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JÉSSICA FERNANDA SENA BONVICINI

Efeito do LED vermelho e infravermelho na produção de radicais livres por células pulpares após estímulo com lipopolissacarídeo

Effect of red and infrared LED on free radicals level produced by pulp cells under stress condition using lipolysaccharide

Dissertação apresenta à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito para obtenção do Título de Mestre em Odontologia na Área de Clínica Odontológica Integrada.

Uberlândia, 2021

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Orientadora: Profª. Drª. Ana Paula Turrioni

Banca Examinadora:
Profª. Drª. Ana Paula Turrioni Hidalgo
Prof. Dr. Guilherme José Pimentel Lopes de Oliveira
Prof. Dr. Carlos Alberto de Souza Costa

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UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenação do Programa de Pós-Graduação em Odontologia
Av. Pará, 1720, Bloco 4L, Anexo B, Sala 35 - Bairro Umuarama, Uberlândia-MG, CEP 38400-902
Telefone: (34) 3225-8115/8108 - www.ppgoufu.com - copod@umuarama.ufu.br

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"Tudo em mim é um desassossego,
sempre crescente e sempre igual.
Tudo me interessa e nada me prende."
Fernando Pessoa

SUMÁRIO

Resumo

RESUMO

Estudos têm apontado efeitos positivos relacionados com o reparo do tecido pulpar, após uso de fontes de luz como laser ou Diodos Emissores de Luz (LED). Entretanto, os parâmetros específicos de irradiação que podem ser benéficos ou deletérios para a modulação inflamatória deste tecido permanecem desconhecidos. Deste modo, o objetivo do estudo foi avaliar o efeito de diferentes parâmetros do LED vermelho e infravermelho na modulação do estresse oxidativo e na viabilidade de células pulpares de dentes decíduos. Para isto, células pulpares foram obtidas de 5 dentes hígidos esfoliados e cultivadas em placas de acrílico de 24 compartimentos (10^5 células/compartimento) utilizando DMEM suplementado com 10% de soro fetal bovino (SFB). Após 24 horas, foi realizada a indução da produção de mediadores inflamatórios pela aplicação de lipopolissacarídeo (LPS) na concentração de 10µg/mL de meio de cultura. Logo após a aplicação do LPS, as células foram irradiadas uma única vez (630nm e 850nm, 40 mW/cm^2 e 80 mW/cm^2) nas doses de 4J/cm^2, 15J/cm^2 e 30J/cm^2. Para avaliação da viabilidade celular, número de células viáveis e morfologia celular, foram realizados os testes de MTT, Trypan Blue e Microscopia Eletrônica de Varredura (MEV) respectivamente. Além disso, foi realizado a quantificação de óxido nítrico (ON), por meio do reagente de Griess, e a quantificação de espécie reativa de oxigênio (EROs) utilizando uma sonda de fluorescência (DCFH-DA). Todas as avaliações foram realizadas 24 horas após a irradiação. Os testes estatísticos de Kruskal-Wallis e Mann-Whitney foram utilizados ($p<0,05$). Para o LED vermelho, um aumento da viabilidade foi observado nas células expostas ao LPS e irradiadas com 15J/cm^2 e 30J/cm^2 a 40mW/cm^2 e 4J/cm^2 e 15J/cm^2 a 80 mW/cm^2 em comparação ao grupo controle ($p<0,05$). As doses de 4J/cm^2 a 40mW/cm^2 e 15J/cm^2 e 30J/cm^2 a 80mW/cm^2 modularam o estresse oxidativo quando comparadas ao grupo apenas tratado com LPS ($p<0,05$). Para o LED

infravermelho, todos os parâmetros de irradiação foram capazes de diminuir as concentrações de EROs após aplicação do LPS quando comparados ao grupo não irradiado ($p<0,05$). As imagens de MEV indicaram células com morfologia normal e aderidas ao substrato. Concluiu-se que o LED vermelho utilizado na dose de 15J/cm² e 30J/cm^2 a 40mW/cm^2 e 15J/cm^2 a 80mW/cm² e o LED infravermelho utilizado na dose de 15J/cm^2 a 40mW/cm^2 foram os parâmetros mais efetivos para o estímulo da viabilidade e a modulação do estresse oxidativo de células pulpares de dentes decíduos.

Palavras-chave: Cavidade pulpar, Células Cultivadas, Estresse Oxidativo, Fototerapia

Abstract

ABSTRACT

Studies have showed positive effects related to the pulp tissue repair, after the use of light sources such as laser or Light Emitting Diodes (LED). However, the specific irradiation parameter that may be beneficial or harmful to the inflammatory modulation of this tissue remains unknown. The aim of the study was to evaluate the effect of different parameters of the red and infrared LED on the oxidative stress modulation and on the viability of pulp cells of primary teeth. Pulp cells were obtained from 5 healthy exfoliated teeth and cultured in 24-compartment acrylic plates (10^5 cells/compartment) using DMEM supplemented with 10% fetal bovine serum (FBS). After 24 hours, the production of inflammatory mediators was induced by the application of lipopolysaccharide (LPS) at a concentration of 10μg/mL in culture medium. Right after the application of LPS, the cells were irradiated only once (630nm and 850nm, 40mW/cm^2 and 80 mW/cm^2) in the energy exposures of 4J/cm^2, 15J/cm^2 and 30J/cm^2. To assess cell viability, number of viable cells and cell morphology, MTT, Trypan Blue and Scanning Electron Microscopy (SEM) tests were performed respectively. In addition, quantification of nitric oxide (NO) was performed using Griess reagent, and the quantification of reactive oxygen species (ROS) using a fluorescence probe (DCFH-DA). All evaluations were performed 24 hours after irradiation. The Kruskal-Wallis and Mann-Whitney statistical tests were used ($p<0.05$). For the red LED, an increase on viability was observed in cells exposed to LPS and irradiated with 15J/cm^2 and 30J/cm^2 at 40mW/cm^2 and 4J/cm^2 and 15J /cm^2 at 80 mW/cm^2 compared to the control group ($p<0.05$). The doses of 4J/cm^2 to 40mW/cm^2 and 15J/cm^2 and 30J/cm^2 at 80mW/cm^2 modulated oxidative stress when compared to the group treated only with LPS ($p<0.05$). For the infrared LED, all irradiation parameters were able to decrease the concentrations of ROS after LPS application when compared to

the non-irradiated group ($p<0.05$). The SEM images indicated cells with normal morphology and adhered to the substrate. It was concluded that the red LED using 15J/cm² and 30J/cm^2 at 40mW/cm^2 and 15J/cm^2 at 80mW/cm² and the infrared LED using 15J/cm^2 at 40mW/cm^2 were the most effective parameters for stimulating viability and modulating the oxidative stress of pulp cells from primary teeth.

Keywords: Cell culture, Dental Pulp Cavity, Phototherapy, Oxidative Stress

Introdução e referencial teórico

INTRODUÇÃO E REFERENCIAL TEÓRICO

O tratamento de dentes apresentando lesões cariosas médias ou profundas ainda se apresenta controverso, levando a diferentes condutas por clínicos nos diversos países, envolvendo procedimentos que variam desde os conservadores (remoção de tecido cariado apenas com instrumentos manuais, proteção pulpar e posterior restauração) até a extração do elemento dentário seguida de reabilitação protética (Peres et al., 2020). Além disso, os procedimentos relacionados ao tratamento de lesões cariosas ainda são extremamente necessários e geram um impacto significante na economia global (Schwendicke et al., 2014, Listl et al., 2015). Deste modo, o desenvolvimento de técnicas e materiais relacionados a tratamentos mais conservadores se torna importante para evitar o desperdício de gastos com tratamentos mais invasivos, e muitas vezes desnecessários (Schwendicke et al., 2014).

A fototerapia utilizando diversos parâmetros do laser ou LED tem sido apontada como uma interessante opção de tratamento para o reparo de tecidos e, especificamente, tem sido sugerida como etapa de tratamento para lesões cariosas, com o intuito de estimular o processo de reparo pulpar (Turrioni et al., 2013, 2014, 2015a, Alonso et al 2016, de Almeida et al., 2016). A irradiação utilizando comprimentos de onda no espectro vermelho ou infravermelho tem se mostrado eficiente no estímulo da viabilidade de células pulpares obtidas de roedores e em culturas imortalizadas de células odontoblastóides (Holder et al., 2012, Turrioni et al 2015a). Além disso, esta modalidade de terapia tem apresentado resultados promissores no que diz respeito à expressão e produção de proteínas envolvidas na formação de matriz dentinária e sua mineralização,

tanto em estudos *in vitro* como em estudos utilizando modelo animal (Segovia et al., 2006, Turrioni et al., 2014, Turrioni et al, 2015b).

Clinicamente, sugere-se que a luz possa ser utilizada após a remoção do tecido cariado (em cavidades médias e profundas), antes da realização do procedimento restaurador, objetivando o estímulo do reparo pulpar e, consequentemente, evitando procedimentos curativos mais invasivos. A polpa possui uma característica única, pois está confinada entre paredes mineralizadas de dentina, inextensíveis, que não permitem o extravasamento do edema inflamatório. Além disso, a circulação colateral e o sistema de drenagem linfática estão comprometidos, uma vez que tanto os vasos sanguíneos e linfáticos chegam ao interior do tecido via pequenos forames apicais (Bletsa et al., 2006). Deste modo, o grau de inflamação do tecido pulpar pode atingir proporções de difícil controle intrínseco (Tziafas et al., 2000). Sabe-se que a presença de processo inflamatório intenso pode ser prejudicial ao reparo tecidual (Tziafas et al., 2000). Por exemplo, altas concentrações de Óxido Nítrico (ON), que é um radical livre altamente reativo produzido pelas ON sintetases, pode causar efeito citotóxico nos tecidos (Kendall et al. 2001), por isso, o ON, bem como outras espécies reativas de oxigênio (ERO) precisam ter sua produção modulada. Deste modo, seria interessante a realização de uma técnica que, além de estimular a produção de matriz dentinária, também fosse capaz de modular o processo inflamatório agudo instalado após injúria ao tecido.

Até o momento, a informação a respeito do efeito da luz na modulação da inflamação pulpar é escassa. Sabe-se que a irradiação com LED infravermelho pode diminuir os níveis de ON e ERO em cultura de células pulpares submetidas à exposição ao LPS (Montoro et al., 2014). Entretanto, os parâmetros específicos de irradiação que podem ser benéficos ou deletérios para a modulação inflamatória ainda não estão

claramente elucidados. Tampouco, se o comprimento de onda vermelho possui algum efeito positivo nesta modulação.

Com relação aos possíveis efeitos celulares que a fototerapia pode exercer, sabe-se que tanto o espectro vermelho quanto o infravermelho estão relacionados com o estímulo da cascata respiratória, que envolve a enzima terminal da cadeia de transporte de elétrons (citocromo C oxidase) (Karu et al., 2010). Esta enzima parece agir como um fotorreceptor, sendo capaz de utilizar a energia fornecida pela fonte de luz para gerar uma cascata de reações que levam a um aumento de energia (produção de ATP) e consequentemente o estímulo de diversas funções celulares (Karu et al., 2010, de Freitas et al., 2016, Wang et al 2016) . Uma interessante diferença entre a utilização destes dois espectros de luz é a capacidade de penetração em tecidos, onde o infravermelho apresenta-se como espectro com maior capacidade para atingir camadas mais profundas (Turrioni et al., 2013). Apesar da menor capacidade de transpassar os diferentes tecidos biológicos ou materiais quando comparado ao infravermelho, o espectro vermelho ainda apresenta difusão aceitável em tecido dentinário para exercer um possível efeito estimulatório no tecido pulpar (Turrioni et al., 2013).

