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DE BIOMAS TROPICAIS



Programa de Pós-graduação em  
**ECOLOGIA**  
DE BIOMAS TROPICAIS  
UFOP

MASTER`S DISSERTATION

EFFECTS OF THE PHOTOTHERAPY (BLUE LIGHT)  
ON THE HEART INFLAMMATORY RESPONSE INDUCED BY  
*Trypanosoma cruzi* IN MICE

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2019

NATALIA IVANOVA

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Dissertation submitted to the Post graduation program of Ecology of Tropical Biomes at Universidade Federal de Ouro Preto as a requisite for the attainment of the master's degree, under supervision of Prof. André Talvani (UFOP) and co- supervision of Prof. Rodrigo Fernando Bianchi (UFOP).

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**“Efeitos da fototerapia com luz azul (LED) na resposta  
inflamatória cardíaca em camundongos infectados pelo  
*Trypanosoma cruzi*.”**

*Autora: Natalia Ivanova*

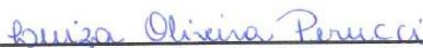
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## DECLARAÇÃO DE PARTICIPAÇÃO À DISTÂNCIA EM BANCA EXAMINADORA

Ribeirão Preto, 29 de novembro de 2019.

Eu, **DRA. MARIA CLÁUDIA DA SILVA**, CPF **334.476.038-60** declaro que participei, de forma síncrona remota, no dia 29/11/2019, com os demais membros que assinaram a ata física deste ato público, da banca para defesa de dissertação de **Natalia Ivanova**, discente do Programa de Pós-graduação em Ecologia de Biomas Tropicais - nível Mestrado Acadêmico, da Universidade Federal de Ouro Preto.

Considerando a avaliação do trabalho intitulado *Efeitos da fototerapia com luz azul (LED) na resposta inflamatória cardíaca em camundongos infectados pelo Trypanosoma cruzi* e os questionamentos da banca examinadora, ao preencher e assinar este documento, reitero minha decisão que o estudante pode ser considerado:

- Aprovado.  
 Aprovado com condicionamentos e/ou modificações.  
 Reprovado.

Atenciosamente,

  
**Dra. Maria Cláudia da Silva**  
Pós-doutoranda da Universidade de São Paulo

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## ABSTRACT

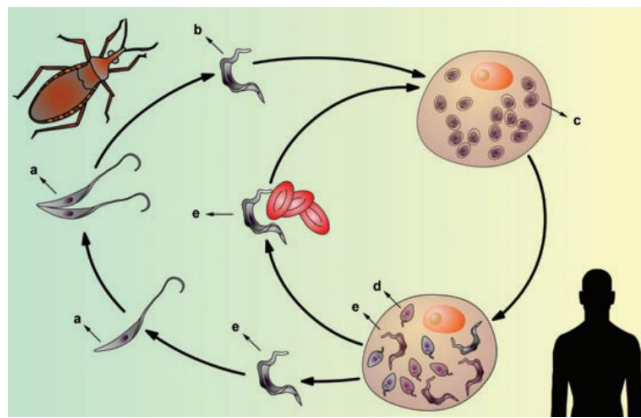
The *Trypanosoma cruzi*, is the causative agent of Chagas disease. According to WHO (2019), currently about 7 million people are infected with *T. cruzi* mainly in Latin America. Despite the fact that pharmaceutical industry releases various new drugs every year, Chagas disease has only two standard substances for treatment, Benznidazole and Nifurtimox, which were recognized as chemotherapy. However, at the same time these medicines have many side effects and are inefficient in chronic phase. Nowadays the blue light is commonly used in medicine practice around the world. It was proved its antimicrobial characteristics. In the current study, were evaluated the influence of the blue light on the inflammatory parameters in C57BL6 mice (*in vivo*) infected with *T. cruzi* and as well as development of parasites *in vitro*. For *in vitro* experiment we used Y and CL strain of *T. cruzi*. The blue light was applied (with the pic 460 nm and 80  $\mu\text{W}/\text{cm}^2$ ) on parasite cell culture for 5 days, 6h per day, at room temperature. The parasites were counted in 10  $\mu\text{L}$  every day using the Neubauer Chamber. Treatment with the blue light led to the reduction of replication of *T. cruzi* *in vitro*. Data showed 50% reduction of parasites in experiment after 5 days of exposition, in comparison to control. For *in vivo* experiment was used Y strain of *T. cruzi*. 40 animals were separated into 4 groups: non-infected + conventional light, non-infected + blue light, infected + conventional light, and infected + blue light. Blue light was applied (with the pic 460 nm and 7  $\mu\text{W}/\text{cm}^2$ ) for 9 days, 12 h per day. Was found that phototherapy with blue light reduce quantity of parasites circulating in the blood of animals comparing with conventional light. Blue light altered the plasma pattern of the inflammatory and regulatory mediators. After morphological analysis, was found that both infected groups exhibited parasitized cardiomyocytes with intense myocarditis with predominance of pericellular and perivascular mononuclear inflammatory infiltrate. Phototherapy with blue light reduced the quantity amastigote in the nests in cardiac tissue. These data can be used for the development of a new way of treatment for Chagas disease in the future.

Key words: *Trypanosoma cruzi*, inflammation, blue light, phototherapy.



# 1 INTRODUCTION

According to World health organization (WHO) (2019), currently, about 7 million people are infected with *Trypanosoma cruzi* mainly in Latin America, where Chagas disease is one of the biggest problems in the public health. Every year around 10 000 people die because of *T. cruzi* infection (WHO, 2019). As was shown by Pérez-Molina *et al.* (2018), migration helps in the process of spreading disease beyond its natural geographical borders and becoming a global issue (PÉREZ-MOLINA *et al.*, 2018). Mainly transmission occurs by the insect vector (blood-sucking triatomine bugs) (WHO, 2019). The life cycle of *T. cruzi* is complex and includes vector of transition (bugs) and host (mammalians) (Figure 1).



