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Effects of photobiomodulation on the growth of intestinal bacteria

Efeitos da fotobiomodulação no crescimento de bactérias intestinais

Efectos de la fotobiomodulación sobre el crecimiento de bacterias intestinales

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Abstract
Necrotizing enterocolitis is an inflammatory bowel disease that occurs in newborns, more commonly in preterm infants. It is the leading cause of death from gastrointestinal diseases in neonates, and is characterized by the development of diffuse intestinal necrosis in premature infants subjected to stress. The high incidence and lack of effective treatment strategies suggest that new approaches to treating the disease are needed. It is in this context that the possibility of using photobiomodulation as a therapeutic modality arises. However, studies on the use of photobiomodulation in intestinal bacteria are scarce. To study the effect of photobiomodulation used in clinical parameters on the growth of bacteria commonly present in the newborn microbiota. Four strains of bacteria were chosen to be studied, two belonging to healthy intestinal microbiota, *Lactobacillus acidophilus* and *Lactobacillus reuteri*, and two pathogenic bacteria, *Escherichia coli* and *Staphylococcus aureus*. These bacteria were cultivated in planktonic growth and irradiated with LED at a wavelength of 660 nm and a power density of 0.025 W/cm^2, at fluences of 1, 5 and 10 J/cm^2. The evaluation of cell growth was performed through absorbance readings in the periods of 4h, 24h and 48h after irradiation. The growth of *L. acidophilus*, *L. reuteri* and *S. aureus* did not undergo biomodulation in any of the fluences. The growth of *E. coli* was stimulated at 1 J/cm^2 when compared to the Control group, with statistical significance ($p<0.005$). In the other fluencies there was no biostimulation for the *E. coli* bacteria. The use of a wavelength of 660 nm in the fluences of 5 J/cm^2 and 10 J/cm^2 in the studied bacteria did not lead to a significant change in the growth rate.
Keywords: Necrotizing enterocolitis; Photobiomodulation; Intestinal bacteria.

Resumo
A enterocolite necrotizante é uma doença inflamatória intestinal que ocorre no récem-nascido, mais comumente no prematuro. É a principal causa de morte por doenças gastrointestinais em neonatos, e caracterizada pelo desenvolvimento de necrose intestinal difusa no prematuro submetido a estresse. A alta incidência e a falta de estratégias de tratamentos efetivas sugerem que novas abordagens para o tratamento da doença são necessárias. É neste contexto que surge a possibilidade do uso da fotobiomodulação como modalidade terapêutica. Porém, os estudos sobre a utilização da fotobiomodulação em bactérias intestinais são escassos. Estudar o efeito da fotobiomodulação utilizado em parâmetros clínicos no crescimento de bactérias comumente presentes na microbiota do recém nascido. Foram escolhidos 4 cepas de bactérias para serem estudadas, sendo duas pertencentes a microbiota intestinal saudável, *Lactobacillus acidophilus* e *Lactobacillus reuteri* e duas bactérias patogênicas, *Escherichia coli* e *Staphylococcus aureus*. Essas bactérias foram cultivadas em crescimento planctônico e irradiadas com LED em comprimento de onda 660 nm e uma densidade de potência de 0,025 W/cm^2, em fluências de 1, 5 e 10 J/cm^2. A avaliação do crescimento celular foi realizada através de leituras por absorvância nos períodos de 4h, 24h e 48h após irradiação. O crescimento de *L. acidophilus, L. reuteri* e *S. aureus* não sofreu biomodulação em nenhuma das fluências. O crescimento de *E. coli* foi estimulado em 1 J/cm^2 quando comparado ao grupo Controle, com significância estatística ($p<0,005$). Nas demais fluências não houve bioestimulação para a bactéria *E.coli.* O uso do comprimento de onda de 660 nm nas fluências de 5 J/cm^2 e 10 J/cm^2 nas bactérias estudadas não levou a uma alteração na velocidade de crescimento significativo.
Palavras-chave: Enterocolite necrotizante; Fotobiomodulação; Bactérias intestinais.