Considerando o efeito bioestimulatório do LED vermelho e infravermelho em células pulpares (Holder et al., 2012; Turrioni et al., 2014; Turrioni et al., 2015a), a facilidade de uso e o baixo custo desta fonte de luz, ressalta-se a importância de se investigar o efeito da irradiação LED vermelha e infravermelha na modulação inflamatória de células pulpares de dentes decíduos, gerando resultados que direcionarão futuros estudos laboratoriais e clínicos que foquem na padronização desta inovadora técnica de bioestimulação do tecido pulpar.

Capítulos

1. CAPÍTULO 1

ARTIGO 1

Photobiomodulation effect of 630nm red LED on free radicals level produced by pulp cells under stress condition

***Artigo em consideração no Lasers in Medical Science com Minor Revision, em 14 de Janeiro de 2021.**

Photobiomodulation effect of 630nm red LED on free radicals level produced by pulp cells under stress condition

Jéssica Fernanda Sena Bonvicini[1], Fernanda Gonçalves Basso[2], Carlos Alberto de Souza Costa[3], Carlos José Soares[4] & Ana Paula Turrioni [1]

[1]Department of Pediatric Dentistry, Federal University of Uberlândia, UFU, Uberlândia, MG, Brazil

[2]Department of Oral Pathology, University of Ribeirão Preto, UNAERP, Ribeirão Preto, SP, Brazil

[3]Department of Physiology and Pathology, Araraquara School of Dentistry, UNESP, Araraquara, SP, Brazil

[4]Department of Operative Dentistry and Dental Materials, Federal University of Uberlândia, UFU, Uberlândia, MG, Brazil

Corresponding Author
Dr. Ana Paula Turrioni
DDS, MSc, PhD, Cell Biology Research Group, Department of Pediatric Dentistry, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.
Av. Pará 1720, Umuarama.
CEP: 38400902, Uberlândia, MG, Brasil
E-mail:apturrioni@ufu.br
Tel: +55-16-3301-64

Photobiomodulation effect of 630nm red LED on free radicals level produced by pulp cells under stress condition

Abstract

The aim of this study was to evaluate the capacity of red LED at different parameters of irradiation to modulate oxidative stress of human dental pulp fibroblasts (HDPFs). HDPFs obtained from primary teeth were seeded (100.000 cells/well) in 24-well plates using culture medium (DMEM). After 24-hour incubation, the culture medium was replaced by DMEM with 10 μg/mL of lipopolysaccharide (LPS). Then, the cells were irradiated (LED 630 nm, 0.04 W/cm² and 0.08 W/cm²) at 0 J/cm² (control group), 4 J/cm², 15 J/cm² and 30 J/cm², and assessed regarding their viability (MTT assay), number (Trypan Blue), synthesis of NO (Griess reagent) and ROS (fluorescence probe, DCFH-DA). The Kruskal-Wallis and Mann-Whytney statistical tests using Bonferroni correction were used (significance level of 5%). Increased viability was observed in those HDPFs exposed to LPS, irradiated with 15 J/cm^2 and 30 J/cm^2 at 0.04 W/cm^2 and 4 J/cm^2 and 15 J/cm^2 at 0.08 W/cm^2 in comparison with control group ($p<0.05$). Radiant exposures of 4 J/cm^2 at 0.04 W/cm^2 and 15 J/cm^2 and 30 J/cm^2 at 0.08 W/cm^2 modulated the oxidative stress when compared with non-irradiated LPS-treated pulp cells ($p<0.05$). It was concluded that the irradiation strategy of using red LED with radiant exposures of 15 J/cm² and 30 J/cm^2 at 0.04 W/cm^2 and 15 J/cm^2 at 0.08 W/cm² were the best parameters to decrease NO and ROS concentration and to stimulate viability of human dental pulp fibroblasts exposed to LPS challenge.

Keywords: Cell culture, Photobiomodulation, Dental Pulp, Oxidative Stress

Introduction

For treatment of tooth with very deep decay, dental materials with specific properties, such as biocompatibility and potential to stimulate the healing of inflamed pulp, should be applied, after caries removal, on the cavity floor [1]. However, the development of such dental products is still a challenge for the Restorative Dentistry field. In this way, the strategy of photo-stimulating pulp cells through a thin remaining dentin between the cavity floor and the subjacent pulp has been investigated and considered as a potential adjuvant therapy for such clinical condition [2]. Untreated dental caries in permanent teeth affect about 2.4 billion people worldwide and 7.8% of children are affected in primary dentition [3]. Additionally, direct treatment costs due to dental diseases worldwide were estimated at $297.67B, causing a significant impact on the global economy [4]. Therefore, the importance of more conservative treatments is emphasized, avoiding excessive expenses with more invasive dental procedures [5].

Different modalities of photobiomodulation have been used in healthcare field as adjuvant treatments of bone loss, myalgia, skin burns, spinal cord injury and pulp repair [6, 7, 8, 9, 10]. Clinical and laboratory studies have evaluated the effect of photobiomodulation on healing of different tissues such as muscle and cartilage [11,12], neuronal cells [13, 14], bone [15, 16], and the dentin-pulp complex [2, 17, 18]. Photobiomodulation using Light Emitting Diodes (LED) has been widely assessed in Dentistry, being considered as a potential adjuvant treatment to stimulate pulpal healing [2, 18, 19, 20, 21, 22, 23]. LED irradiation with either infrared or red light enhances the proliferation of human and rodent pulp cells [21, 22, 23] as well as cultured odontoblast-like cells [2]. Moreover, dentin protein expression and production have also increased after the use of these wavelengths [20, 23]. Photobiomodulation has also induced *in vivo* mineralized tissue formation, decreased inflammatory cell infiltration and stimulated tissue vascularization after pulpal exposure performed in rat molars [19].

Particularly for the red LED, studies have shown positive effects on different cell lines such as fibroblasts [24], odontoblast-like cells [24] and pulp cells [21]. Red LED has also stimulated proliferation and angiogenesis in rodents [26]. Moreover, Turrioni *et al*. (2013) [27] demonstrated that red LED did not differ from infrared LED for 0.2 mm and 0.5 mm dentin disc thickness regarding light attenuation, indicating that red light also has good capacity of penetration in dentin tissue. Considering all these encouraging in vitro and in vivo data concerning the biological effects of red, one may suggest the clinical

application of photobiomodulation to stimulates pulp tissue recovery after caries removal or dental trauma, modulates the local inflammation and even to induces mineralized tissue formation on pulp exposure site.

It is known that pulp inflammation may be aggravated by the restriction of blood and lymphatic circulation that only occurs through the apical foramen of tooth [28, 29]. Specific molecules play a key role during the inflammatory process. For example, high concentrations of the free radical nitric oxide (NO), which is produced by NO synthetase, can damage healthy tissues and surrounding cells [30]. Therefore, one may consider that to regulate the intensity and time of inflammation, unstable molecules like NO and reactive oxygen species (ROS) should be modulated in injured tissues.

Even though infrared LED light can reduce NO and ROS concentrations in pulp cells exposed to LPS [31], optimal irradiation parameters have not yet been defined [32]. Additionally, it is also important to highlight the physiological cell mechanism of photobiomodulation in the red and near-infrared spectrum, which involves the terminal enzyme of the electron transport chain (Cytochrome C oxidase - COX). Briefly, COX seems to be the primary photoacceptor, being able to oxidize using photon-energy from LEDs. This process can trigger the enhanced oxygenation of cell metabolism and ATP production, further modulating the ROS and NO concentrations [33, 34, 35].

Given that LEDs are low cost, easily manipulated and may stimulate cell proliferation within the dental pulp [2, 21], the magnitude of the effect of red LED irradiation on the modulation of pulp inflammation should be determined. Therefore, the aim of this study was to evaluate the capacity of red LED at different parameters of irradiation to modulate oxidative stress of human dental pulp fibroblasts.

Methods

Obtaining and maintaining human dental pulp fibroblasts

This study was developed using a culture of human dental pulp fibroblasts (HDPFs), reported by previously studies [36, 37, 38], obtained from two exfoliated and healthy primary teeth from the Children's Clinic at the School of Dentistry of the Federal University of Uberlândia (UFU), Brazil, after approval of the research project by the Ethics Committee of the Institution (protocol number 54488816.2.0000.5152). The teeth were donated by the patients themselves after signing the informed assent form and by

those responsible after signing the free and informed consent term. The epidemiologic information about donators is shown in Table 1.

After extraction, the teeth were placed in a Falcon tube with culture media and immediately taken to the Laboratory of Biomaterials and Cell Culture. Next, the pulp was mechanically extracted within a vertical laminar flow from the interior of the pulp chamber using a sharp sterilized dentine spoon. The time of tooth extraction at the clinic and the removal of pulp tissue at the laboratory was from 10 to 20 minutes. The pulp tissue was immersed for 1 h in the following solution: 3 mg/mL collagenase type I (Sigma-Aldrich, Saint Louis, MO, US) and 4 mg/mL dispase (Sigma-Aldrich). The solution was then centrifuged at 1200 rpm for 2 minutes. The pellet formed at the bottom of the Falcon tube was re-suspended in a basal medium. The resulting cells were plated in 25 cm² flasks and incubated for 4 days at 37°C with 5% CO_2 [39]. The culture medium was initially changed after 3 days of incubation, and thereafter changed twice a week.

The culture and expansion of the cells isolated from pulp of each deciduous tooth were separately performed to obtain the number of cells enough to perform this investigation. Therefore, after isolating the human dental pulp fibroblasts, these cells were incubated at 37ºC until to reach approximately 80% confluence. At this point, cells were sub-cultured using trypsin 0.25% (passages) [22].

The experiments were performed in two distinct moments. In the first moment, three 75cm^2 bottles obtained from each donor at 3rd passage (equivalent to 8x10^6 pulp cells attached to the surface of each bottle) were used. Therefore, a total of 24x10^6 cells per donor were available for the tests. The number of cells requested for all laboratory protocols performed in this study, considering each donor and group was: ROS quantification – 4x10^5 cells (four compartments of each donor), ROS image – 2x10^5 cells (two compartments of each donor), MTT Assay – 4x10^5 cells (four compartments of each donor), TB Assay – 4x10^5 cells (four compartments of each donor). For NO, the supernatant of cells seeded for MTT Assay was used. Then, the total number of cells per donor and moment necessary to carry out the laboratory tests, considering the 14 groups established in this study was: 14x10^5 cells x 14 groups = 196x10^5 cells = 19.6x10^6 cells per donor. In the second moment, the same number of cells at 4th passage was used. Experimental and control groups that were stablished in this study according to the exposure or not of cells to LPS and the irradiation parameters are shown in Table 2.

Irradiation device

The irradiation of cultured pulp cells was performed using a LED Table, which is a device widely employed in laboratorial studies of this research field (Figure 1) [2, 22, 23, 25, 40, 41]. The LED Table was developed by the Optical Group (OG) of the Optics and Photonics Research Center (Centro de Pesquisa em Óptica e Fotônica - CePOF) of the São Carlos Institute of Physics (IFSC) at the University of São Paulo (USP). The 24 InGaN (Indium gallium nitride) diodes were positioned in the LEDTable at a standardized distance from the culture plate (1.4 cm) so that each could homogeneously irradiate all the cells seeded at the bottom of each compartment (2 cm^2).

Before starting this in vitro study, the LEDtable was calibrated to ensure that each LED diode was viable and operating within established parameters requested. Based on the measurements performed at the moment of LEDtable calibration/evaluation, it is important to highlight that 5% of power loss occurred because of the acrylic interposition and the distance (standardized in 1.4 cm) between the diode and the bottom of the well-plate. Therefore, the output power of the dispositive was adjusted in such way that a sensor placed at the bottom of compartment indicated the exact power of 0.16 W/cm^2 and 0.08 W/cm^2. In this way, the output power of the device was readjusted to reach the irradiances tested. The output power to reach the irradiance of 0.08 W/cm^2 (total area of each compartment - 2 cm^2) was 0.168 W and the output power to reach 0.04 W/cm^2 for the same area was 0.084 W.