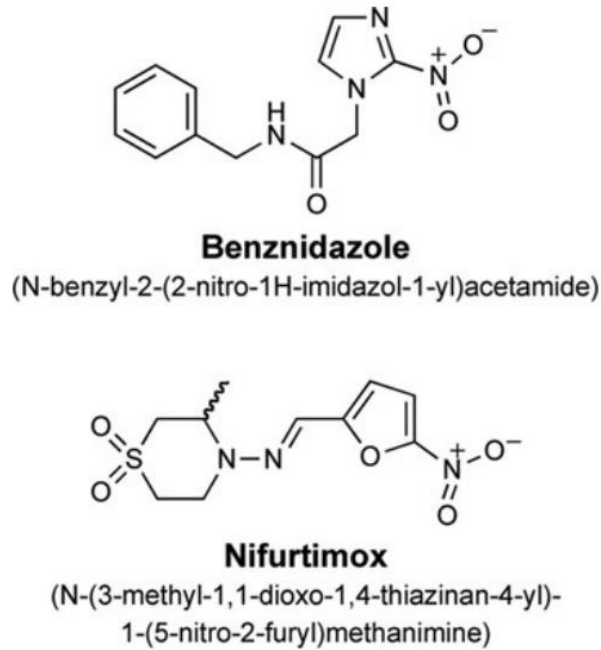
**Figure 1. Illustration of the *Trypanosoma cruzi* life cycle.** Replicative, non-infective epimastigote forms (in the vector) (a), transform into infective metacyclic trypomastigote forms (which lose the capacity to replicate) (b). After that metacyclic forms invade the host cells and differentiate into amastigote forms with a replicative capacity (c), this is establishing of the infection. These forms transform into a transient stage - intracellular epimastigote forms (d), after some time they differentiate into trypomastigote forms (e). Trypomastigotes can spread in the host body through the bloodstream. The cycle ends when the trypomastigotes, which was consumed with the blood by the vector, differentiate into epimastigote forms (a), which colonize the digestive tube of the insect (BOSCARDIN *et al.*, 2010).

In the mammalian hosts, the protozoa cause an intense immune response during acute and chronic phase. This can manifest itself in an alteration in level of some cytokines such as IL-10, IL-6, and CCL2. IL-10 is immuno-modulatory cytokine with an anti-inflammatory activity which plays important role in the process of inflammation in the human body (ESKDALE *et al.*, 1997). Usually it is synthesized by immune cells and glia and serves as a negative feedback during inflammation (KWILASZ *et al.*, 2015). It controls the immune response caused by pathogens and prevents harm of the host body. It modulates inflammatory responses by suppressing monocyte/macrophage function. Also, it can influence on growth/differentiation of some immune cells. In addition, it can influence on immunoglobulin secretion, and promotes

antibody class switching (ACUNER-OZBABACAN *et al.*, 2014). IL-6 is a multifunctional cytokine which can affect peripheral tissues and the central nervous system. In the body, it can play a role of pro-inflammatory cytokine and an anti-inflammatory myokine. It can inhibit TNF and IL-1, and activate of IL-1 and IL-10 expressions (PETERSEN AND PEDERSEN 2005). It was showed that IL-6 is produced by cardiomyocytes it plays a protective role in heart injuries. For example, Sanmarco *et al.*, 2017 showed that IL-6 mediates the regulation of nitric oxide which plays a significant role in defense mechanism against pathogens including *T. cruzi* infection (SANMARCO *et al.*, 2017). CCL2, also known as monocyte chemoattractant protein 1 (MCP1), is a pro-inflammatory cytokine which is responsible for recruiting different cells (monocytes, memory T cells, dendritic cells) in the areas of inflammation which were caused by injury or infection (CARR *et al.*, 1994, XU *et al.*, 1996).

Usually it is possible to comprehend the *T. cruzi* infection into two phases: acute and chronic (WHO, 2019). The acute phase can be characterized by many symptoms such as skin lesion or a purplish swelling in the eyes, also fever, headache, muscle pain and others. The chronic phase usually presents by cardiac and/or digestive disorders of various severities (BOSCARDIN *et al.*, 2010, WHO, 2019).

Currently exist only two substances, which were recognized as chemotherapy standard for treatment Chagas disease: Benznidazole (BZ) and Nifurtimox (NF) (Figure 2). Treatment with BZ is preferred over NF; it is because BZ shows the benefits in tolerability profile and better ability to deliver into the tissue (COURA *et al.*, 2002). Although, from the medical practice we know that both have many side effects and show their inefficiency in chronic phase (PÉREZ-MOLINA *et al.*, 2018). The most common side effects of BZ treatment are hypersensitivity, mainly in the form of skin rash (29–50%), digestive intolerance (5–15%), anorexia, asthenia, headache, and sleeping disorders (40%). In 9-29% of cases, the treatment with BZ was stopped (PÉREZ-MOLINA *et al.*, 2018). According to Coura *et al.* (2002), the side effects of NF treatment occur in 43.0–97.5% cases. The most common are anorexia, neurological disorders, nausea and vomit. The NF treatment was stopped in 14.5–75.0% of cases (COURA *et al.*, 2002).



**Figure 2. Molecular formulas of benznidazole and nifurtimox** (BOSCARDIN *et al.*, 2010).

In the situation of inefficiency of current method of treatment *T. cruzi* infection, there is a necessity for the studying and analysis of new compounds and new methods of treatment. Last decades scientists have studied the blue light as a way of treatment in different experimental models for understanding the potential of light application in medical practice (DOLMANS *et al.*, 2003).

The blue light is a part of the high-energy visible spectrum, with the short wavelength from 450 to 495 nm (KUSE *et al.*, 2014). From 1958, the blue light has been used for the treatment of neonatal jaundice (hyperbilirubinemia) (MCDONAGH, 2001). The efficacy of blue light phototherapy is dependent on the fundamental laws of photobiology and photochemistry. The blue light causes rapid oxidative reactions and intermolecular rearrangements of bilirubin to produce mutant isomers. These isomers are more polar, and it facilitates their extraction from the body by bile and urine without conjugation (VREMAN, *et al.* 2004).