Resumen
La enterocolitis necrotizante es una enfermedad inflamatoria intestinal que se presenta en recién nacidos, más comúnmente en bebés prematuros. Es la principal causa de muerte por enfermedades gastrointestinales en recién nacidos y se caracteriza por el desarrollo de necrosis intestinal difusa en bebés prematuros sometidos a estrés. La alta incidencia y la falta de estrategias de tratamiento efectivas sugieren que se necesitan nuevos enfoques para tratar la enfermedad. Es en este contexto que surge la posibilidad de utilizar la fotobiomodulación como modalidad terapéutica. Sin embargo, los estudios sobre el uso de la fotobiomodulación en bacterias intestinales son escasos. Estudiar el efecto de la fotobiomodulación utilizada en parámetros clínicos sobre el crecimiento de bacterias comúnmente presentes en la microbiota neonatal. Se eligieron cuatro cepas de bacterias para su estudio, dos pertenecientes a la microbiota intestinal sana, *Lactobacillus acidophilus* y *Lactobacillus reuteri*, y dos bacterias patógenas, *Escherichia coli* y *Staphylococcus aureus*. Estas bacterias se cultivaron en crecimiento planctónico y se irradiaron con LED a una longitud de onda de 660 nm y una densidad de potencia de 0,025 W/cm^2, a fluencias de 1, 5 y 10 J/cm^2. La evaluación del crecimiento celular se realizó mediante lecturas de absorbancia en los periodos de 4h, 24h y 48h posteriores a la irradiación. El crecimiento de *L. acidophilus*, *L. reuteri* y *S. aureus* no experimentó biomodulación en ninguno de los flujos. El crecimiento de *E. coli* se estimuló a 1 J/cm^2 en comparación con el grupo Control, con significación estadística (p <0,005). En las otras fluencias no hubo bioestimulación para la bacteria *E. coli*. El uso de una longitud de onda de 660 nm en las fluencias de 5 J/cm^2 y 10 J/cm^2 en las bacterias estudiadas no produjo un cambio significativo en la tasa de crecimiento.
Palabras clave: Enterocolitis necrotizante; Fotobiomodulación; Bacterias intestinales.

1. Introduction

Necrotizing enterocolitis (NEC) is an inflammatory bowel disease that occurs in the newborn, more commonly in the premature infant, with a high mortality and morbidity rate. It has been one of the most difficult diseases to eradicate and has become a priority for research. The mortality rate varies from 20 to 30%. The intense inflammatory process that begins in the highly immunoreactive intestine extends the disease systemically, affecting other organs, such as the brain. Despite many studies, prevention strategies do not present a satisfactory result, showing the lack of clear definitions of what constitutes the diagnosis of classic NEC (Obladen, 2009).

The pathophysiology of the disease is not fully understood. Epidemiological observations suggest that it is a multifactorial disease. The condition is probably related to the newborn's gastrointestinal tract's immaturity, which facilitates the multiplication of pathogenic bacteria and their translocation. It happens through mixed colonization of the child's intestine, and pathogens frequently found are *Staphylococcus aureus* and *Escherichia coli*. Treatment consists of antibiotic therapy and fasting. The condition is associated with an intense inflammatory process and mesenteric vasoconstriction, leading to a state of intestinal ischemia and necrosis with extremely short and long-term sequelae (Sueh et al., 2002; Samuels et al., 2017).

Photobiomodulation by light in the red to the spectrum's infrared region (630-1000 nm) modulates numerous cellular functions. The clinical and experimental applications of photobiomodulation have expanded over the past 30 years. Low-power lasers and light-emitting diodes (LEDs) are well-accepted therapeutic tools for treating infected, ischemic, and hypoxic wounds and other soft tissue injuries. The positive effects of photobiomodulation include accelerated healing, better recovery from ischemic lesions in the heart, and attenuated degeneration of the injured optic nerve (Desmet et al., 2006). At the cellular level, photobiomodulation can modulate fibroblasts' proliferation, the fixation and synthesis of collagen and procollagen, promote angiogenesis and stimulate macrophages and lymphocytes, improving energy metabolism in mitochondria. Besides, photobiomodulation has demonstrated the ability to increase the production of growth factors, such as keratinocyte growth factor (KGF), transforming growth factor (TGF), and platelet-derived growth factor (PDGF) (Desmet et al., 2006; Hamblin, 2017).