Then, the possible beneficial parameters: 4 J/cm^2, 15 J/cm^2 and 30 J/cm^2, at 0.04 W/cm^2 [20, 23, 25, 31, 40], as well as more intense parameters, which may be considered as potentially harmful to the cells: 4 J/cm^2, 15 J/cm^2 and 30 J/cm^2, at 0.08 W/cm^2, were assessed. The irradiation parameters used in this study are shown in Table 2 and 3.

Induction of inflammatory mediator production by LPS

Lipopolysaccharide (LPS) is a toxic molecule derived from the outer cell membrane of gram-negative bacteria that are expressed in inflamed pulp tissue [42, 43]. Therefore, 10 μg/ml of LPS from *Escherichia coli* was used to cause oxidative stress without exacerbating pulp cell damages [31, 44]. The methodology involving the induction of inflammatory mediator production by LPS was previously described [31].

After this process, cells were irradiated according to Table 2 and Table 3 information. All tests were performed 24 hours post irradiation.

Cell viability (MTT assay)

Cell viability was determined using the methyl tetrazolium assay (tetrazoline 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl bromide). This widely used method determines the succinic dehydrogenase activity, which is a measure of cellular (mitochondrial) respiration and represents the rate of viability. This well standardized protocol was previously described in detail [2,18,20,31].

Number of viable cells (Trypan Blue)

The number of viable cells was determined by Trypan Blue assay [25]. For this purpose, the culture medium was aspirated and the pulp cells were trypsinized for 10 minutes using 0.25% trypsin (300 µL, Invitrogen). Afterwards, 50 µL of cell suspension and 50 µL of 0.4% Trypan Blue solution (Sigma–Aldrich) were transferred to wells of a 96-well plate. Next, the cells were incubated for 2 minutes at room temperature and then 10 µL of each sample was transferred to a hemocytometer, where the total numbers of viable and nonviable cells were counted using an inverted light microscope (Nikon Eclipse TS 100, Nikon Corporation, Tokyo, Japan). Cell proliferation was determined by subtracting the number of nonviable cells from the total number of cells in the wells.

Nitric Oxide (NO) Quantification

Nitric oxide (NO) production was measured using the supernatant of the cell culture, as previously described [31]. Nitrite is formed by a diazotization reaction with Griess's reagent, which is composed of 1g sulfanilamide (Merck KGaA, Darmstadt, HE, Germany), 0.1 g N (1-naphthyl) ethylenediamine dichloride (Merck KGaA), 2.5 ml orthophosphoric acid (Mallinckrodt Chemical, St. Louis, MO, US) and 100 mL deionized water. At the moment of evaluation, three aliquots (50µL) of the supernatant were added to 50 µL of Griess reagent in compartments of a 96-well plate. After 10 minute-

incubation, the absorbance reading was performed in a dark room using a spectrophotometer (ThermoPlate, Shenzhen, China) with a 540 nm filter.

Quantification of Reactive Oxygen Species

Quantitative analysis of the formation of reactive oxygen species (ROS) was performed after the application of the 2 ', 7' - dichlorodihydrofluorescein diacetate (DCFH-DA; InvitroGen, San Diego, CA, USA) which is converted via ROS during the increase of oxidative metabolism. In a 24-well plate, the culture medium in contact with the cells was replaced by 300 μL of a solution containing the fluorescent probe (5 μmol/L in PBS). After 5-minute incubation, the cells were washed twice with PBS and then analyzed using a GLOMAX® multimodal fluorescence microplate reader (Promega Corporation, São Paulo, Brazil). For qualitative assessment, the same protocol was performed and two samples were analyzed using the EVOS FL cell imaging system (Thermo Fisher Scientific, São Paulo, Brazil).

Statistical analysis

The data were initially analyzed for normality and homoscedasticity by the Shapiro-Wilk test. As the data did not show adherence to the normal curve (non-parametric data), the Kruskal-Wallis complemented by Mann-Whitney tests were used. As the present study performed multiple comparisons, to minimize the probability of observing a type I error, all p values were adjusted using the Bonferroni correction (which multiplies the raw P values by the number of comparisons) for statistical inferences [45]. All statistical tests carried out using SPSS 19.0 software (IBM, Armonk, NY, USA) were considered at the pre-established significance level of 5%.

Results

Cell viability assessment (MTT)

Figure 2a shows the results of the cell viability evaluation. When compared to the non-irradiated control group, enhanced cell viability was observed in all those groups in which cells were only submitted to photobiomodulation ($p=0.0012$ to $p=0.0072$, Mann-

Whitney), except for 15 J/cm^2 at 0.08 W/cm^2 (p=1.000). However, greater viability was determined in those groups in which the cultured cells were exposed to LPS and then LED-irradiated in comparison to the groups in which the cells were not treated with LPS (p= 0.0024 to p=0.036, Mann-Whitney). In the presence of LPS, the following LED irradiation parameters resulted in significantly higher cell viability than in non-irradiated control group (p=0.0012 to p= 0.0072, Mann-Whitney): 15 J/cm² at 0.04 W/cm²; 30 J/cm² at 0.04 W/cm²; 4 J/cm² at 0.08 W/cm²; and 15 J/cm² at 0.08 W/cm².

Viable cell counts (Trypan Blue)

Concerning the viable cell count, in which the Trypan Blue assay was used, it was observed that the number of viable cells did not vary among all experimental and control groups (Figure 2b; p=1.000, Kruskal-Wallis).

Nitric oxide quantification (Griess's reagent)

Regarding comparison between control groups (LPS- or LPS+), NO quantification was higher in the control group with LPS (Figure 3a; p=0.0036, Mann-Whitney). In the absence of LPS, NO production was higher in all irradiated groups than in the control group (non-irradiated) (p=0.0012 to p=0.0144, Mann-Whitney) excepted for 30 J/cm^2 at 0.04 W/cm^2 (p=1.000, Mann-Whitney). The highest NO values were observed in those groups in which cells were irradiated with 0.08 W/cm^2. Cells exposed to LPS and LED-irradiated exhibited lower NO values, with significant difference between the irradiation parameters and the positive control group (p=0.0072 to p=0.024, Mann-Whitney), except for 4 J/cm^2 at 0.08 W/cm^2 (p=0.144, Mann-Whitney).

ROS quantification and imaging

In control and LED-irradiated with 4 J/cm^2 at 0.08 W/cm^2 groups, cells exposed to LPS showed higher ROS values than those cells not exposed to LPS (p=0.0012 and p=0.0036 respectively, Mann-Whitney). Among other groups, ROS did not differ significantly by LPS treatment (Figure 3b; p=0.144 to p=1.000, Mann-Whitney).

Regarding groups without LPS, ROS was highest at the 4 J/cm^2 at 0.08 W/cm^2 and 15 J/cm² at 0.08 W/cm² irradiation rates (p=0.0012 and p=0.024, respectively, Mann-Whitney). Among LPS groups, all irradiation rates modulated ROS concentration (p=0.00012 to p=0.036, Mann-Whitney). The images generated after ROS detection confirmed the quantitative findings that the highest fluorescence levels were produced by the LPS control group, followed by the groups irradiated with 0.08 W/cm^2, regardless the radiant exposure. The control group without LPS and the groups irradiated with 0.04 W/cm^2 presented the lowest fluorescence levels (Figure 4).

The summarized results found for all the response variables analyzed in this study is shown in Table 4.

Discussion

Interest in the effects of irradiation on pulp tissue has increased in recent years. Several clinical studies, as well as in vitro and in vivo investigations using animal models have been carried out to define optimum irradiation parameters for the photobiostimulation of dental pulp cells [7, 17, 18, 19, 21, 40]. Along with the purpose of determining irradiation parameters capable of reestablishing pulp tissue vitality, researchers have also assessed how photobiomodulation may regulate pulp inflammation [31]. Thus, the present study aimed to provide preliminary data regarding the effects of red LED irradiation on the viability and inflammatory modulation of human dental pulp fibroblasts submitted to LPS stimulus [31, 44].

The results of the MTT assay demonstrated an increased viability in those cells exposed to LPS and that the concentrations of LPS used in the present study did not cause cell death. Additionally, the number of viable cells also confirmed that the pulp cells were not damaged by LPS or by the irradiation parameters used in this study. Regarding the influence of irradiation on the HDPFs viability, all irradiation parameters tested in this study increased the viability of the LPS non-exposed pulp cells, corroborating data of previous studies in which the authors reported that light irradiation enhances pulp cells viability [2, 20, 21, 22, 23, 31]. Turrioni *et al.,* 2015(a) found that the best parameter of red LED to stimulate pulp cells viability was 25 J/cm^2 (0.088 W/cm^2). However, the authors have used a different cell line (MDPC-23) seeded on 0.2 mm dentin discs, to simulate a clinical situation of non-exposed pulp tissue [2]. Different parameters of red and infrared wavelengths have achieved encouraging results regarding pulp cells

stimulation and differentiation. The irradiation parameters used in some of these studies included energy exposure range from 1.5 J/cm^2 [41] to 25 J/cm^2 [2] and irradiance from 0.0037 W/cm^2 [21] to 0.088 W/cm^2 [2]. Although Holder et al. (2012) did not use the LPS to induce the inflammatory process and also have used a different cell line (rodent pulp cells); the authors demonstrated that the levels of adenosine triphosphate (ATP) and cell viability (MTT) were significantly elevated after the irradiation using red LED for 60 seconds (224 mJ/cm^2, 0.003733 W/cm^2) [21].

In the presence of LPS, the following parameters produced greater cell viability in comparison with control group: 15 J/cm^2 at 0.04 W/cm^2, 30 J/cm^2 at 0.04 W/cm^2, 4 J/cm^2 at 0.08 W/cm^2, and 15 J/cm^2 at 0.08 W/cm^2. Overall, the LED-irradiation of pulp cells with 15 J/cm² at 0.04 W/cm² presented the best viability results. Montoro *et al*. (2014) exposed pulp cells to a protocol that was similar to that used in the present study, however using different LED parameters (855 nm, 0.04 W/cm^2); the authors found that all radiant exposures tested stimulated cells viability in the absence of LPS, but such LED-therapy did not increase significantly the viability of LPS-exposed HDPFs [31]. It may be suggested that, in case of challenging situation, where pulp cells are directly exposed and subjected to aggression by bacterial products, red LED could be more effective for biostimulation of human pulp cells.

MTT and Trypan Blue assays are both laboratory protocols widely used to measure cell viability and to determine cell damage, respectively [2,18, 20, 21, 22, 23, 25]. As shown in Figure 2a, enhanced viability occurred in those LPS-exposed pulp cells irradiated with some energy doses (specially 15 J/cm^2 – 0.04 W/cm^2). On the other hand, the LPS exposition and irradiation doses used in this study did not influence the number of viable cells (Figure 2b). These data determined that the cultured pulp cells were not damaged by LPS or irradiation parameters, showing positive effects in terms of cell integrity and/or proliferation.

Previous studies stimulated the pathways that activate NO synthase to demonstrate the relationship between photobiomodulation and NO/ROS production [21, 46, 47]. Holder *et al.* (2012) blocked NO synthase in vitro and detected that NO production still increased after irradiation of cells with red LEDs, suggesting that NO could also be released via respiratory cascade (i.e., by cytochrome c oxidase) [21]. Depending on tissue concentrations, ROS/NO may be either pro-inflammatory or anti-inflammatory [47]. According to Karu (2010), high levels of ROS present in inflammation site or produced by stressed cells prevent the activity of cytochrome c

oxidase and thereby limiting the healing potential of damaged tissues [33]. In these situations, pro-apoptotic enzymes that cause cell death can also be activated [49]. However, at moderate concentrations, ROS/NO may have positive vasodilation action during the first vascular inflammatory response [50] as well as stimulatory effect on cytosine expression [51] or cell differentiation [21]. Therefore, one may suggest that moderate concentrations of NO/ROS should be maintained at injured tissue sites in order to prevent cell damage or death after local photobiomodulation.