Nowadays, almost every hospital has the equipment for blue light phototherapy. Therefore, scientists are actively exploring the possibility of using blue light for other purposes. For example, they study antimicrobial effect of blue light. In the work of DAI *et al.* (2012) was shown antimicrobial (*Propionibacterium acnes* and *Helicobacter pylori*) effect of blue light in the absent of exogenous photosensitizers. It seems to be less harmful for the mammalian cells than ultraviolet irradiation. However, scientists still do not have a clear answer about the

mechanism of the antimicrobial effect of blue light. There is an hypothesis which was discussed by several research groups such as Ashkenazi *et al.* (2003); Hamblin *et al.* (2005); Maclean *et al.*, (2008), that in the cells, blue light activates endogenous porphyrins, that provoke of the reactive oxygen species (ROS) synthesis, which is known by its high cytotoxicity (DAI *et al.*, 2012; ASHKENAZI *et al.*, 2003; HAMBLIN *et al.*, 2005; MACLEAN *et al.*, 2008). Also, in *in vivo* models of bacterial infections was shown efficiency of blue light application. In the work carried out by Fan *et al.* (2013), in an animal model (rats) of acne caused by intradermal injection in auricular tissue using of *P. acnes*. The blue light therapy reduced TNF and matrix metalloproteinase 2 (MMP-2), markers of inflammation implicated in acne and general condition of animals (FAN *et al.*, 2013). However, there were no studies about the influence of blue light on parasitic organisms, in particular, the *T. cruzi*. But it seems like a promising way of application of blue light therapy especially in regions with high risk of Chagas disease, like Brazil.

## **2 OBJECTIVES**

### **2.1 General objective**

Determine whether blue light can affect the inflammatory parameters in C57BL6 mice (*in vivo*) infected with *T. cruzi* as well as affect development of parasites *in vitro*.

### **2.2 Specific objectives**

- (i) Evaluate *in vitro* the survival of the different strains of *T. cruzi* under phototherapy with the blue light;
- (ii) Evaluate the parasitemia in isogenic (C57BL6) mice infected by the Y strain of *T. cruzi* in the presence and absence of blue light;
- (iii) Evaluate whether the blue light affects the plasma pattern of the inflammatory and regulatory mediators (IL-6, CCL2 and IL-10);
- (iv) Evaluate whether the blue light can control the cardiac tissue inflammation as well control the tissue parasites (amastigote forms);
- (v) Evaluate whether the blue light can affect the cellular parameters (morphometric) in the infected animals.

### 3 MATERIALS AND METHODS

#### 3.1 Parasites

In current work were used strains of *T. cruzi*: Y strain which was classified as *T. cruzi* II (ZINGALES *et al.*, 2009). CL strain which was classified as *T. cruzi* VI (ZINGALES *et al.*, 2009). These strains were maintained by successive passage in Swiss mice in Laboratory of Immunobiology of Inflammation, at Federal University of Ouro Preto (UFOP), Minas Gerais, Brazil.

#### 3.2 *In vitro* experiment with *T. cruzi*

Pure cell culture of *T. cruzi* (Y and CL strains) were cultured at 25 °C in LIT medium (liver infusion-tryptose) supplemented with 10% (v/v) inactivated fetal bovine serum (ESPINOZA *et al.*, 2010; MARTÍNEZ *et al.*, 2013). These cultures were exposed to blue LED light (with the peak 460 nm and 80  $\mu\text{W}/\text{cm}^2$ ) during 5 days, 6h per day, at room temperature. The parasites were counted in 10  $\mu\text{L}$  every day for analyzing their survival using the Neubauer Chamber (Figure 3). Experiments were repeated twice.

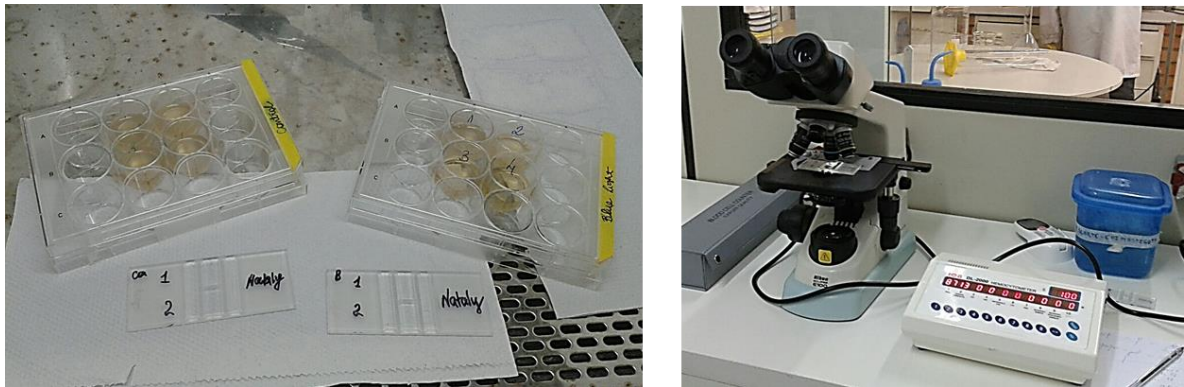


Figure 3. Equipment used for *in vitro* experiments.

#### 3.3 *In vivo* experiment with *T. cruzi*

C57BL/6 male mice of age 20-22 weeks and mass around 30 g, were used for our study. Animals were separated into 4 groups, 10 animal per group: non-infected + conventional light, non-infected + blue light, infected + conventional light, and infected + blue light. Infection of

mice were made through the intraperitoneal injection with Y strain (100 trypomastigotes). In this part of experiment, groups with “blue light” were exposed to blue light (with the peak 460 nm and 7  $\mu\text{W}/\text{cm}^2$ ) for 9 days, 12 h per day (from 07:00h to 19:00h), at room temperature. 5  $\mu\text{l}$  of animal blood were analyzed every day using optic microscopy for creation of parasitemia curve according Brener (BRENER 1962). Also, during experiment were evaluated the survival of animals. On 9th days of infection (acute phase), animals were euthanized with collection of blood for immune assays. The heart and spleen mass were measured. The heart of each animal was fixed with 10% formalin to make histopathology analysis. Mice were housed and maintained at the animal central facility at UFOP. All animal experiments and procedures were approved by the institution’s committee on the ethical handling of laboratory animals (CEUA Protocol- CEUA 2018/02).