The advances in the clinical use of photobiomodulation and its anti-inflammatory, neovascularization, and cell regeneration effects, transform this therapeutic modality into an excellent option for the treatment of this pathology, being non-invasive and easy to apply (Desmet et al., 2006). The possibility of a new therapeutic modality, without side effects, reducing antibiotic therapy time, the need for surgical intervention, decreased use of antibiotic therapy, parenteral nutrition, and shorter hospital stay is very positive in treating these children. The prospect of a satisfactory biomodulation response in this disease could revolutionize the treatment and survival of premature babies and the quality of life of these children in the future.

The limiting factor at the moment for the use of laser therapy in intestinal pathologies is the potential biostimulating effect on intestinal bacteria, which would make its use unfeasible. Both an increase in pathogenic bacteria and a massive increase in flora bacteria in immunocompromised patients is not tolerable. The number of photobiomodulation studies in intestinal pathologies is scarce, but with a potential for promising clinical application. In *in vitro* studies on the influence of photobiomodulation on bacterial growth, the results are diverse. Depending on the dose and parameters used, bacterial growth can be stimulated or inhibited, varying with bacteria species (Nussbaum et al., 2003).

In order to study the effect of photobiomodulation on the growth of some bacteria that colonize the newborn's intestine, we conducted this study.

2. Methodology

That experimental study, *in vitro*, used a deductive hypothetical method, with a quantitative approach through statistical analysis of the collected data (Pereira et al., 2018).

Four species of bacteria were selected to be studied. Two belonging to healthy intestinal flora (*Lactobacillus acidophilus* and *Lactobacillus reuteri)* and two pathogenic bacteria that commonly infect newborns' intestines (*Escherichia coli* and *Staphylococcus aureus*). Strains of *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, grown in BHI medium, and *Lactobacillus reuteri* DSM17938, *Lactobacillus acidophilus* LA14, grown in MRS medium were used to carry out the research. After bacterial growth in 24 hours, the cells were added to a culture plate and irradiated by the Irrad LED 5 660 Biopdi equipment (São Carlos Brasil) with diodes at 660 nm wavelengths and a power of 0.025 W/cm^2, in doses of 1, 5 and 10 J/cm^2 for 40 seconds, 3 minutes 20 seconds and 6 minutes and 40 seconds, respectively. The readings were taken at 4h, 24h, and 48h.

In each experiment, four groups were prepared: initial reading, control, 1J/cm^2, 5 J/cm^2, and 10 J/cm^2, and four experiments were repeated. The initial number of bacteria was read by optical spectrophotometry, using the SpectraCount® Spectrophotometer (Packard) with a 570nm filter to measure the optical density of bacterial suspensions (Aacos et al., 2004).

3. Results

The growth of *L. acidophilus* (Table1) and *L. reuteri* (Table 2) did not undergo biomodulation in any of the fluences.

Table 1 - Average and standard deviation of the colony-forming units x 10^8 of *Lactobacillus acidophilus* in the control and irradiated groups at 660nm wavelength, with fluences 1, 5, and 10 J/cm^2

	Control n=24	**1J/cm^2 n=23**	**5J/cm^2 n=24**	**10J/cm^2 n=24**
0h	2,963 (0,462)	2,986 (0,458)	2,963 (0,463)	2,963 (0,463)
4h	2,990 (0,581)	2,906 (0,467)	2,881 (0,493)	2,837 (0,442)
24h	4,578 (0,790)	4,382 (0,551)	4,266 (0,724)	4,145 (0,733)
48h	4,141 (0,627)	4,177 (0,473)	4,330 (0,450)	4,246 (0,331)

Source: Authors.

Table 2 - Average and standard deviation of the colony-forming units x 10^8 of *Lactobacillus reuteri* in the control and irradiated groups at 660nm wavelength, in fluences 1, 5 and 10 J/cm^2

	Control n=23	**1J/cm^2 n=24**	**5J/cm^2 n=23**	**10J/cm^2 n=23**
0h	3,093 (0,518)	3,116 (0,519)	3,095 (0,520)	3,093 (0,518)
4h	4,536 (1,165)	4,328 (0,903)	4,357 (0,890)	4,897 (1,085)
24h	17,257 (2,754)	17,245 (2,515)	17,181 (1,595)	18,189 (1,718)
48h	14,307 (3,709)	14,633 (3,047)	16,114 (2,576)	15,754 (2,230)

Source: Authors.