In the present study, all irradiation parameters tested (with exception of 4 J/cm^2 at 0.08 W/cm^2) decreased NO/ROS production by pulp cells exposed to LPS stimulus. Montoro *et al.* (2014) used LPS-induced stress on pulp cells of human teeth and also observed that LED irradiation parameters were effective to decrease NO/ROS production by cultured pulp cells after LPS exposure, concluding that among the investigated parameters, 15 J/cm^2 resulted in the lowest NO production (0.04 W/cm^2) [31]. Also, in the ROS qualitative analysis, the authors observed that the irradiation of human dental pulp cells stimulated by LPS, regardless of the dose used, reduced the production of ROS in comparison with the group that was LPS stimulated and not irradiated [31]. Moreover, Dong et al. (2015) reported that low level light therapy (LLLT) could reverse the increased formation of ROS after cell/tissue injury. The authors suggested that light irradiation could support mitochondrial functions during cell/tissue injuries, preventing the intense release of cytochrome c from mitochondria [13].

In general, the aim of this laboratorial study was to assess the hypothesis that irradiation parameters of low intensity (at 0.04 W/cm^2) would be less harmful to pulp cells than more intense parameters (at 0.08 W/cm^2). However, we found that even the highest parameter (30J/cm² - 0.08 W/cm²) did not cause cell damage. According to the methodology used in the present investigation, we found that 15J/cm² and 30 J/cm^2 at 0.04 W/cm^2 and 15 J/cm^2 at 0.08 W/cm² were the most effective LED irradiation parameters to modulate LPS-induced pulp cells. These irradiation levels were more effective at reducing NO/ROS concentrations when compared with positive control group (with LPS). In addition, these LED photobiomodulation parameters did not decrease the number of cells and promoted an increase on the cell viability.

The process of defining specific optimal parameters for tissue biostimulation is hindered by many factors. Chief among these is an incomplete picture of the parameters used in other studies, and a lack of information on the measurement or calibration of irradiation equipment in these studies [32]. In the present well-controlled laboratorial

study, we clearly demonstrated some interesting data concerning the potential of applying the LED-therapy for treatment of inflamed pulps of deciduous teeth. These results are important to guide further other in vitro and in vivo preclinical studies aiming a clinical application of phototherapy to stimulate pulp repair after caries removal or even to treat traumatic tooth injuries, preserving the pulp vitality. Additionally, it is important to highlight that this technique may present low cost, a relatively easy application for dental treatment and good acceptance by patients, especially in cases of pediatric patients or adult patients who have dental treatment phobia.

Regarding limitations of this study, it is important to consider that the results from in vitro investigations cannot be directly extrapolated to clinical situations [1]. The data of this study only suggest the possible benefits of LED-therapy to pulp cells. Besides the limitations of the resemblance of pulp cells population using only primary cultures of two individuals, slight standard deviation was observed when the data was obtained, indicating that cells exhibited similar biological behavior. Moreover, other studies have reported original data using a single-source of primary pulp cells and demonstrated positive reproducibility of the data [52,52]. Also, different cell lines can present different response, depending on the irradiation protocol and methodology applied. Systematic reviews evaluating in vitro and animals protocols involving phototherapy for pulp tissue are also important and strongly suggested.

Conclusion

It was concluded that the irradiation strategy of using red LED with radiant exposures of 15 J/cm² and 30 J/cm^2 at 0.04 W/cm^2 and 15 J/cm^2 at 0.08 W/cm² were the best parameters to decrease NO and ROS concentration and to stimulate viability of pulp cells exposed to LPS challenge.

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Ethical approval

The study was approved by the Institutional Research Ethics Committee of the Faculty of Dentistry of the Federal University of Uberlândia (process 54488816.2.0000.5152).

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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TABLES

Table 1. Donators Epidemiologic information

Gender	Age	Race	Tooth	General health conditions	Medications
M	10	White	Upper Canine	Good	None
F	11	White	Upper First Molar	Good	None

Table 2. Distribution of experimental and control groups according to exposure to LPS and irradiation parameters used

Power density (W/cm^2)	Energy densities (J/cm^2)	Irradiation Times	LPS	
			-	+
0.04	4	3 min and 20 sec	G1 (control)	G8 (experimental)
0.04	15	12 min and 30 sec	G2 (control)	G9 (experimental)
0.04	30	25 min	G3 (control)	G10 (experimental)
0.08	4	1 min and 40 sec	G4 (control)	G11 (experimental)
0.08	15	6 min and 15 sec	G5 (control)	G12 (experimental)
0.08	30	12 min and 30 sec	G6 (control)	G13 (experimental)
0	0	-------	G7 (control)	G14 (control)

Table 3. Irradiation parameters used in the present study

Parameter (unit)	Value
Wavelength (nm)	630
Power (W)	0.08 and 0.16
Power density (W/cm^2)	0.04 and 0.08
Distance between diodes and culture plate (cm)	1.4
Beam area (cm^2)	2
Energy Densities (J/cm^2)	4, 15 and 30
Number of treatments	1

Table 4. Cellular response found for each test (24 hours after irradiation) according to the different irradiation parameters tested when compared to the control group in the presence of LPS (non-irradiated)

	Tests (24 hours after LPS application and LED irradiation)			
Tested parameters (630 nm)	Cell Viability (MTT Assay)	Number of viable cells (TB Assay)	Nitric oxide quantification (NO)	Reactive oxygen species quantification (ROS)
4J/cm^2 – 0.04 W/cm^2	= C*	=C	<C (-75%)	<C (-70%)
15J/cm^2 – 0.04 W/cm^2	>C (+50%)	=C	<C (-75%)	<C (-60%)
30/cm^2 – 0.04 W/cm^2	>C (+10%)	=C	<C (-75%)	<C (-70%)
4J/cm^2 – 0.80 W/cm^2	>C (+25%)	=C	=C	< C (-10%)
15J/cm^2 – 0.08 W/cm^2	>C (+10%)	=C	<C (-30%)	<C (-50%)
30/cm^2 – 0.08 W/cm^2	=C	=C	<C (-30%)	<C (60%)

*C=control group. The differences showed considerable significance level of 5% ($p<0.05$). Mann Whitney test using Bonferroni correction, n=8.

FIGURE LEGENDS

Fig 1 (a) Top view of the LEDtable on which a sterilized 24-well plate is placed. (b) The InGaN diodes (arrows) and the collimators (red circles) are positioned at the base of the device in such way that each of them irradiates homogeneously the pulp cells attached to the bottom of compartments of the 24-well plate.

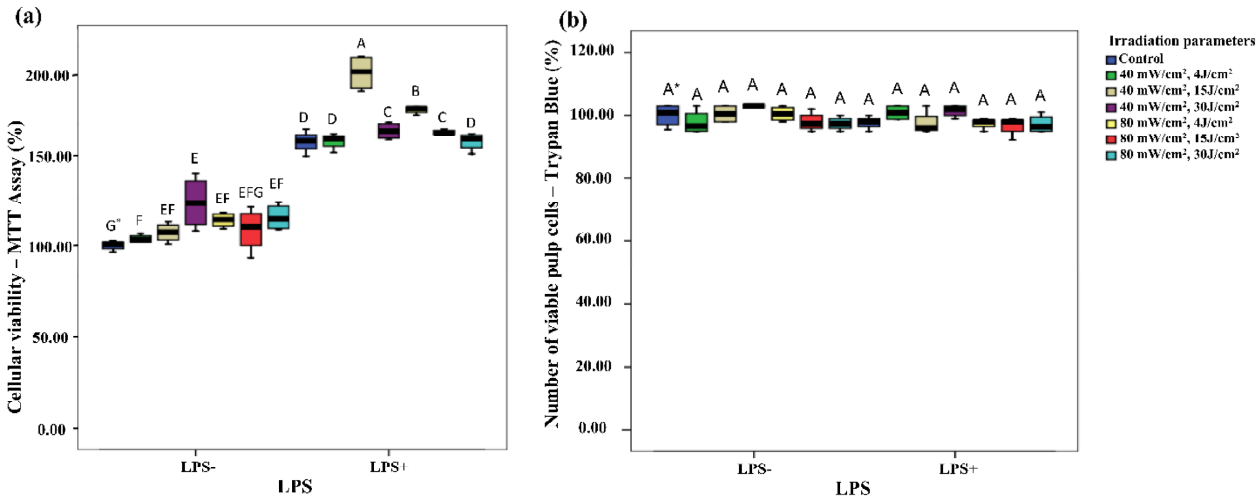

Fig 2 Data of cell viability (MTT Assay - a) and number of viable pulp cells (Trypan blue assay - b), considering the presence of LPS and the different irradiation parameters used in the study.

*Different letters represent statistical difference. Mann-Whitney test ($p < 0.05$), n=8

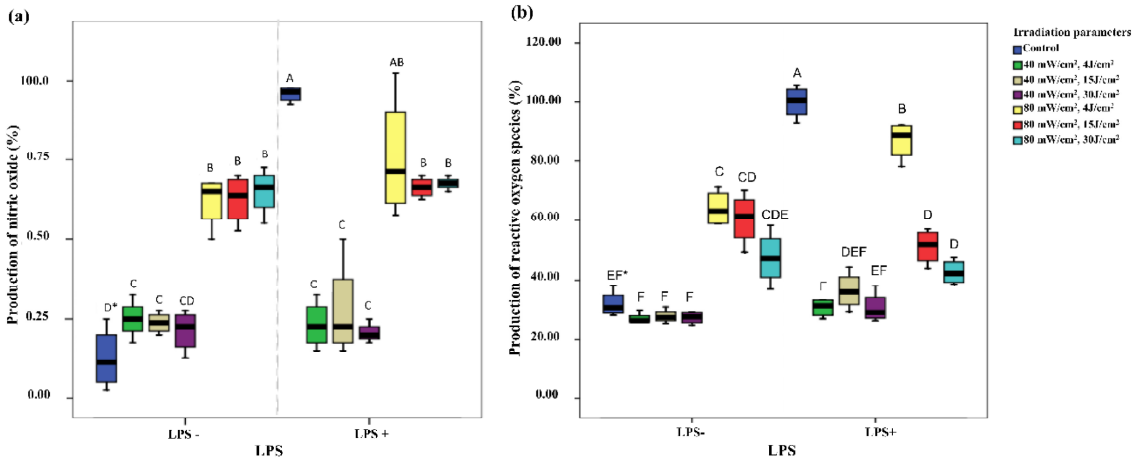

Fig 3 Production of NO (a) and ROS (b) by cultured pulp cells exposed or not to LPS and submitted to different irradiation parameters.