### **3.4 Enzyme-linked immunosorbent assay (ELISA)**

Immunoenzymatic assays were used to evaluate the concentrations of the inflammatory cytokines IL-6, the chemokine CCL2, and the regulatory cytokine IL-10. Plasma were isolated from blood and used according to the manufacturer’s protocol (PeproTech Inc., Rocky Hill, NJ, USA). Samples were read in a spectrophotometer (Emax Molecular Devices, Sunnyvale, CA, USA) using wavelengths of 405 and 630 nm.

### **3.5 Heart processing and histopathology**

Heart samples of all animals were fixed in formalin for 24h, dehydrated in ethanol, embedded in glycol methacrylate resin and cut at 3- $\mu\text{m}$  thick sections using glass knives (RM2125RTS1, Leica Biosystems, Wetzlar, Germany). Two histological slides with four heart sections collected in semi-series were obtained, using one out of every 50 sections to avoid evaluate the same histological area. In the first slide, the sections were stained with hematoxylin and eosin (H&E) at 60°C (40 min) for general histopathology. Distribution and organization of the heart parenchyma and connective stroma, tissue necrosis, as well as morphology and distribution of cardiomyocytes, blood vessels and inflammatory foci were evaluated in the histopathological analysis (NOVAES *et al.*, 2013). Heart microstructure was analyzed in by bright field microscopy by using a 40 $\times$  objective lens (400 $\times$  magnification; Axioscope A1, Carl Zeiss, Germany) (NOVAES *et al.*, 2013)

### 3.6 Heart stereology and histomorphometry: myocardial compartments and tissue cellularity

In the same sections used for histopathology, cardiac microstructural reorganization was analyzed from stereological principles (NOVAES *et al.*, 2013). Volume density ( $V_v$ , %) of the heart stroma (connective tissue [CT]), parenchyma (cardiomyocytes [CMY]), and blood vessels (BV), was estimated as  $V_v = \Sigma PP / PT$ ; where  $\Sigma PP$  is the number of test points hitting on the structure of interest, and  $PT$  is the total number of test points. A quadratic test system with 100 test points distributed in a standard test area of ( $A_t = 25 \times 10^3 \mu m^2$ ) was used. Volume density was estimated from 20 randomly sampled histological fields of heart sections from each animal using a  $\times 40$  objective lens ( $400\times$  magnification; Axioscope A1, Carl Zeiss, Germany). A total tissue area of  $25.0 \times 10^5 \mu m^2$  was analyzed for each group. In the second slide, heart sections were stained with hematoxylin at  $60^\circ C$  to evaluate myocardial cellularity and cardiomyocytes parasitism. The number density (QA,  $n/mm^2$ ) of mononuclear (MN) and polymorphonuclear (PMN) interstitial cells per myocardial area was estimated as  $Q_{AMN/PMN} = \Sigma Q_{MN/PMN} / AT$ ; where  $\Sigma Q_{MN/PMN}$  is the number of MN or PMN in the microscopic focal plane, and  $AT$  is the dimension ( $mm^2$ ) of the two-dimension test area ( $AT = 0.009 mm^2$ ). QA was estimated from 20 randomly sampled histological fields from each animal using a  $\times 100$  objective lens ( $1000\times$  magnification; Axioscope A1, Carl Zeiss, Germany). A total tissue area of  $85,600 \mu m^2$  was analyzed for each group. The number of amastigotes per area of *T. cruzi* nest was estimated by computational panimetry. Briefly, the number of amastigotes were counted and normalized by the nest area, which was determined by using a contour function applied in the image analysis software. Were analyzed 50 amastigote nests per animal, using a  $\times 100$  objective lens ( $1000\times$  magnification; Axioscope A1, Carl Zeiss, Germany). All morphological parameters were quantified by using the image analysis software Image-Pro Plus 4.5 (Media Cybernetics, MD, USA)

### 3.7 Statistical analysis

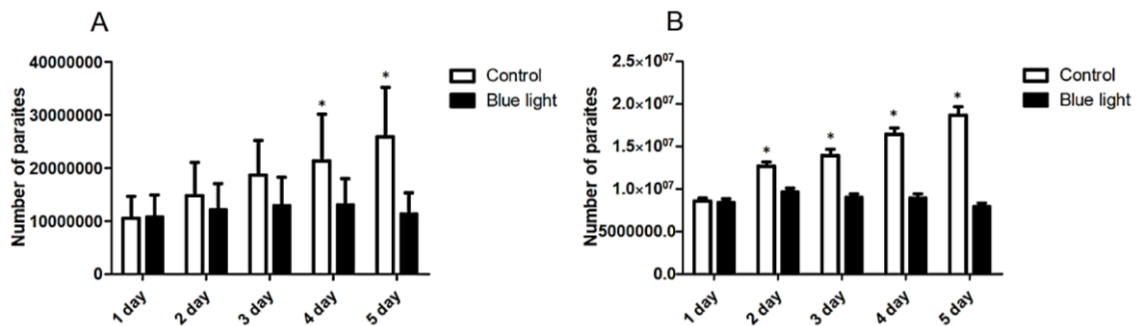
The statistical analysis of the results were carried out using a Shapiro –Wilk test to verify the normality among data and one-way analysis of variance test followed by a Bonferroni's

multiple comparison test and Wilcoxon rank sum test for multiple comparisons through the GraphPrism v.5 (GraphPad Software, Inc., La Jolla, CA). Results represent mean  $\pm$  standard error of the mean (SEM) of 6–10 survivors per experimental group and differences with a P value of  $< 0.05$  were considered statistically significant.

## 4 RESULTS

### 4.1 *In vitro* experiments

During the *in vitro* experiments we found that phototherapy with blue light influenced on growth and the mobility of the *T. cruzi*. Our experiments show that in both strains CL (Figure 4 A) and Y (Figure 4 B) exposure under blue light, there were inhibition the parasite growth. There were no significant differences between the strains, they both show similar pattern of growth inhibition in the presence of blue light. On the 5th day of phototherapy, blue light inhibited the parasite growth with more than 50% in comparing with control group (conventional light). Also, it was noticed that in groups with phototherapy, blue light influenced on mobility of parasites. Visually, parasites in these groups moved slowly in comparison with control group (conventional light).