The growth of *Escherichia coli* was stimulated (Table 3) when we compared the 1 J/cm^2 group to the Control group, with statistical significance.

Table 3 - Average and standard deviation of colony-forming units x 10^8 of *Escherichia coli* in the control and irradiated groups at 660nm wavelength, in fluences 1, 5 and 10 J/cm^2

	Control n=23	**1J/cm^2 n=24**	**5J/cm^2 n=24**	**10J/cm^2 n=23**
0h	1,108 (0,014)	1,108 (0,014)	1,108 (0,014)	1,108 (0,014)
4h	9,513 (2,008)	9,950 (0,930)	9,751 (1,579)	9,589 (2,250)
24h	16,652 (2,079)	18,119 (1,954)	18,006 (1,724)	17,871 (1,457)
48h	18,307 (1,860)	19,023 (1,963)	18,632 (1,982)	18,840 (1,176)

Source: Authors.

The growth of *Staphylococcus aureus* did not undergo biostimulation (Table 4) in any of the fluences.

Table 4 - Average and standard deviation of the colony-forming units x 10^8 (CFU) of *Staphylococcus aureus* in the control and irradiated groups at 660nm wavelength, in fluences 1, 5 and 10 J/cm^2

	Controle n=22	**1J/cm^2 n=24**	**5J/cm^2 n=23**	**10J/cm^2 n=23**
0h	1,137 (0,049)	1,137 (0,049)	1,137 (0,049)	1,137 (0,049)
4h	6,420 (1,382)	5,916 (1,526)	6,100 (1,540)	5,976 (1,071)
24h	15,189 (1,260)	15,265 (2,471)	15,748 (1,935)	15,160 (2,608)
48h	16,286 (2,730)	16,682 (3,712)	15,871 (3,097)	15,664 (3,111)

Source: Authors.

4. Discussion

Necrotizing Enterocolitis (NEC) is the leading cause of death from gastrointestinal diseases in neonates and is characterized by the development of diffuse intestinal necrosis in preterm infants subjected to stress. Still, it is the leading cause of intestinal failure in premature infants, which leads to Short Bowel Syndrome. The high incidence of NEC, and the lack of effective treatment strategies suggest that new approaches to the treatment of the disease are needed (Hackam et al., 2005). It is in this context that the possibility of using photobiomodulation as a therapeutic modality arises.

Current treatment for NEC involves antibiotics, fasting bowel rest, total parenteral nutrition, and surgical resection or peritoneal drainage when indicated. Each of these modalities is used to prevent the patient's septic condition from getting worse. However, none of these modalities effectively deals with the biological process involved in ECN. This may explain why the ECN mortality rate has changed so little over the past 40 years (Hackam et al., 2005; Hackam & Caplan, 2018).

Possible new treatment fronts would neutralize the intestinal nitric oxide levels, minimizing the enterocytes' apoptosis, and restoring the epithelial restitution and proliferation. Photobiomodulation has already proven effective in this function, protecting cells against NO-induced apoptosis (Hackam et al., 2005; Farivar et al., 2014; Rizzi et al., 2018). Besides, therapeutic agents that act on RhoA-GTPase in the intestine, or restore basal sodium-proton exchange in the enterocyte, could mitigate endotoxins' effects on the intestinal barrier function. Photobiomodulation proved to be effective in activating RhoA-GTPase, stimulating corneal epithelial cells (Hackam et al., 2005; Rhee et al., 2017).

The fact that enterocytes exposed to bacterial endotoxins express high levels of cyclooxygenase-2 leads us to an intriguing possibility that agents that modify COX-2 activity, or its expression may reduce the extent and severity of NEC in these patients. Tatmatsu-Rocha et al. (2018) showed in their work that the use of photobiomodulation in skin lesions of diabetic rats decreases the expression of COX-2. Interestingly, in this work, that comparing irradiation with Laser and LED, the latter showed better results in inhibiting the expression of COX-2 (Hackam et al., 2005; Tatmatsu-Rocha et al., 2018).

The choice of parameters used for irradiation was based on previous data from the literature, showing promising results in endothelial cells and fibroblasts in the red wavelength (Moore et al., 2005). Wavelengths of 500-700 nm are effective on more superficial tissues, while wavelengths of 800 to 1000 nm are indicated for deeper tissues (Kuffler, 2016). For the treatment of abdominal pathologies in newborns, wavelengths in the red band are sufficient. Another aspect is the behavior of different wavelengths in bacteria. While infrared irradiation alters bacteria's growth rate, in some cases stimulating them, irradiation in the red band has little effect on bacterial growth (Liebert et al., 2019; Nussbaum et al., 2002).

