Photobiomodulation reduces the cytokine storm syndrome associated with Covid-19 in the zebrafish model

Supplementary files

RNA extraction and gene expression (qPCR)

Gene expression of factors involved in signaling and responding to inflammatory processes was examined in zebrafish treated with COVID-19 (rSpike) to assess the therapeutic effect of low-level laser. For this, different tissues (brain, testicle, intestine, liver, and muscle) were collected from adult males (n = 25) in each experimental group: (1) laser (control), (2) rSpike, and (3) Spike+laser. The tissues were frozen in liquid nitrogen and stored at –80 °C until RNA extraction. The total RNA extraction process from each organ was performed according to the manufacturer's specifications for the Trizol method (Invitrogen, Carlsbad, CA, EUA). cDNA was synthesized using a Superscript II kit according to the manufacturer's protocols (Bio-Rad, Hercules, CA, USA).

The qPCR reactions were conducted with 10 μL 2X SYBRGreen Universal Master Mix, 2 μL forward primer (9 mM), 2 μL reverse primer (9 mM), 1 μL DEPC water, and 5 μL cDNA. The Cts were determined using StepOne Plus (Applied Biosystems), and reactions were subjected to 40 cycles (5 min at 95 °C, 40 cycles of denaturation at 95 °C, for 30 s at 60 °C, and 30 s at 72 °C). The relative mRNA levels of genes involved in signaling processes and inflammatory responses were evaluated in the different experiments (Supplementary Table 1). Primers were made using Primer Express 3.0 software (Software for Windows) based on sequences deposited in the public International Database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The relative expression of the genes studied was determined by the ddCt method. The mRNA levels (Cts) were calculated and normalized using the reference gene β-actin.

In silico Bioinformatics Analysis

Danio rerio predicted protein-protein interactions with SARS-Cov-2 related to the subcellular location: cytoplasm, membrane, and nucleus were recovered according to Ventura and collaborators [1]. The genes of interest to the study were added for each

subcellular location (denominated the **related genes** (*Slc2a1a, Slc2a1b, Romo1, Nfkb1, Il1b, Il6, Cox1*). The interacted proteins and the genes were submitted to functional enrichment to identify gene ontology (GO) using the G: Profiler software [2]. Based on the database of the *D. rerio*, the sub-networks of proteins and pathways (KEGG and REACTOME) were obtained with the Cytoscape software [3]. The sub-networks were extracted according to the first neighbor of the **related genes** (*Slc2a1a, Slc2a1b, Il1b, Il6, Nfkb1, Cox1*).

Photobiomodulation (PBM) with Low-Level Laser Therapy

To elucidate the role of PBM with low-level laser as a potential therapeutic in pathologies originating from SARS-COV-2, adult males injected with rSpike (n = 40) were subjected to PBM after 5 hours of inoculation. Adult males (n = 40) were subjected only to treatment with a low-power laser as a control group. For this purpose, the low-power laser device, Therapy XT (DMC, São Carlos, Brazil), with an output power of 100 mW, emitting at 660 nm and energy density of 2 J cm^{-2}, was used. After treatment with PBM, the fish were kept in a 10-liter tank at 28 °C, pH 7.6, and under a 14h:10 h (light, dark) photoperiod. After a 6 and 24 h period, collections of different tissues (brain, testicle, intestine, liver, and muscle) were performed for each experimental group (1) control, (2) rSpike, (3) rSpike+laser for gene expression, as well as collections from adult subjects for histopathological analysis.

1. Results

Treatment with rSpike significantly increased the mRNA levels of pro-inflammatory markers such as nuclear factor-kappa B (*nf-kB*) and interleukins (*il-1β* and *il-6*) in brain tissue after 6 h when compared with the control group (**supplementary figure** 1 A).

Additionally, the genes ROS (reactive oxygen species), mt-co1 (cytochrome c oxidase 1), and *glut* (glucose transporter 2) involved in the processes of reactive oxygen species production, mitochondrial respiratory chain, and cell membrane glucose transport, were also positively regulated by rSpike after 6 h. (**supplementary figure 1**). After 24 h, rSpike promoted a significant increase in tumor necrosis factor, *tnf-α*, *il-1β*, *il-6*, and *ros* compared to the control group (**supplementary figure 1**).