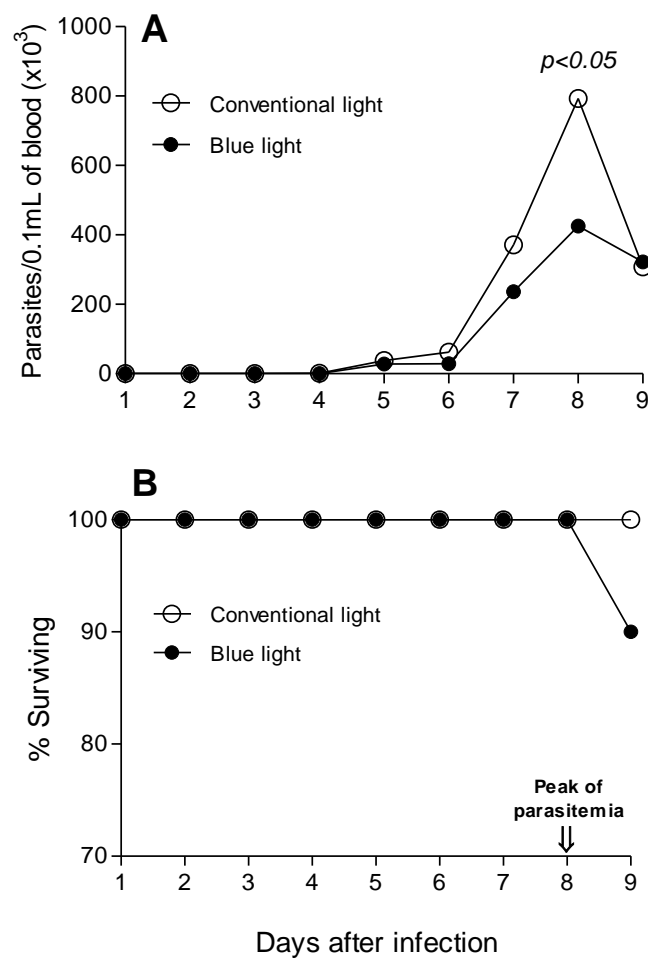
**Figure 4. Growth inhibition of the CL (A) and Y (B) strains of *T. cruzi*.** The parasites cultures were exposed to phototherapy with blue light for 5 days 6h per day, at room temperature. The growth inhibition was evaluated in 10  $\mu$ L every day using the Neubauer Chamber and a light microscope. \* -  $p < 0.05$  means differences between control groups and blue light therapy.

### 4.2 *In vivo* experiments

#### Parasitemia and animals` survival



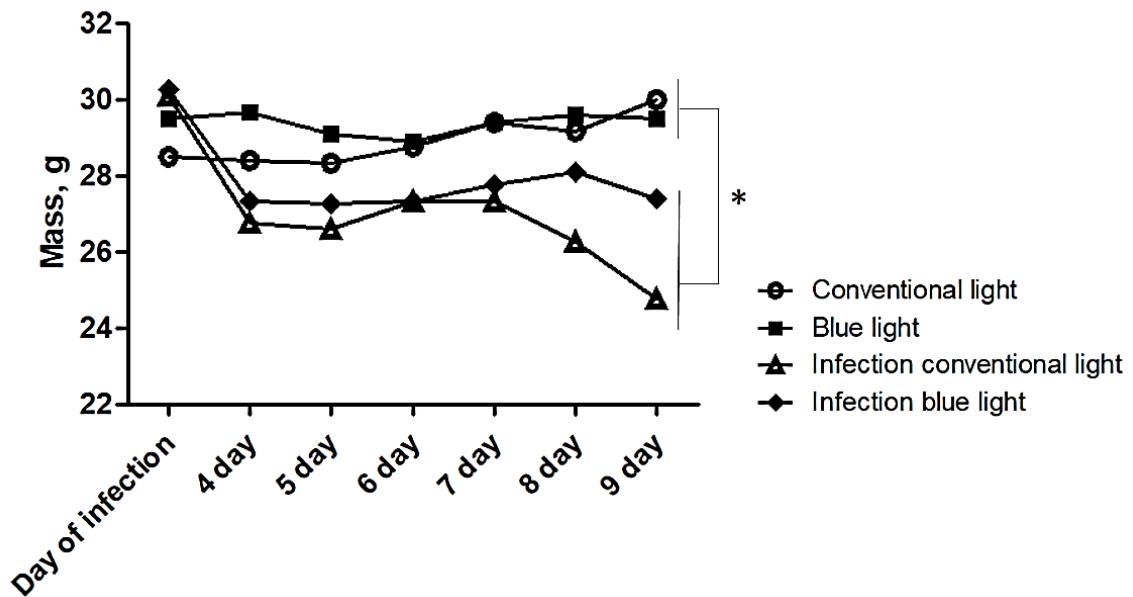
During *in vivo* experiments, the parasitemia and animals' survival were analyzed. The curve of survival was plotted during whole acute phase of the *T. cruzi* infection (Figure 5 B). There was no significant difference between infected group with conventional light and blue light groups. Curve of parasitemia shows that blue light influenced on quantity of parasites circulating in the blood of animals. Starting with 7th day of infection was observed reduction quantity of parasites in the groups with blue light. On 8th day was registered peak of parasitemia and on this day was observed a significant reduction in the number of parasites in the groups with phototherapy of blue light (Figure 5 A). These results are compared with the results obtained during the *in vitro* experiments.