Regarding the choice of bacteria, *Escherichia coli* and *Staphylococcus aureus* are among the bacteria most commonly associated with NEC, one of which is Gram-negative and the other Gram-positive, allowing the study of the response of different bacterial types to the same treatment with photobiomodulation. Still, *Lactobacilli* are found in breast milk and the newborn's flora, but when supplemented with probiotic pills, they can lead to sepsis, showing that their increase during photobiomodulation would also be harmful (Hunter et al., 2008).

We observed in previous studies that very high fluences inhibit bacterial growth. Despite the positive result for pathogenic bacteria, irradiation would probably inhibit the growth of commensal bacteria (Sousa et al., 2016; Barboza et al., 2015).

The growth of *L. acidophillus* was slower compared to the growth of *L. reuteri*. Ribeiro in 2012 showed that the LA14 strain has a slower growth when compared to other strains of *Lactobacillus spp (*Ribeiro et al., 2012). Our study demonstrated that there is no influence of photobiomodulation on the growth of *Lactobacillus*. In 2020, Nemeth et al (2020) showed a decrease in the number of CFU of *Lactobacillus* irradiated with LEDs at wavelengths 625, 660, and 850 nm and an energy density of 16 mW /cm^2. The difference found can be explained by the fact that the salivary glands were irradiated by the extra-oral route (Nemeth et al., 2020).

In our study, in *E. coli* bacteria, irradiation with fluency of 1J/cm^2 stimulated bacterial growth in relation to the Control group. In the other fluences, growth was stimulated, but without statistical significance. Nussbaum in 2002 showed no general effect on the growth of *E. coli* after irradiation with wavelengths 630, 660, 810, and 905 nm. The most significant inhibition occurred with 630 nm irradiation at a radiant exposure of 1 J/cm^2 (Nussbaum et al., 2002). Sousa in 2016 showed that *E. coli* had similar growth inhibition at a wavelength of 830 nm at fluencies of 3, 6, 12, and 24 J/cm^2. At 660 and 904 nm wavelengths, growth inhibition was observed only at 12 and 18 J/cm^2 fluences, respectively (Sousa et al., 2016).

In our study at 660 nm 0.025 W/cm^2, at doses of 5 and 10J/cm^2, there was a decrease in the growth of *S. aureus*, but without statistical significance. Ranjbar & Takhtfooladi (2016), in his *in vivo* study using photobiomodulation at parameters 685 nm InGaAlP 15 mW, 3 J/cm^2, obtained inhibition of the bacterial growth of *S. aureus (*Ranjbar & Takhtfooladi, 2016). Sousa in 2012 showed that the infrared spectrum was more effective when compared to red, mainly for 904 nm, since 660 nm did not promote global inhibition (Sousa et al., 2012).

The results obtained were optimistic. If there is no significant change in the intestinal flora, the potential for using photobiomodulation in intestinal pathologies, including necrotizing enterocolitis, begins to appear promising. The use of the 660nm wavelength in the fluences of 5 J/cm^2 and 10 J/cm^2 in pathogenic bacteria did not lead to a significant change in the speed of growth, as well as in *Lactobacillus*.

5. Conclusion

Cultures of bacteria *Lactobacillus acidophillus*, *L. reuteri*, *S. aureus* irradiated at a wavelength of 660 nm and a power of 0.025 W/cm^2, in doses of 1, 5, and 10 J/cm^2 did not undergo biostimulation. *E. coli* growth was stimulated when we compared the 1 J/cm^2 group to the Control group, with statistical significance ($p < 0.005$). In the other fluencies, there was no biostimulation. The use of the 660nm wavelength in the fluences of 5 J/cm^2 and 10 J/cm^2 in pathogenic bacteria did not lead to a significant change in the speed of growth, as well as in *Lactobacillus*.

The results obtained were optimistic. If there is no significant change in the intestinal flora, the potential for using photobiomodulation in intestinal pathologies, including necrotizing enterocolitis, begins to appear promising.

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