In the intestine, gene expression of *ros*, n*f-kB*, *il-6*, and *tnf-α* was positively regulated by rSpike in both periods (6 h and 24 h) compared to the healthy group (Figure 1. B and **supplementary figure 1**). In contrast, IL-1β mRNA levels increased significantly only after 24 h of rSpike inoculation (**supplementary figure 1**). We also observed a variable expression profile in liver tissue, with increased gene expression of ROS, nfk-β, and mt-co1 after 6 h and 24 h (Figure 1.C and **supplementary figure 1**), while IL1β and IL-6 genes were positively regulated by rSpike only after 24 h (**supplementary figure 1**). Finally, no statistical difference was observed in the relative mRNA levels of TNF-α and glut genes in both groups evaluated (**supplementary figure 1**).

Similar to liver tissue, ROS, nfk-β, and cox1 (**supplementary figure 1**) were also positively regulated by rSpike in testicular tissue after 6 h and 24 h, including interleukin IL1β. Furthermore, IL-6 mRNA levels increased significantly only after 6 h of inoculation (**supplementary figure 1**). However, TNF-α and glut transcripts were not modulated by rSpike in any group evaluated (**supplementary figure 1**). Finally, we observed that rSpike also can modulate the expression of pro-inflammatory markers (IL1β, TNF-α) and glut and ROS genes after 6 h and 24 h in muscle tissue (**supplementary figure 1**), as well as of IL-6 and cox1 after 24 h of inoculation (**supplementary figure 1**).

Similarly, the protein-protein interaction prediction among SARS-CoV-2 and *D. rerio* proteins shows the biological pathways that can modify the normal function in infection of SARS-CoV-2 and the alteration of the expression of related genes. Among them, we can highlight the involvement of *Slc2a1a, Slc2a1b (*cell membrane glucose transport), interleukins-*Il1b, and Il6* (pro-inflammatory pathway), *Nfkb1*, and *Cox1* (mitochondrial respiratory chain) in COVID-19.

Our findings indicate that the inflammatory process is an incisive feature of COVID-19. Thus, excessive inflammation by oxidative stress, and stimulation of pro-inflammatory factors, including a depressed immune system, contribute substantially to the negative consequences of COVID-19. Controlling hyper glycolysis and oxidative stress in patients would help control the deregulated or excessive production of pro-inflammatory cytokines, improving the inflammatory process systemically. In this context, applying a low-power laser may be a strategy to minimize the adverse effects of COVID-19.

Photobiomodulation (PBM) using a low-intensity laser emitting at 660 nm has been widely used in different fields in dentistry, such as oral surgery, endodontics, and periodontology [4, 5, 6, 7]. Mainly for promoting tissue repair effects and increasing local circulation [8, 4, 9, 10, 11]. Light energy absorbed by intracellular photoreceptors initiates a

cascade of intracellular photochemical reactions that enhance cellular activity, promoting anti-inflammatory effects and enhancing the healing process in injured tissues [12, 13, 14].