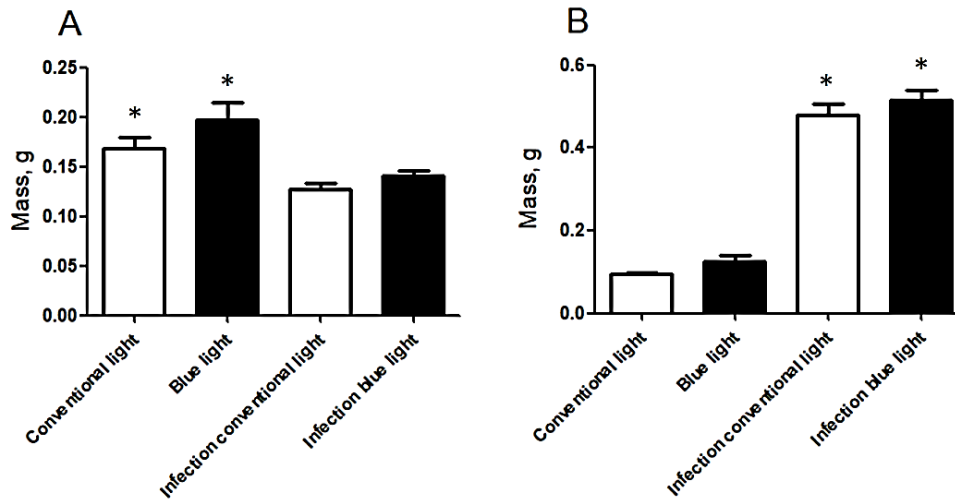
**Figure 5. Curves of parasitemia and animals' survival *in vivo* experiment.** A - curve of parasitemia; B – curve of animals' survival. C57BL/6 male mice of age 20-22 weeks and mass around 30 g were infected with Y strain of the *T. cruzi*. Then groups with “blue light” were exposed to blue light (with the peak 460 nm and 7  $\mu\text{W}/\text{cm}^2$ ) for 9 days, 12 h per day (from 07:00h to 19:00h). For creating parasitemia curve, blood samples were collected every day and parasites number presented as average.  $p < 0.05$  means differences between groups under conventional and blue light therapy.

## Mass of heart, spleen and body

The body mass of animals was daily analyzed during all experiment. The groups under conventional and blue light showed similar pattern of body mass (Figure 6). However, in the groups with infection, the body mass decreased during the experiment comparing to non-infected groups. But there were no statistical differences inside groups with infection and inside non-infected groups. In the last day of experiment animals were euthanized and their heart and spleen mass were measured (Figure 7). Significant statistical differences were not found between groups with phototherapy of blue light and groups with conventional light. It seems that in our model of *T. cruzi* infection, the blue light did not have interference on the parameters of body, heart and spleen mass.



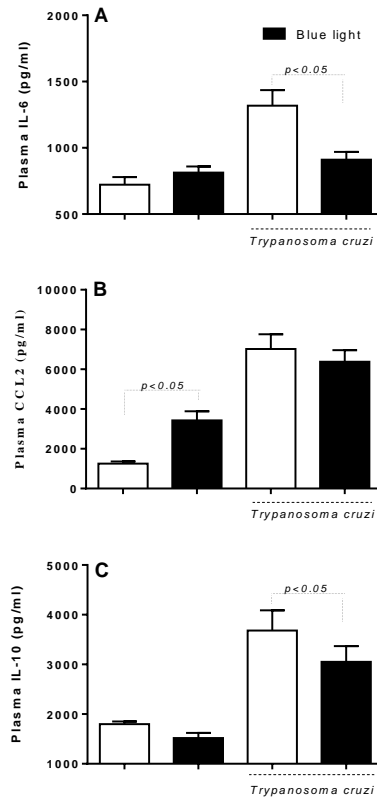
**Figure 6. Curves of body mass during *in vivo* experiment.** Body mass of experimental animals was daily analyzed. Control groups were exposed to conventional light and experimental groups to blue light, for 9 days, 12 hr. per day. For creating the average of receiving data was used. \*-  $p < 0.05$  means differences between non-infected and infected groups.



**Figure 7. Mass of internal organs (heart and spleen) in C57BL/6 mice during phototherapy with blue light.** **A** – mass of heart; **B** – mass of spleen. Data were obtained after animal euthanasia on the 9th day after infection. \*- $p < 0.05$  means differences between non-infected and infected groups.

### **Level of inflammatory cytokines in plasma of C57BL/6 mice**

The plasma level of inflammatory cytokines such as IL-6, IL-10, CCL2, were analyzed by performing ELISA (Figure 8). According to our results, the level of IL-6 was higher in group with infection (conventional light) in comparison with non-infected animals and in those infected under blue light phototherapy. The levels of the regulatory cytokine IL-10 was lower in the non-infected animals when compared with the infected mice. There were no differences in the levels of IL-10 inside the groups with infection and non-infected groups with both types of light (conventional and blue). Level of the chemokine CCL2 was higher in the non-infected animals under blue light. However, no difference was found between the infected groups.

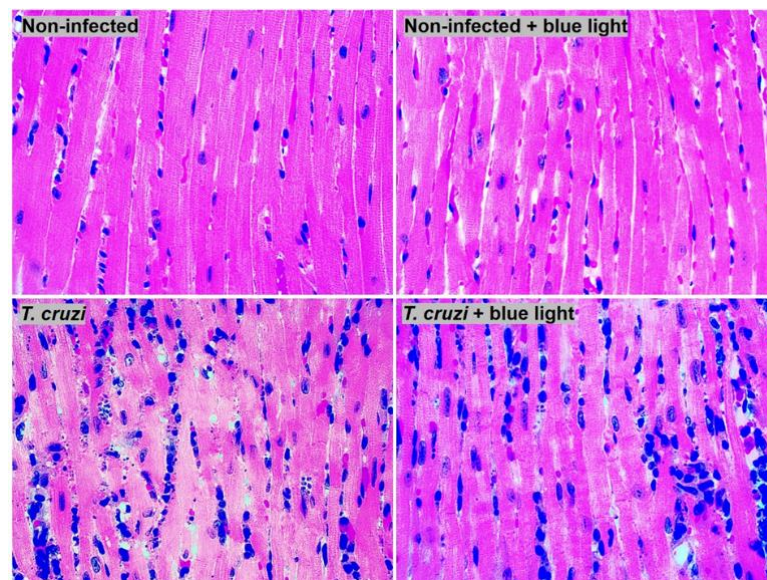


**Figure 8. Level of cytokines in plasma of C57BL/6 mice.** Animals were separated into 4 groups (40 animal total): non-infected + conventional light, non-infected + blue light, infected + conventional light, and infected + blue light. For infection was used Y strain of the *T. cruzi*. The groups with “blue light” were exposed to blue light (with the peak 460 nm and 40  $\mu$ W/cm<sup>2</sup>) for 9 days, 12 h per day. The groups with conventional light were exposed to conventional light for 9 days, 12 h per day. The plasma was collected to evaluate the cytokines IL-6 (A), CCL2 (B) and IL-10 (C) production.  $p < 0.05$  means differences between infected groups under blue light and conventional light.

