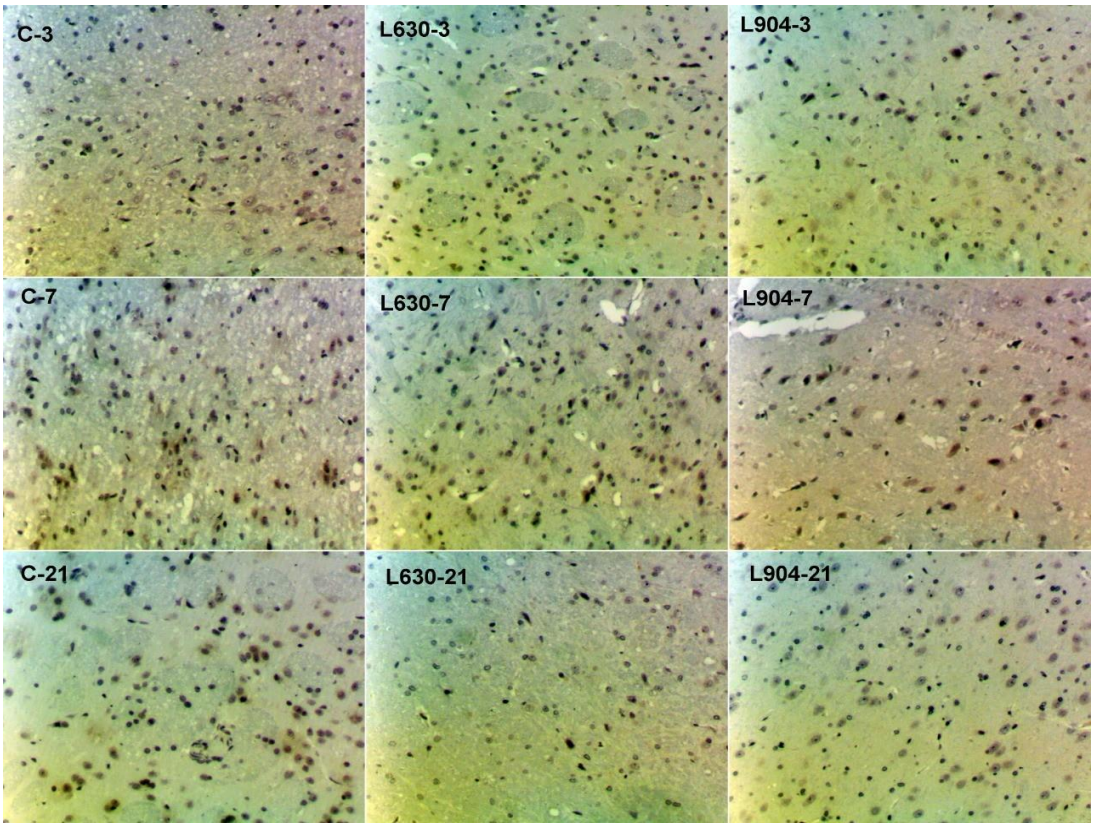


**Fig. 2. Photomicrograph of the immunohistochemical sections of the CG, LED 630nm, and LED 904nm at 3, 7, and 21 days of treatment, with CD68 marker**

The present study evaluated the photobiomodulation after stroke induction through treatment protocols with two LEDs of different wavelengths and at different experimental times of treatments. In general, the results demonstrated that the inflammatory response was more controlled in the treated groups than the untreated control group [22].
Disorders of the central nervous system (CNS) may cause changes in glial cells and trigger reactive astrogliosis, which can be observed by the increased expression of GFAP. GFAP are filamentous proteins expressed by the CNS cells, such as astrocytes, that participate in physiological and metabolic processes that maintain homeostasis [23,24].

Reactive astrogliosis is important for astrocytes to act in the protection of the CNS. This process is associated with morphological, molecular, biochemical, and physiological changes of astrocytes so that the formation of a scarring border occurs to seal the tissue against injuries [21,25].

In general, although GFAP is not an absolute marker of this process and is not strictly correlated with astrocytic reactivity, its increase is a strong indication of reactive astrogliosis [26].

In this context, based on the results observed in the analysis obtained, it can be said that the control group remained longer with increased marking, while the treated groups showed lower expression of GFAP after 7 days, with emphasis on the group treated with 904nm LED, suggesting that the response to the injury, after this time of treatment, was already more controlled.

After the stroke, harmful agents are released and trigger pro-inflammatory responses that cause damage to the local tissue and activate the microglia present [27]. Microglia are the main immune cells of the brain; however, it is still not well understood how these cells influence the activity and survival of neurons against CNS lesions (SZALAY et al., 2016). According to Zhang et al (2019) [28], different studies showed

that microglial phenotypic transformation is related to different cellular functions, with the M1 phenotype being pro-inflammatory and the M2 phenotype anti-inflammatory and tissue repair. The authors reported that M2 has neuroprotective effects after stroke and promotes tissue repair, debris phagocytosis, and regeneration after cerebral ischemia. M1 may aggravate brain damage due to the production of pro-inflammatory cytokines [28].

A greater marking was observed in the control group, which can be attributed to the presence of the pro-inflammatory microglial phenotypic due to the inferior performance presented in other aspects studied. High marking was also observed in the group treated with 904nm LED; however, this group showed better results than the other analyses. Therefore, it is believed that the greater presence of this phenotype may be related to the function of the anti-inflammatory and tissue repair microglia at this time.

## 4. CONCLUSION

Based on the results presented in this study, it is concluded that the treatments with LED 630nm and LED 904nm showed promising results after stroke in the analyzed periods, showing improvements related to inflammatory response and tissue repair. The findings suggest that the application of phototherapy as treatment may have promising results not only for stroke but also for other neurodegenerative diseases, being an accessible, easy-to-apply, and non-invasive therapy.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

The procedures were performed at the Laboratory of Neuroanatomy and Neurophysiology of University of the Midwest (UNICENTRO), according to the Ethics Committee on the Use of Animals – CEUA of UNICENTRO, approved by protocol number 034/2017.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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