*Different letters represent statistical difference. Mann-Whitney test ($p < 0.05$), n=8

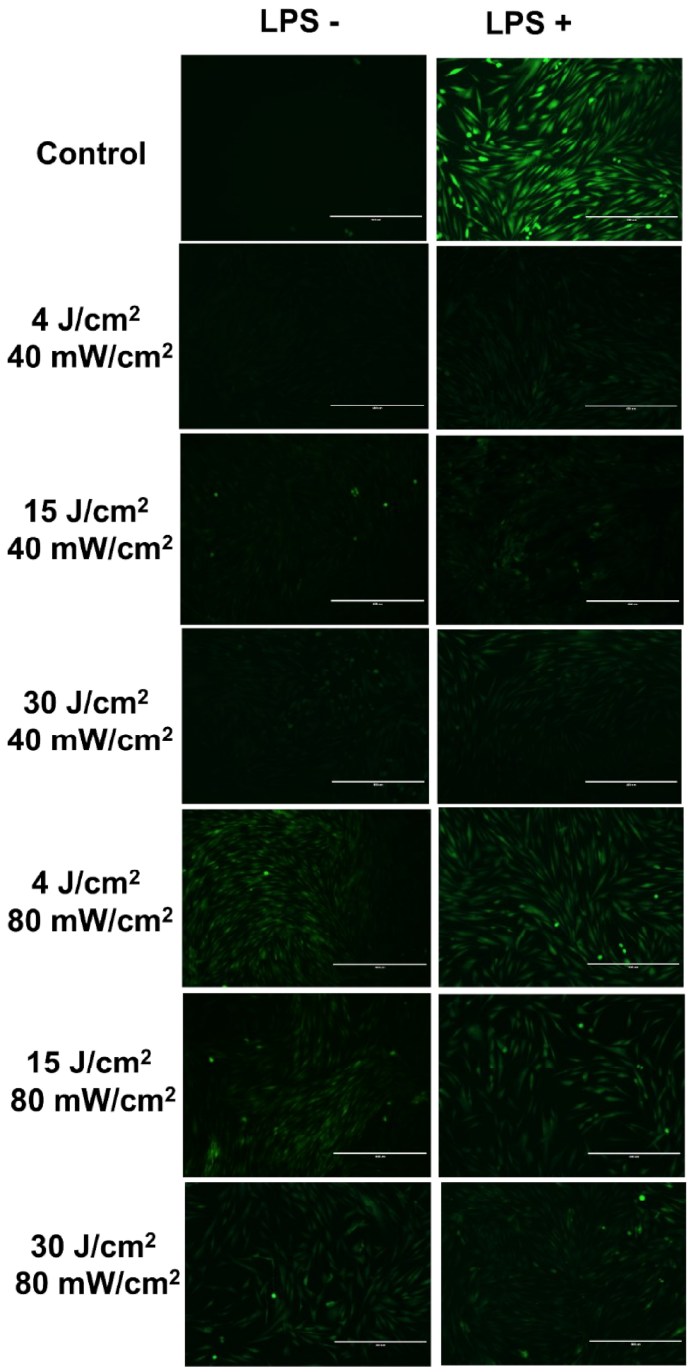

Fig 4 Representative images of reactive oxygen species (ROS) produced by pulp cells exposed or not to LPS and submitted to different parameters of LED irradiation at red wavelength (630 nm). The intensity of the fluorescence emitted by the DCFH-DA probe capable of penetrating the cell membranes determines the intensity of ROS produced by cells. Therefore, the higher the fluorescence, the greater the production of the oxidizing agents (Fluorescence Microscope EVOS FL Cell Imaging System; scale, 400 μm). The images in the left column represent the groups in which the pulp cells were not exposed to LPS (LPS -) before LED irradiation; the images in the right column show the LPS-exposed cells (LPS +) that were submitted to different parameters of LED irradiation.

2. CAPÍTULO 2

ARTIGO 2

Specific parameters of infrared LED irradiation promote the inhibition of oxidative stress in dental pulp cells

***Artigo a ser enviado para o periódico Archives of Oral Biology**

Specific parameters of infrared LED irradiation promote the inhibition of oxidative stress in dental pulp cells

Jéssica F. S. Bonvicini[a], Gabriela L. de Souza[b], Marcelo J. B. Silva[c], Carlos A. de Souza Costa[d], Fernanda G. Basso[e], Camilla C. G. Moura[b], Ana P. Turrioni[a]

[a] Department of Pediatric Dentistry, Federal University of Uberlândia, UFU, Uberlândia, MG, Brazil

[b] Department of Endodontics, Federal University of Uberlândia, UFU, Uberlândia, MG, Brazil

[c] Department of Immunology, Federal University of Uberlândia, UFU, Uberlândia, MG, Brazil.

[d] Department of Physiology and Pathology, Araraquara School of Dentistry, UNESP, Araraquara, SP, Brazil

[e] Department of Oral Pathology, University of Ribeirão Preto, UNAERP, Ribeirão Preto, SP, Brazil

Corresponding Author
Dr. Ana Paula Turrioni
DDS, MSc, PhD, Cell Biology Research Group, Department of Pediatric Dentistry, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.
Av. Pará 1720, Umuarama.
CEP: 38400902, Uberlândia, MG, Brasil
E-mail:apturrioni@ufu.br
Tel: +55-16-3301-64

Specific parameters of infrared LED irradiation promote the inhibition of oxidative stress in dental pulp cells

Objectives: The aim of this study was to evaluate the effect of irradiation by infrared LED (850 nm) on the metabolism and inhibition of oxidative stress of dental pulp cells stimulated with LPS.

Methods: Three healthy deciduous teeth (n=3) were collected and seeded in 24-well plates with 10 µg/mL of LPS to induce inflammatory mediators formation. The cells were irradiated (850nm, 40mW/cm^2 and 80mW/cm^2) at the proposed radiant exposures of 0 (control), 4 J/cm^2, 15 J/cm^2 and 30 J/cm2 shortly after LPS application. Tests were performed 24h after irradiation for mitochondrial activity (MTT assay), number of viable cells (Trypan Blue), quantification of Nitric Oxide (ON), Reactive Oxygen Species (ROS) and cell morphology (Scanning Electron Microscopy - SEM). The data were analyzed using Kruskal-Wallis and Mann-Whitney ($p<0.05$).

Results: It was observed that irradiated groups showed larger number of viable cells than non-irradiated group with LPS ($p<0,0001$). All irradiation parameters were able to decrease ROS concentrations after LPS application compared to non-irradiated group ($p<0,05$). Concerning NO results, it was observed no difference among groups ($p>0,05$). SEM images showed cells with regular morphology and adhered to the substrate.

Conclusions: According to the parameters used in the study, the radiant exposure of 15 J/cm² and irradiance of 40 mW/cm² was the most effective irradiation parameter to stimulate and modulate oxidative stress in the pulp cells from primary teeth.

Keywords: Dental pulp cells; Irradiation; Infrared LED; Reactive oxygen spec

1.Introduction

Low-intensity light therapies (LLLT), using laser or light-emitting diode (LED), have been widely employed in dentistry to stimulate tissues and pulp cells from human and rodent teeth [1–6]. The use of human dental pulp cells (HDPCs) to assess different protocols of photobiomodulation (PBM) has become an increasingly viable option, since these primary cell culture is easily established and have the positive characteristics of proliferation and differentiation in vitro [7–9].

Several studies have showh that different protocols of PBM applied to the dentin-pulp complex increase dentin matrix deposition [10–12], cells viability and proliferation [13], as well as regulate inflammatory cytokines and reactive oxygen species production by pulp cells [14].

Hamblin et al. [15] showed that PBM using low intense light levels moderately enhances the production of reactive oxygen species (ROS) with no harmful effects to the cell by stimulating the transport of mitochondrial electrons (redox mechanism), favoring cell signaling related to the repair and survival of cells. The authors also reported that high intense levels of light can impair the beneficial action of ROS, exacerbating their intracellular volume and even activating pro-apoptotic enzymes. Despite the encouraging results of cells PBM [2,6,11], the influence of specific parameters of infrared LED irradiation on the viability and modulation of oxidative stress of HDPCs is missing [14, 16]. Only a few studies on irradiance, wavelength, radiant exposure, and incidence time have been conducted, indicating the relevane of performing laboratory studies to standardize irradiation parameters used for low-intensity light PBM [17]. Although the limitations of data obtained from in vitro studies, one may consider that this laboratorial investigation has clinical relevance for the restorative dentistry since it may predict the potential of PBM to stimulate pulpo-dentin regeneration in cases of deep cavities and/or

micro-exposure of the pulp. In these situations, infrared light could be applied to the cavity floor before the restorative protocol, stimulating the subjacent pulp cells.

The aim of the present study was to assess the viability and oxidative stress of pulp cells stimulated by lipopolysaccharide (LPS) and submitted to PBM with infrared LED (850 nm). The authors hypothesized that irradiation of pulp cells with infrared LED using a high irradiance (80 mW/cm^2) inhibits the viability and exacerbate the cell oxidative stress, and that a low irradiance (40 mW/cm^2) stimulates the viability and decrease ROS and NO production by cells previously exposed to LPS.

2. Material and methods

2.1. Human dental pulp cells culture

HDPCs cultures were obtained from three exfoliated and healthy primary teeth donated from 3-10 years-old patients after signing the informed consent form by the guardians (approved by the Research Ethics Committee [protocol number 54488816.2.0000.5152], at the School of Dentistry of the Federal University of Uberlândia/UFU, Minas Gerais, Brazil). Cells were isolated using enzymatic disaggregation in a solution containing 3 mg/mL type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) and 4 mg/mL dispase (Sigma-Aldrich) [3,18].

The pulp tissue was immersed in the enzymatic solution for 1 h, at 37°C and 5% CO_2. The solution was centrifuged at 1200 rpm for 2 min and the cell suspension was seeded in 25 cm² flasks. For further expansion, after the cell cultures reach 80-90% confluence, they were sub-cultivated using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA, USA), L-glutamine, and 1% penicillin–streptomycin (Gibco,). For this study, cells were used between 1st and 4th passage.

2.2. Induction of inflammatory mediator production by LPS

The *Escherichia coli* LPS stimulus on HDPCs was peformed to simulate a microenvironment of pulp aggression such as previously decribed [14,19]. The cells were seeded ($1x10^5$ cell/well) in 24-well plates (TPP), using DMEM containing 10% FBS, and incubated for 24 h in a humid atmosphere containing 5% CO_2 and 37 °C. After this period, the culture medium was replaced by FBS-free DMEM with LPS (Ultra-pure grade, *Escherichia coli* O111, B4, Invitrogen, San Diego, CA, USA) at the concentration of 10 µg/mL.

2.3. Irradiation device

After being exposed to LPS inflammatory stimulus, the cells were immediately irradiated using a device previously reported in the literature - LEDTable (InGaN, 850 nm, ± 20, 40mW and 80mW) [3, 14, 20, 21, 22, 23]. Twenty-four infrared diodes are coppled to this irradiation device. Between the diodes and the bottom of the wells of a 24-well plate, lenses are positioned in such way that the ligh is simultaneously delivery to the whole bottom area (2 cm^2) on which the cells are attached.

The energy densities were fixed at 0J (control), 4J/cm², 15J/cm² and 30 J0/cm² [3, 14, 21, 22, 23, 24] and the exposure times for each energy dose were determined based on the formula: density of energy = power × exposure time × $area^{-1}$ and the fixed parameters (power and area). The exposure times were 1 min 40 s, 3 min 20 s, 6 min 15s, 12 min 30 s, and 25 min.

2.4. Analysis of mitochondrial activity

Mitochondrial activity was assessed by the methyl tetrazolium (MTT) assay, performed 24 h after irradiation, for the analysis of cell viability. This method determines the activity of succinic dehydrogenase enzyme, which is a measure of cellular respiration (mitochondrial) and can be considered as the metabolic rate of a cell. This protocol has been previously described in detail [12, 22, 25].

2.5. Analysis of the numbers of viable cells (Trypan Blue)

The cell viability assay was based on the integrity of the cellular membrane by Trypan Blue staining, which identifies non-viable cells by the cytoplasmic incorporation of trypan blue dye (Sigma-Aldrich) [26]. The culture medium was removed, and the cells were trypsinized by the addition of 300 µL of trypsin 0.25% (Invitrogen) for 10 min. After this period, 50 µL of the cell suspension and 50 µL of 0.04% trypan blue solution (Sigma-Aldrich) were transferred to a 96-compartment plate.

After incubating cells for 2 min at room temperature, a 10-µL quantity of each sample was transferred to a hemocytometer for quantification of the total number of viable and non-viable cells, by inverted light microscopy (Nikon Eclipse TS 100, Nikon Corporation, Tokyo, Japan).