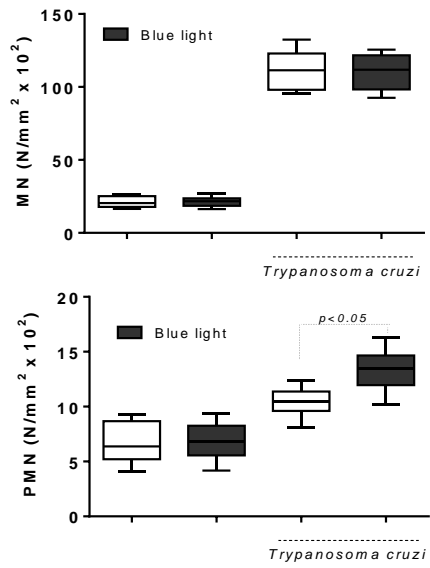
### The cardiac inflammation and morphological analysis

The morphological analysis indicated that non-infected groups both with conventional light and blue light presented a normal myocardial microstructure. In these groups were noticed reduction of interstitial cellularity. Also, were observed well-defined cardiomyocytes arranged in parallel beams. Both infected groups exhibited parasitized cardiomyocytes with intense myocarditis (Figure. 9) with predominance of pericellular and perivascular mononuclear inflammatory infiltrate (Figure 10). Analysis showed that there were difference in the quantity of mononuclear cells between non-infected and infected groups but there were no difference

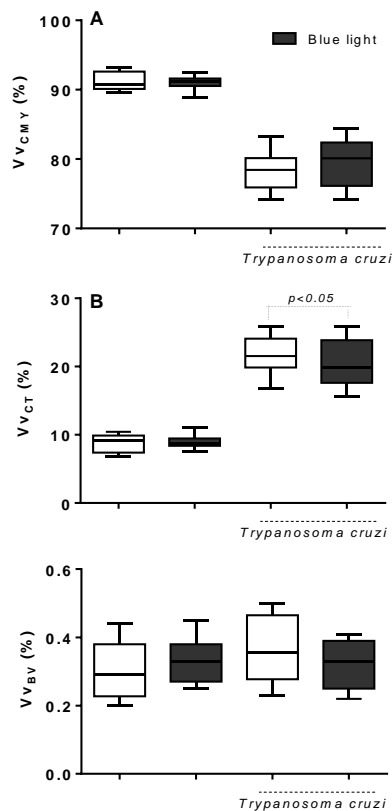
between convectional and blue light. However, there was a difference in the quantity of polymorphonuclear cells in infected group under blue light compare to infected group (convectional light). We found difference in cardiac microstructural reorganization (Figure 11). The volume density of cardiomyocytes in infected groups were lower than in control groups, but there were no difference between lights. In opposite, the volume density of connective tissue in infected groups were higher compare to non-infected and there were difference between lights in infected groups. Though, there were no difference in the volume density of blood vessels between non-infected and infected animals under both type of lights. It was also observed that foci of tissue necrosis, cardiac stroma expansion and evident the *T. cruzi* amastigote nests (Figure 12). After analysis of the quantification of amastigote nests in cardiac tissue were found reduction of visual parasites in the infected groups under blue light therapy compared to convectional light.



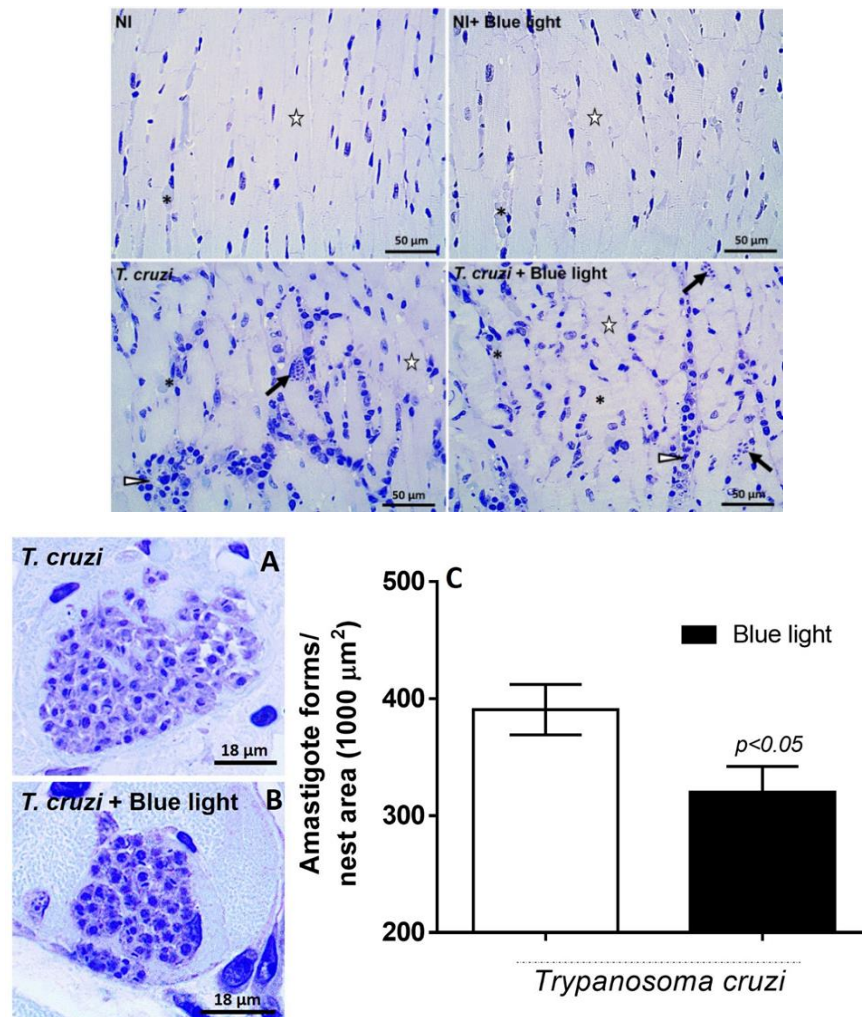
**Figure 9. Photomicrography of cardiac tissue.** Cardiac tissue from non-infected and *T. cruzi*-infected animals were analyzed under conventional light and blue light phototherapy. Inflammatory cells and loss of tissue integrity are evidenced in those images related to parasite infection.