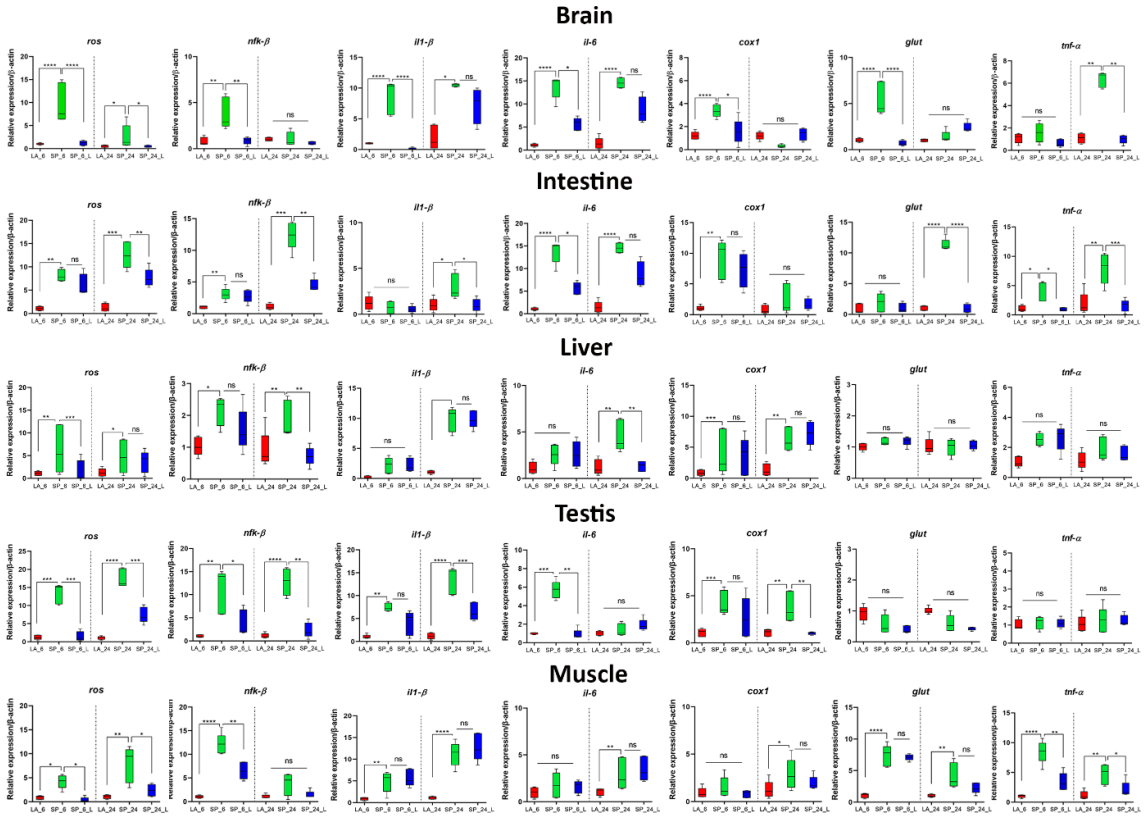
Considering the pathophysiology of COVID-19 and the anti-inflammatory and biostimulatory effects of PBM [15, 16, 17], this treatment modality could be a promising new approach to be applied in controlling COVID-19 inflammatory processes. Therefore, in the present study, we evaluated the effect of red PBM (660 nm, 2 J cm^{-2}) on inflammation generated by SARS-CoV-2 rSpike protein in various tissues. Furthermore, we evaluated its direct effect on cell and tissue regeneration processes and indirect effects on modulating pro-inflammatory factors and the immune system. In recent study randomized performed by Marashian et al., (2022) [18], demonstrate a decrease in the levels of IL-6, IL-8, and TNF-α in PBM patients covid-19 positive. In the literature, many *in vitro* and *in vivo* clinical studies also report the action of PBM on other inflammatory processes [12, 13, 14].

Positive effects are mainly found in down regulation of pro-inflammatory cytokines (IL-1β, IL-6, IL-2), markers of cellular respiration (cox2) at both 660 nm and 684 nm wavelengths [19, 20, 21, 22, 23, 24, 25]. Aimbire et al. (2006) [22], also denote that PBM significantly reduces TNFα and Nfk β levels compared to the control group, suggesting therapeutic potential in suppressing TNFα in various inflammatory pathologies in the clinical area.

Similarly, recent systematic reviews also suggest that PBM at blue wavelengths (450, 454, and 470 nm) favor lung tissue regeneration in patients with asthma as well as pneumonia triggered by COVID-19 [26, 27, 28, 29, 30, 31]. Patients treated with PBM showed rapid recovery of pulmonary status without the need for ICU admission or mechanical ventilation after treatment [31].