2.6. Quantification of nitric oxide (NO)

NO cells production was quantified by spectrophotometry (ThermoPlate, Shenzhen, China) 24 h after irradiation. For this purpose, it was assessed the accumulation of nitrite in the cell culture supernatant caused by the diazotization reaction with the Griess reagent, composed of 1 g of sulfanilamide (Merck KGaA, Darmstadt, Germany), 0.1 g of N (1-naphthyl) ethylenediamine dichloride (Merck KGaA), 2.5 mL of

orthophosphoric acid (Mallinckrodt Chemical, St. Louis, MO, USA), and 100 mL of deionized water [14].

2.7. Quantification of reactive oxygen species (ROS)

The production of reactive oxygen species (ROS) was quantified by the analysis of the fluorescence intensity rate of the sample after using 2′,7′-dichlorodihydrofluorescein diacetate probe (DCFH-DA; Invitrogen) [14]. The culture medium was removed, and a 300-µL quantity of a solution containing the fluorescent probe (5 µmol/L on PBS) was added to each well. After 5 min, the samples were washed twice with PBS and analyzed with GLOMAX® multimodal fluorescence microplate reader equipment (Promega Corporation, São Paulo, Brazil) to acquire quantitative data. For the qualitative analysis of the cells (images), the samples were evaluated by the EVOS FL Cell Imaging System fluorescence microscope (Thermo Fisher Scientific, São Paulo, Brazil).

2.8. Scanning electron microscopy (SEM)

The cells morphology and adhesion to the substrate was assessed by scanning electron microscopy (SEM). For this purpose, cells were seeded on round glass coverslips, fixed for 1 h in 2.5% glutaraldehyde (Sigma-Aldrich), and dehydrated in increasing concentrations of alcohol. The sample preparation steps for this evaluation have been described in previous studies [12, 27].

2.9. Statistical analysis

The data obtained in this laboratorial study were analyzed regarding the normality and homoscedasticity using the Shapiro–Wilk test. Non-parametric test of Kruskal-Wallis complemented by Mann-Whitney were selected. The statistical analysis was performed using the SPSS 18.0 software (IBM, Armonk, NY, USA). All statistical tests were considered at the pre-established significance level of 5%.

3. Results

3.1 Mitochondrial activity

Mitochondrial activity, determined by the MTT assay, is shown in Table 1.

When groups with and without LPS were compared, it was possible to observe that, in almost all irradiation parameters, LPS reduced pulp cell metabolism ($p<0.05$), with the exception of the parameters "40 mW/cm^2 - 4 J/cm^2" and "80 mW/cm^2 - 30 J/cm^2", where the presence of LPS had no effect on cell viability ($p>0.05$).

When the different irradiation parameters were considered for the same condition (with or without LPS), it was observed that, in the absence of LPS, the only parameter that presented higher values of viability with a statistically significant difference in relation to the control group (non-irradiated) was "40 mW/cm^2 - 15 J/cm^2" ($p<0.05$). The parameter "40 mW/cm^2 - 30 J cm^2" showed no statistically significant difference in relation to the control group ($p>0.05$). The other parameters tested showed viability values statistically significantly lower when compared with those of the control group ($p<0.05$). In the presence of LPS, the parameters that showed greater viability in relation to the control group were "40 mW/cm^2 - 15 J/cm^2" and "80 mW/cm^2 - 30 J/cm^2" ($p<0.05$). The parameter "40 mW/cm^2 - 30 J/cm^2" did not differ statistically significantly from that of the control group

($p<0.05$), and the other parameters showed lower viability values when compared with the control group ($p<0.05$).

3.2. Number of viable cells

The analysis of the number of viable cells by Trypan Blue is shown in Table 2.

When the groups were compared in the presence or absence of LPS, it was possible to observe that, for the "control" groups, "40 mW/cm^2 - 30 J/cm^2", "80 mW/cm^2 - 15 J/cm^2", and "80 mW/cm^2 - 30 J/cm^2", the presence of LPS promoted a lower number of viable cells ($p<0.05$). For the other parameters, LPS had no effect on this variable.

When the different irradiation parameters were compared for the same condition (with or without LPS), it was observed that, in the absence of LPS, all irradiation parameters showed a higher number of viable cells in relation to that in the control group ($p<0.05$), and the "40 mW/cm^2 - 30 J/cm^2" group showed the highest number of viable cells. In the presence of LPS, almost all irradiation parameters showed a higher number of viable cells when compared with the control group, with the exception of the "80 mW/cm^2 - 30 J/cm^2" parameter, which did not differ from the control group ($p>0.05$). The parameter with the highest number of viable cells was "40 mW/cm^2 - 4 J/cm^2".

3.3. Quantification of nitric oxide

Table 3 presents the values for quantification of nitric oxide (NO) by pulp cells.

The presence of LPS promoted an increase in NO values for all tested parameters ($p<0.05$), and when the condition "presence of LPS" was considered, all irradiation parameters promoted an increase in NO values compared with those of the control group ($p < 0.05$).

3.4. Quantification of reactive oxygen species (ROS)

The results generated after the quantification of ROS are shown in Table 4 and representative photomicrographs in Fig. 1.

When the groups were compared in the presence or absence of LPS, it was possible to observe that, for "Control", "40mW/cm^2 – 4J/cm^2", "80mW/cm^2 – 15J/cm^2" and "80mW/cm^2 – 30J/cm^2" parameters, there was an increase in the ROS values after the application of LPS ($p<0.05$). For parameters "40 mW/cm^2 - 15 J/cm^2", "40 mW/cm^2 - 30 J/cm^2", and "80 mW/cm^2 - 4 J/cm^2", the LPS exposure had no effect on the ROS concentration ($p>0.05$).

When the different irradiation parameters were compared for the same condition (with or without LPS), it was observed that, for the condition without LPS, there was no statistically significant difference between irradiated and control groups ($p>0.05$). As for the condition in the presence of LPS, all tested irradiation parameters were able to decrease the amount of ROS, with values lower than those of the control group ($p<0.05$).

According to the images generated by the fluorescence microscope, confirmation of the quantitative data was observed, where the control group with LPS showed greater fluorescence, followed by the other irradiation parameters, at all radiant exposures. The groups "control without LPS", "40 mW/cm^2 - 4 J/cm^2", and "80 mW/cm^2 - 4 J/cm^2"

showed less fluorescence (with and without LPS).

3.5. Scanning electron microscopy (SEM)

The analysis of cell morphology obtained through representative images by scanning electron microscopy complemented the MTT and Trypan Blue tests for cell viability, as shown in Fig. 2 (40 mW/cm^2 and 80 mW/cm^2).

For both irradiances used (40 mW/cm² and 80 mW/cm^2), normal pulp cells (arrows), with fusiform shape, could be observed, covering the glass coverslip with cytoplasmic extensions intact. It was possible to record mitosis cells in all groups (circles), confirming the positive results on cell viability.

4. Discussion

Irradiation with red and infrared LED has shown promising results in the synthesis of dentin proteins [3], modulation of oxidative stress [14], and biostimulation of pulp cells [2,23]. These results are dependent on the irradiation parameter used. Considering the methodology used in the present study, the use of infrared LED was evaluated in relation to stimulation of cell viability and modulation of molecules from the inflammatory process, with specific parameters: 4 J/cm², 15 J/cm², or 30 J/cm² (40 mW and 80 mW/cm²). The null hypothesi was rejected, since the cell response was not directly dependent on the irradiance used, even though lower irradiance presented better results in general. The parameter of 15 J/cm^2 at 40 mW/cm^2 showed higher values of cell viability and simultaneous inhibition of oxidative stress when the cells were stimulated by LPS.

In the field of phototherapy, the ideal set of specific irradiation parameters for

clinical treatment in dentin-pulp complex repair has not yet been defined [28, 29]. It is known that there is an ideal radiant exposure for any specific application of phototherapy, called a biphasic dose. Thus, a radiant exposure that is less than the ideal value will determine a reduced therapeutic response, while increased radiant exposure can lead to harmful effects [29]. In this sense, previous studies have shown that low light levels have better effects on tissue stimulation and repair than higher light levels [15]. Finding a parameter that presents positive results in all evaluations is essential as a reference for the definition of future clinical parameters. In the present study, the irradiation parameters were determined from previous studies where the authors found positive results regarding the proliferation of human dentin and pulp cells, as well as the inflammatory modulation of pulp cells subjected to LPS stimulation [3, 14, 21, 23].

For the results of mitochondrial activity, considering the isolated effect of irradiation, the parameter of 15 J/cm^2 at 40 mW/cm^2 increased the values by 30% compared with the non-irradiated group. The other parameters did not show stimulatory effects in the pulp cells in terms of mitochondrial activity. When irradiation and LPS effect were evaluated together, it was observed that the presence of LPS had a low effect on mitochondrial activity, and the 15 J/cm^2 parameter at 40 mW/cm^2 showed an increase in mitochondrial activty. In contrast, the isolated action of irradiation in groups without LPS promoted a considerable increase of more than 74% in the number of viable cells, except for the parameter of 30 J/cm^2 – 80 mW/cm^2, when an increase of 17% was observed. Although the presence of LPS generated a 28% decrease in the number of viable cells, the irradiation parameters managed to reverse this effect, with radiant exposures of 15 and 30 J/cm² - 40 mW/cm² showing positive effects in terms of cell integrity and/or cell proliferation.

Among the methods used to measure cell viability rates, the tests for the exclusion

of dead cells by Trypan Blue and the colorimetric MTT reaction are widely used. Briefly, the trypan blue exclusion test principle consists of the penetration of the dye into damaged cell membranes, excluding cells with functional membranes [26]. In the formazan MTT method, the MTT reagent enters cells via endocytosis and is reduced to colored formazan crystals by active mitochondria [30]. In general, the results of the present study showed that, with the trypan blue exclusion method, all parameters evaluated showed an increase in viable cells when compared with the control, whereas by the formazan MTT method, this occurred only with specific parameters. This could be related to the fact that the trypan blue method assesses viability indirectly, so it is possible that the viability/mitochondrial activity of a cell may have been compromised, even if the integrity of its membrane is, at least temporarily, maintained, or that the integrity of the cell membrane is abnormal and the cell can still repair itself and become fully viable [26]. Therefore, the mechanism of the cell damage caused by each irradiation parameter selected in the present study is not yet clear. The combination of these methods allows for the evaluation of complementary principles, for detecting any possible negative effect and increasing the validity of the results [31].

Cellular stress by LPS resulted in an increase in the production of ROS, as already reported in the literature [32, 33]. Reactive oxygen species are highly unstable and reactive free-radical molecules that can oxidize proteins and lipids or break DNA bonds [34]. ROS can have beneficial effects in low or moderate concentrations, acting on cell signaling in the face of stimuli caused by infectious agents, as well as in the induction of mitogenic responses. As harmful effects, ROS cause considerable biological damage through oxidative stress, affecting cellular structures, including lipids and membranes, proteins, and nucleic acids [34, 35]. The present study indicated that the presence of LPS caused an increase of 50% in the production of ROS for the control group; however, when

the irradiated groups were compared, all irradiation parameters managed to reverse this effect, inhibiting the concentrations of this agent induced by LPS from 27 to 42%, without a sharp decrease in these values. Through the qualitative analysis of intracellular ROS, it was possible to confirm the quantitative findings, demonstrating that, in the absence of LPS, the control group emitted low fluorescence. For the positive control group (presence of LPS), there was intense fluorescence, followed by all irradiation parameters, with less intense fluorescence mainly in the parameters of 4 J/cm^2 - 40 and 80 mW/cm^2. The radiant exposure of 15 J/cm^2-40 mW responded more positively to the biomodulator effect generated by the use of light. There are still no studies reported in the literature that have evaluated ROS photobiomodulation in pulp cells; however, light was able to decrease the levels of oxidative stress generated by ROS in other types of cells, such as neuronal cells, human gingival fibroblasts, and osteoblastoid cells [36, 37, 38]. Montoro et al. (2014) [14] also found that infrared LED (855 nm) promoted ROS inhibition in pulp cells, using the same probe as in our experimental protocol, and concluded that the radiant exposure of 15 J/cm², at the power of 40 mW/cm², was the best parameter to modulate stress oxidation with cell viability maintained.