**Figure 10. Analysis of the inflammatory infiltration.** Cardiac tissue from animals under conventional and blue light phototherapy was analyzed and blood mononuclear cells (A) polymorphonuclear cells (B) quantified.  $p < 0.05$  means differences between infected groups under blue light and conventional light.



**Figure 11. Cardiac microstructural reorganization according to stereological principles.** Volume density ( $V_v$ , %) of the heart stroma (connective tissue [CT]), parenchyma (cardiomyocytes [CMY]), and blood vessels (BV).  $p < 0.05$  means differences between groups under conventional and blue light therapy.



**Figure 12. Quantification of amastigote nests in cardiac tissue.** The amastigote nests of the *T. cruzi* were evidenced in the upper images with reduction of visual parasites in the infected group under blue light therapy. (A) an amastigote nest from animals under conventional light therapy, (B) an amastigote nest from animals under blue light therapy and (C) quantification of the area of the amastigote nests.  $p < 0.05$  means differences between infected groups under conventional and blue light therapy. Arrow – the *T. cruzi* amastigote nest, arrowhead – the inflammatory foci, asterisk – the blood vessel.

## 5 DISCUSSION

The limitation of current ways of treatment to Chagas disease pushes medical and research communities constantly to investigate other therapy alternatives. One of them is “chemical”, discovery new compounds and drugs or a combination of already existing compounds with Benznidazole (URBINA *et al.* 2010). This chemical therapy has found a list of limitations in the last decades for the Chagas disease treatment: (i) side-effects which conduct patients to abandon treatment, (ii) resistance of some genetic populations of the *T. cruzi*, (iii) different actions on the evolutive forms of the parasites in blood and tissue, (iv) particularities

of treatment in non-adult individuals and in congenital transmission (ISSA *et al.* 2010; GUEDES *et al.* 2011; MORILLO *et al.* 2015). Besides there are other limitations when the chemical compound/drug is tested in experimental model and then the success is not reproducible in human tests due the pharmacological pathways among the species (OLIVEIRA-SILVA *et al.* 2015; SCARIM *et al.* 2018). Other way is “alternative”, when researchers try to use non-standard solutions, such as physical strategies to affect and eliminate the parasites (BARBOSA *et al.* 2019). Blue light could be one of these alternatives. It has already been included in the standard protocol of treatment of human hyperbilirubinemia because of its safety and efficiency (MCDONAGH, 2001). Also, it has shown the antimicrobial and anti-inflammatory potential of blue light (DAI *et al.* 2012, FAN *et al.* 2013). Based on that, in our current research we demonstrated that *T. cruzi* was vulnerable to the blue light *in vitro*. According our findings, under the blue light photo therapy caused suppression of replication in more than 50% of the parasites and changing their motility in the axenic cultured forms. In our *in vitro* experiments we used two different strains of *T. cruzi* such as CL and Y. These strains presented two different genetic populations but both of them showed similar pattern of reduction quantity of parasites under blue light. As mentioned before, the anti-*T. cruzi* therapy with blue light, that can affect different genetic populations of this parasite and it could be suggested for human treatment, in isolated form or in a drug-combined strategy.

At the beginning of *in vivo* experiments, we had doubts on if the light would be effective to parasites in mice (trypomastigotes in blood) and mainly (amastigotes inside the tissues). Because in the literature there was no evidence of using blue light for using in *T. cruzi* infection or in other protozoa, the majority of experiments were concentrating on photodynamic therapy, which is based on applying a photosensitive substance (as Methylene blue and others) on the target area with subsequent applications of adequate wavelength light. As result activation the cascade of reactions in the presence of oxygen and it led to cell death in targeting area (GIRONÉS *et al.* 2006, VOLPE *et al.* 2018, BARBOSA *et al.* 2019). In our case, the constant blue light was used without any photosensitive substance. We found that blue light did not effect on parameters of body mass, heart and spleen mass. But we could see by analyzing parasitemia that the blue light influenced on the quantity of *T. cruzi* circulating in the blood. It was reduced with a similar pattern as was detected *in vitro* experiments.

Another enthusiastic finding to our group that investigate, for decades, strategies to modulate the immune response in *T. cruzi* infection, was the observation that the phototherapy with blue light influenced on the level of inflammatory cytokines in plasma. Since the immune



response is the “natural strategy” to eliminate the parasites in mammals and, this same inflammation is the most important key for the development of muscles (cardiac, smooth and skeletal) and the nervous system disturbances in the *T. cruzi* infection, any strategy that could control or soften this inflammation represents benefits to the host. In this meaning, we observed that the plasma IL-6, an important inflammatory cytokine, was elevated in the presence of the parasites, but drastically suppressed by blue light therapy. The levels of the regulatory cytokine IL-10 followed this profile of IL-6. The parasites was also able to elevated the plasma level of the chemokine CCL2 as well as other chemokines and inflammatory cytokines in early and chronic phases of the experimental model of *T. cruzi* infection (Talvani et al. 2000). However we did not observe interference of the blue light on this parameter using our proposed designed experiment.

Changing in the production of plasma inflammatory cytokines could be a consequence of the parasites reduction in the blood of the animals - less parasites would cause less immune response. But more exiting result, which was found during *in vivo* part, was the reduction of visual parasites in amastigote nests (the cardiac tissue) of the infected groups under blue light therapy. It is very rare when nonchemical compound can reduce at the same time parasites from blood stream and from tissue (MORILLO *et al.* 2015)

To summarize, the blue light could be a potential treatment in application for *T. cruzi* infection, mainly as additional source to standard chemotherapy. Because it showed its ability to reduce a level of parasites in the blood and tissue of infected animals. It did not alterate dramatically the level of inflammatory cytokines in the plasma. We assume that further studies are necessary to detail the discovery of the mechanism of influence of the blue light to *T. cruzi*, and understand how different strains will react on this type of light.

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