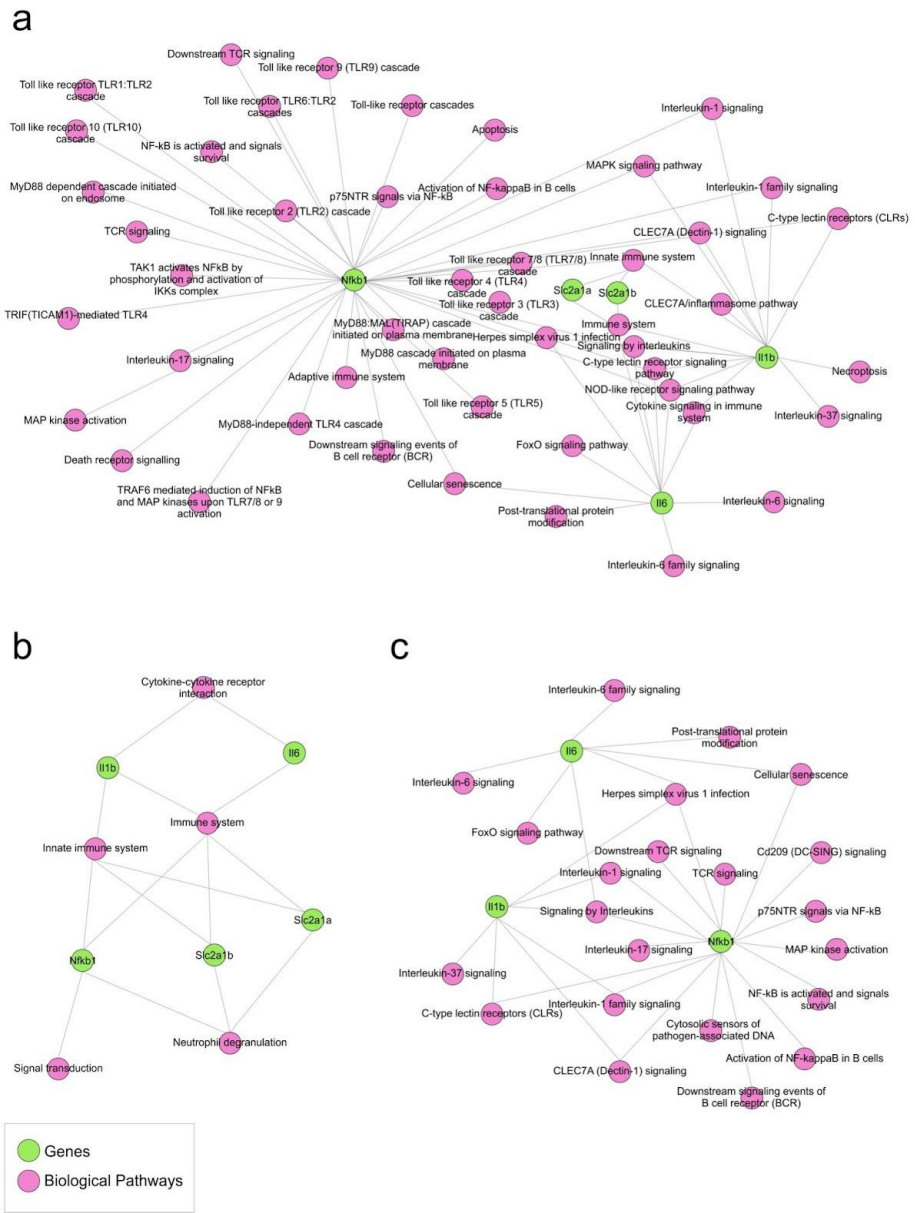
Based on our data, PBM intervention in zebrafish also increased the survival rate (97.5%) of individuals inoculated with rSpike after 24 h of intervention, reversing the inflammatory picture observed in the intestinal tissue. These data demonstrate that PBM can be a valuable therapy for reducing inflammatory processes in injured tissues, accelerating processes of regeneration of cellular and tissue homeostasis. We hypothesize that this tissue restoration can be achieved by directly rehabilitating the damaged tissue or indirectly by increasing blood flow oxygenation by PBM.

In this regard, our data using zebrafish as an experimental model denote that PBM is a safe treatment and may help control hyper glycolysis and oxidative stress and decrease inflammatory processes generated by COVID-19.



supplementary figure 1. .Relative messenger RNA levels of the (A) ROS, (B) nfk-β (C) IL-1β (D) IL-6, (E) TNF-α, (F) cox1, and (G) glut genes in the organs of zebrafish males subjected to PBM (6h and 24h); injected with recombinant Spike protein (rSpike) and subsequently subjected to PBM (6h and 24h). Quantification was performed using the 2-ΔΔCt method and values were normalized by the respective β-actin values. (Asterisks indicate significant difference between groups after Student's test; $p<0.05$, $^{**}p<0.01$, $^{*}p<0.001$, $^{***}p<0.0001$).

The protein-protein interaction prediction among SARS-CoV-2 and *D. rerio* proteins according to subcellular location (cytoplasm, membrane, and nucleus) predicted interactions with 778 proteins for cytoplasm, 2917 proteins for membrane, and 1141 proteins for nucleus (Table 2). The gene ontology (GO) analysis identified 48 related terms for cytoplasm, 5 representative terms for membrane, and 22 for nucleus (Table 2).



Supplementary Figure 10. Network of enrichment function of protein prediction interacted with SARS-CoV-2 and related genes. In a, proteins related to the cytoplasm, in b, proteins related to the membrane; in c, the proteins related to the nucleus. The boxes are the legend: light green represents related genes, and purple represents biological pathways. The networks were obtained with the Cytoscape software (Shannon et al. 2003).

file Table 1. Number of Gene Ontology retrieved from functional enrichment.