Regarding cell stress induced by LPS, nitric oxide, a reactive nitrogen intermediate, is a short-lived free radical that is synthesized by the L-arginine to L-citrulline pathway, which is mediated by NO synthase in most animal cells and is involved in several biological actions, including cell differentiation, proliferation, or death by apoptosis [39, 40]. The actions of this free radical in the pulp cells depend strictly on their concentrations in the tissue. When exposed to a low concentration of NO, cytoprotective proteins, such as heme oxygenase-1 (HO-1), are induced, and cells survive to use NO as a signaling molecule [41]. Such signaling generates the stimulus for the expression of cytokines in inflammatory modulation and consequent cell repair [42]. However, an

excessive increase in NO production generates a cytotoxic effect [43], which can lead to the activation of pro-apoptotic enzymes, making tissue repair difficult [42]. In the present study, all irradiation parameters promoted an increase of between 17% and 41% in the production of NO in the presence of LPS, moderately stimulating the production of this molecule. Holder et al. verified the production of NO via cytochrome c oxidase (CCO), using phototherapy in pulp cells. The intracellular increase of the free radical produced by this pathway can have a positive effect, since it is related to the stimulation of cell differentiation and production of the collagen matrix [2].

The results of SEM made it possible to complement those found in cell viability, ROS, and NO and indicated that even with a significant decrease in mitochondrial activity, there was no morphological change in any of the groups, demonstrating that the quantification of NO, even with its production increased for all irradiated groups, did not cause damage to HDPC; on the contrary, it preserved its format, making it possible for even the occurrence of mitoses to be observed. The results found in the present study confirm the beneficial effects of low-intensity irradiation with infrared LED (850 nm), suggesting clinical applicability after the removal of decayed tissue in medium to deep cavities, before the application of a restorative protocol or in traumatized teeth. In the future, the development of equipment similar to a photopolymerization device is suggested, where the irradiation beam is applied directly over the prepared cavity. The effective use of this protocol must be confirmed in controlled clinical studies for greater safety of use in the dental clinic.

5. Conclusion

According to the tested parameters of irradiation used in this study, the responses of pulp cells were not directly dependent on the irradiance used, even though lower

irradiance (40 mW/cm^2) presented better results in general. The parameter of 15 J/cm^2 at 40 mW/cm^2 showed higher values of cell viability and simultaneously presented modulation of oxidative stress when the cells were stimulated by LPS.

Ethical approval

The study was approved by the Institutional Research Ethics Committee (process 54488816.2.0000.5152).

Declaration of competing interests

The authors declare no conflict of interest.

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Tables

Table 1: Mithocondrial activity (MTT Assay)

	LPS -	LPS +
Control	100.09 (95.13-103.20) Ba*	92.12 (89.89-93.57) Cb
40 mW-4J/cm^2	80.99 (76.26-90.25) Da	82.44 (76.55-87.92) Da
40mW-15J /cm^2	131.05 (125.68-140.22) Aa	108.52 (104.26-111.42) Ab
40 mW-30 J cm^2	101.21 (98.45-105.10) Ba	91.12 (86.63-93.45) Cb
80 mW-4 J/cm^2	87.14 (82.60-90.77) Da	75.74 (73.53-79.23) Eb
80 mW-15 J/cm^2	85.54 (82.55-88.07) Da	75.84 (69.99-81.57) Eb
80 mW-30 J cm^2	93.09 (88.83-97.29) Ca	96.70 (91.30-98.73) Ba

* Values are medians (percentile 25–percentile 75), $n = 8$. Lowercase letters allow comparison in rows and uppercase letters allow comparison in columns. Different letters indicate a statistically significant difference (Mann-Whitney, p<0,05).

Table 2: Analysis of the number of viable cells (Trypan Blue*)*

	LPS -	LPS +
Control	100.88 (87.24-110.64) Fa*	72.35 (55.32-82.13) Db
40 mW-4J/cm^2	225.53 (175.16-344.68) Ba	280.85 (255.32-357.45) Aa
40mW-15J /cm^2	174.47 (112.45-255.32) Da	155.43 (113.99-233.52) Ca
40 mW-30 J cm^2	297.87 (240.43-342.56) Aa	222.35 (142.55-261.70) Bb
80 mW-4 J/cm^2	185.22 (113.19-285.11) Da	132.98 (115.48-152.66) Ca
80 mW-15 J/cm^2	204.26 (144.68-300.00) Ca	116.60 (111.43-157.45) Cb
80 mW-30 J cm^2	117.13 (113.73-165.96) Ea	62.87 (36.17-82.98) Db

*Values are medians (percentile 25–percentile 75), $n = 8$. Lower case letters allow comparison in rows and uppercase letters allow comparison in columns. Different letters indicate a statistically significant difference (Mann-Whitney, p<0,05).

Table 3: *Quantification of Nitric Oxide (NO)*

	LPS -	LPS +
Control	94.06 (87.38-119.06) Ab*	100.43 (100.07-113.12) Ca
40 mW-4J/cm^2	94.56 (90.10-97.53) Ab	134.16 (118.81-146.28) Aa
40mW-15J /cm^2	96.54 (91.09-107.92) Ab	141.58 (138.86-146.53) Aa
40 mW-30 J cm^2	96.54 (84.66-105.94) Ab	134.65 (124.50-144.06) Aa
80 mW-4 J/cm^2	89.11 (83.42-101.49) Ab	129.70 (123.02-145.79) Ba
80 mW-15 J/cm^2	88.62 (81.19-98.02) Ab	122.28 (112.13-138.61) Ba
80 mW-30 J cm^2	98.52 (91.09-119.06) Ab	117.82 (113.37-134.16) Ba

*Values are medians (percentile 25–percentile 75), $n = 8$. Lowercase letters allow comparison in rows and uppercase letters allow comparison in columns. Different letters indicate a statistically significant difference (Mann-Whitney, p<0,05).

Table 4: *Quantification of Reactive Oxygen Species (ROS)*

	LPS -	LPS +
Control	49.41 (44.03-66.11) Ab*	100.08 (93.99-104.47) Aa
40 mW-4J/cm^2	45.83 (39.84-47.74) Ab	58.70 (46.99-65.41) Ca
40mW-15J /cm^2	62.11 (56.21-65.96) Aa	73.58 (65.64-79.11) Ba
40 mW-30 J cm^2	61.76 (54.80-65.23) Aa	59.57 (57.10-68.68) Ca
80 mW-4 J/cm^2	52.28 (43.41-63.29) Aa	57.29 (54.62-65.17) Ca
80 mW-15 J/cm^2	60.47 (57.64-65.21) Ab	69.26 (67.17-72.88) Ba
80 mW-30 J cm^2	63.24 (53.67-66.13) Ab	73.58 (68.48-76.83) Ba

*Values are medians (percentile 25–percentile 75), n = 8. Lowercase letters allow comparison in rows and uppercase letters allow comparison in columns. Different letters indicate a statistically significant difference (Mann-Whitney, p<0,05).

Figure legends

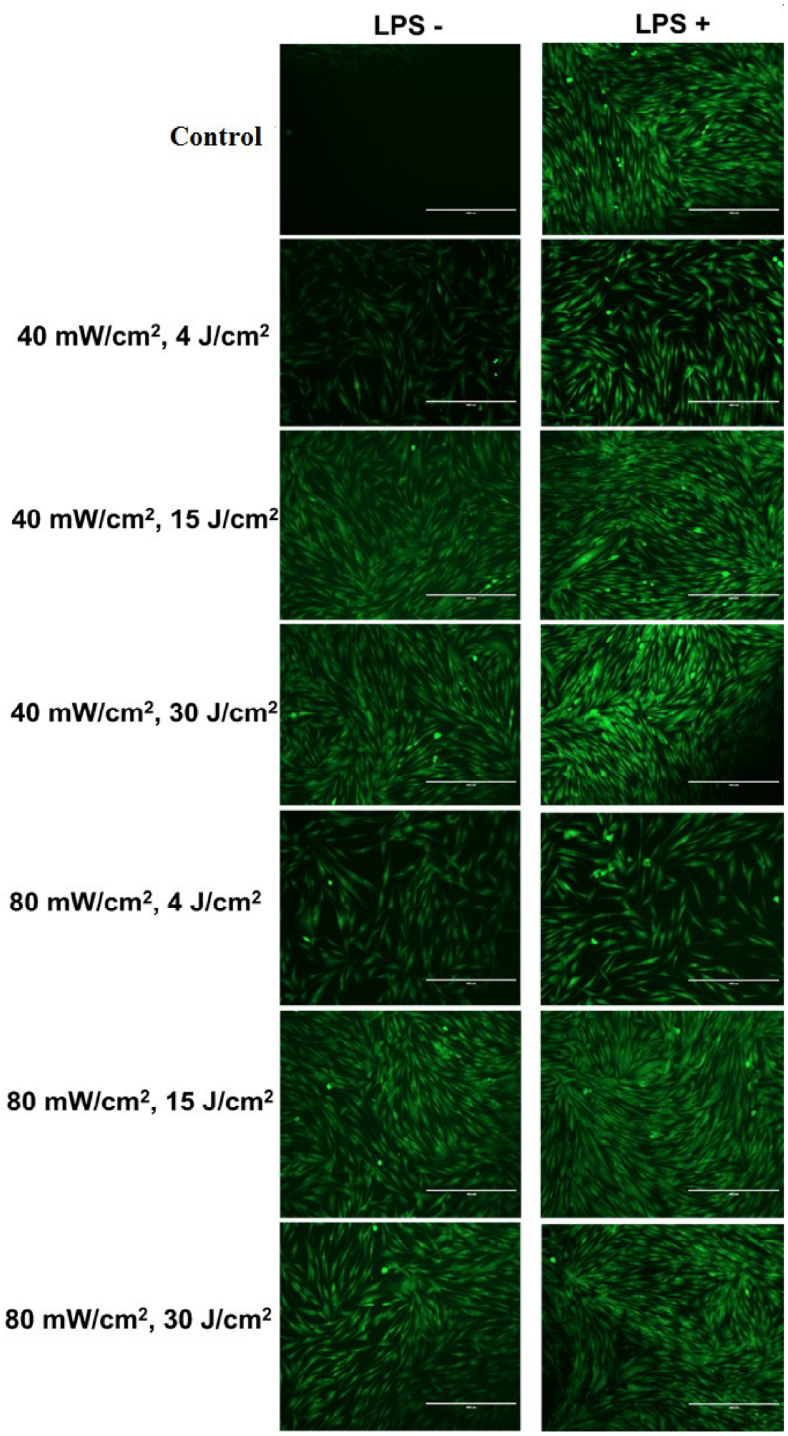
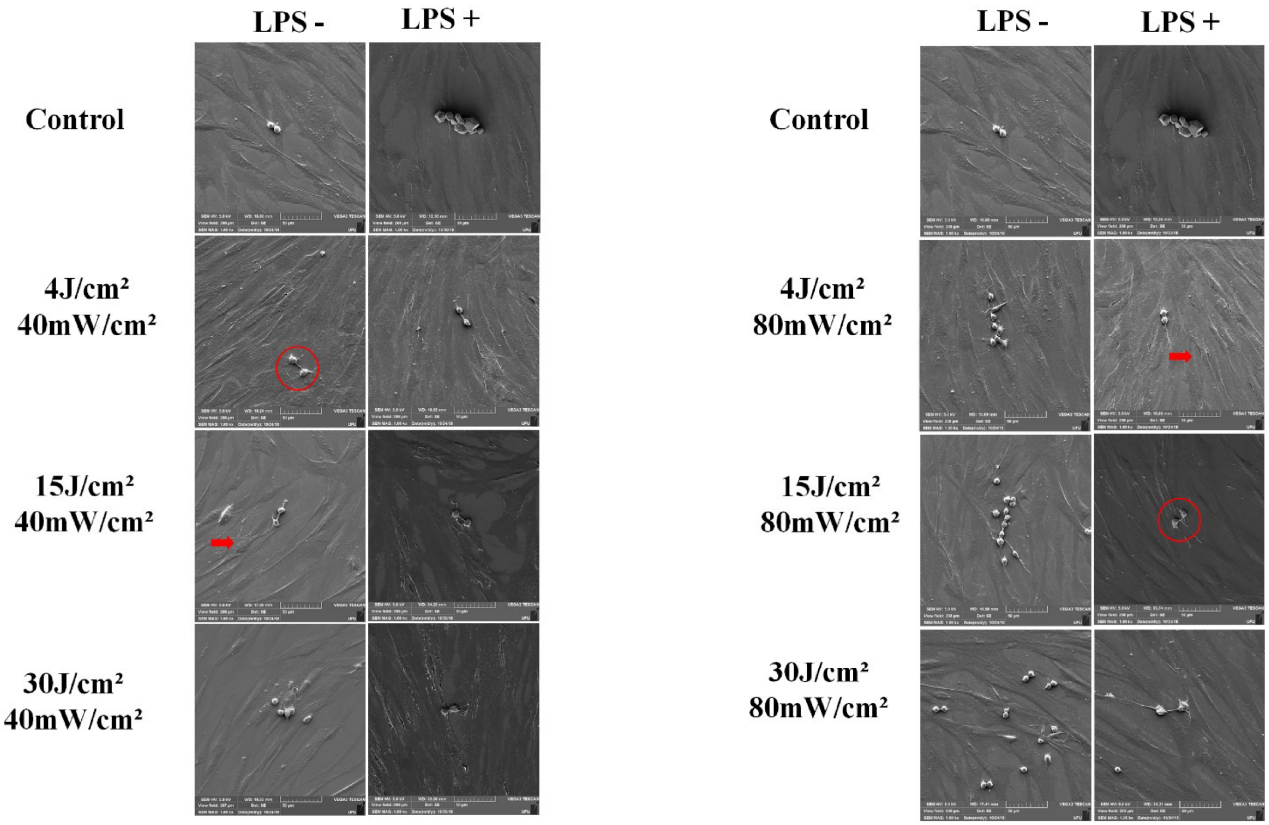