Subcellular localization	N° GO – functional enrichment	N° GO – related
Cytoplasm	116	48
Membrane	128	5
Nucleus	143	22

N° GO – related: functional enrichment terms that present one of the genes of interest.

2. Discussão

Herein, the inflammatory response is characterized by the enlightening of the increased metabolic differences caused by r-Spike, followed by the gene overexpression and the production of proteins associated with the inflammatory process, gene expression modulates various metabolic pathways.

Other aspects of COVID-19 infection include the expression of the SARS-CoV-2 receptor, ACE2 [32, 33, 34, 35, 36]. As in the lung, studies have reported elevated ACE2 receptor expression in several organs, including in gastrointestinal epithelial cells and hepatocytes in liver tissue [37, 38, 39, 40, 41]. More precisely, in liver tissue, the presence of the rSpike protein in the cytoplasm of hepatocytes has been demonstrated, as well as the ability of the virus to be replicated in this cell type [42]. In this sense, the pathologies observed in zebrafish inoculated by rSpike may be closely related to the high expression of ACE2 in these tissues.

In a study performed by Tyrkalska et al. (2022) [43] observed that S protein induced the local and systemic expression of genes encoding proinflammatory mediators. Several other studies have shown that zebrafish is an excellent model to study the inflammatory process caused by COVID-19 [44, 43], According to Fernandes et al., 2021 [36]. The human receptor of angiotensin converting enzyme 2 (ACE2) shares 72% sequence similarity, in addition, zebrafish show tissue distribution of ACE2 RNA. Our study demonstrated that inoculation of the recombinant viral protein rSpike promoted the favoring of inflammatory processes in the liver and intestinal tissue compared to healthy subjects.

The process of virus infection and replication in host cells induces cellular oxidative stress, which is responsible for promoting the secretion of critical pro-inflammatory factors "cytokine storms" [45, 46, 15, 16, 17]. The induction of these cytokines is tightly controlled to prevent tissue damage and maintain immune homeostasis [47]. However, if the synthesis process is too excessive, this exaggerated increase can trigger high immune and inflammatory response, causing cell and tissue damage in multiple organs [48, 49, 50, 51, 52, 53].

Here we observed elevated levels of IL-1β, IL-6, TNF-α, and NFKb in subjects injected with the rSpike protein compared to healthy subjects in most tissues. Interestingly, studies on SARS-CoV-2-mediated cytokine induction also report positive regulation of these markers [54, 52, 53], as well as the association of the NF-κB marker to activate the "cytokine storm" [55, 56, 20]. The secretion of IL-1β, IL-6, and mediators such as tumor necrosis factor (TNF-α), are also associated with the stimulation of a hyper tissue inflammation and rapid disease progression in high-risk patients [57, 58, 54, 52, 53]. Our data suggest that the infection generated by rSpike stimulated the "cytokine storm", substantially promoting disease worsening through the high mortality rate and the histopathological changes observed.

(a Supplementary Discussion and Supplementary References) SARS-CoV-2 induces a set of changes at various cellular levels, ranging from alterations in cellular respiration pathways, and energy metabolism (glycolytic), to inflammatory pathways [59, 20, 60]. It is also accepted that activation of the cellular immune system, as well as increased glucose uptake (hyper glucose) in COVID-19 patients, worsen the inflammatory response by increasing intracellular ROS production and stimulating the secretion of pro-inflammatory cytokines [61, 62, 63, 64, 56, 65]. Increased ROS in mitochondria and its consequent cellular toxicity is also related to *Cox1* enzyme dysfunction [66, 67, 68]. Our results showed that rSpike positively modulated glut (cell membrane glucose transporter) and ROS and COX1 in various tissues. Thus, our data support the hypothesis that the pathogenesis of COVID-19 is also related to the glycolytic pathway and oxidative stress in zebrafish.

Similarly, the protein-protein interaction prediction among SARS-CoV-2 and *D. rerio* proteins shows the biological pathways that can modify the normal function in infection of SARS-CoV-2 and the alteration of the expression of related genes. Among them, we can highlight the involvement of *Slc2a1a, Slc2a1b (*cell membrane glucose transport), interleukins-*Il1b, and Il6* (pro-inflammatory pathway), *Nfkb1,* and *Cox1* (mitochondrial respiratory chain) in COVID-19.

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