Fig. 1. Representative images of the production of reactive oxygen species (ROS) by pulp cells of primary teeth irradiated by LED at infrared wavelength (850 nm) at different

doses of energy (J/cm²) and power (mW/cm²). The intensity of the fluorescence emitted by the probe DCFH-DA qualified the production of ROS so that the higher the fluorescence, the greater the production of these reactive species (Fluorescence Microscope EVOS FL Cell Imaging System; scale, 400 µm).

Fig. 2. Representative images of cell morphology obtained by scanning electron microscopy (SEM), considering the presence or absence of LPS for the different irradiances (40 mW/cm² and 80 mW/cm^2 [1000x magnification]). Normal-looking pulp cells are observed, with intact cytoplasmic extensions, covering the glass coverslip.



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4. ANEXOS

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Instructions for Authors

Types of papers

- Original Article – limited to 4000 words, 45 references, no more than 5 figures
- Review Article – limited to 5000 words, 50 references, no more than 5 figures
- Brief Report - limited to 2000 words, 25 references, no more than 4 figures - Case Reports will not be accepted!
- Letter to the Editor – up to 600 words

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Manuscript Submission

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Please follow the hyperlink “Submit manuscript” on the right and upload all of your manuscript files following the instructions given on the screen.

Please ensure you provide all relevant editable source files. Failing to submit these source files might cause unnecessary delays in the review and production process.

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Editorial Procedure

Double-blind peer review

This journal follows a double-blind reviewing procedure. Authors are therefore requested to submit:

A blinded manuscript without any author names and affiliations in the text or on the title page. Self-identifying citations and references in the article text should be avoided.

A separate title page, containing title, all author names, affiliations, and the contact information of the corresponding author. Any acknowledgements, disclosures, or funding information should also be included on this page.

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Title page

Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

- The name(s) of the author(s)
- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
- A clear indication and an active e-mail address of the corresponding author
- If available, the 16-digit ORCID of the author(s)

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Abstract

Please provide a structured abstract of 150 to 250 words which should be divided into the following sections:

- Purpose (stating the main purposes and research question)
- Methods
- Results
- Conclusion

For life science journals only (when applicable)

Trial registration number and date of registration

Trial registration number, date of registration followed by “retrospectively registered”

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations'.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

To be used for all articles, including articles with biological applications

Funding (information that explains whether and by whom the research was supported)

Conflicts of interest/Competing interests (include appropriate disclosures)

Availability of data and material (data transparency)

Code availability (software application or custom code)

Authors' contributions (optional: please review the submission guidelines from the journal whether statements are mandatory)

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

Ethics approval (include appropriate approvals or waivers)

Consent to participate (include appropriate statements)

Consent for publication (include appropriate statements)

Please see the relevant sections in the submission guidelines for further information as well as various examples of wording. Please revise/customize the sample statements according to your own needs.

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Text

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

LaTeX macro package (Download zip, 188 kB)

Headings

Please use no more than three levels of displayed headings.

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Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

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Scientific style

Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

Units and abbreviations

- Please adhere to internationally agreed standards such as those adopted by the commission of the International Union of Pure and Applied Physics (IUPAP) or defined by the International Organization of Standardization (ISO). Metric SI units should be used throughout except where non-SI units are more common [e.g. litre (l) for volume].
- Abbreviations (not standardized) should be defined at first mention in the abstract and again in the main body of the text and used consistently thereafter.

Drugs

- When drugs are mentioned, the international (generic) name should be used. The proprietary name, chemical composition, and manufacturer should be stated in full in Materials and methods.

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References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

- Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN.org LTWA

If you are unsure, please use the full journal title.

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

EndNote style (Download zip, 4 kB)

Authors preparing their manuscript in LaTeX can use the bibtex file spbasic.bst which is included in Springer's LaTeX macro package.

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Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

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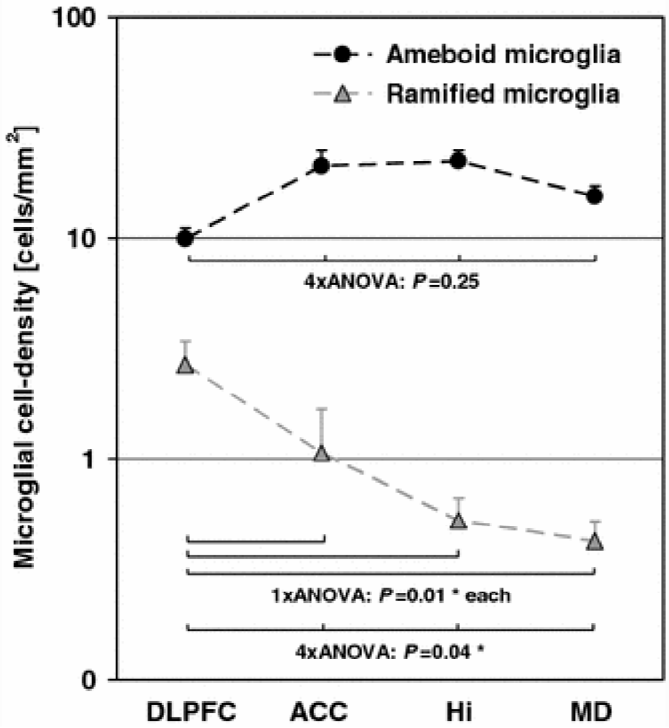
Artwork and Illustrations Guidelines

Electronic Figure Submission

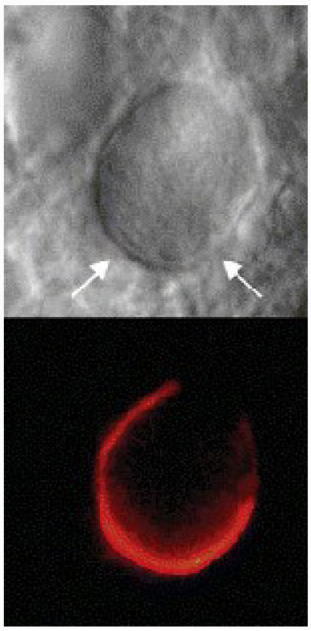
- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art



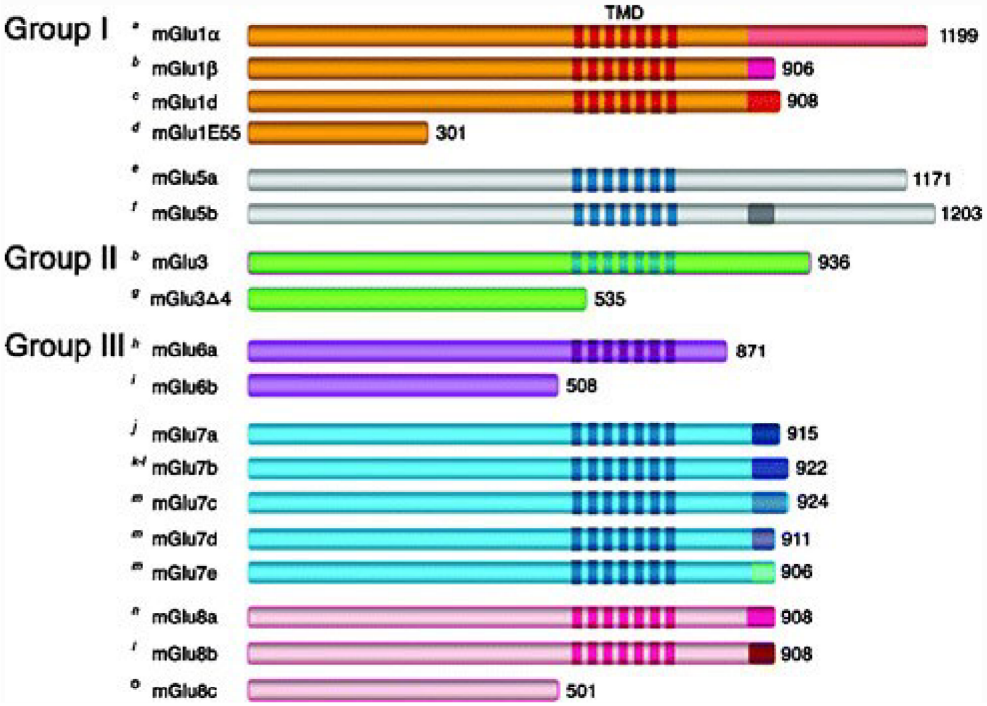
- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art



- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color art is free of charge for online publication.
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- If the figures will be printed in black and white, do not refer to color in the captions.
- Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

Figure Numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
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- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,"A1, A2, A3, etc." Figures in online appendices [Supplementary Information (SI)] should, however, be numbered separately.

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- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

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- Figures should be submitted separately from the text, if possible.
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- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.
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Spreadsheets

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- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

Collecting Multiple Files

- It is possible to collect multiple files in a .zip or .gz file.

Numbering

- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
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- Name the files consecutively, e.g. “ESM_3.mpg”, “ESM_4.pdf”.

Captions

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Authorship principles

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2) drafted the work or revised it critically for important intellectual content;

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* Based on/adapted from:

ICMJE, Defining the Role of Authors and Contributors,

Transparency in authors' contributions and responsibilities to promote integrity in scientific publication, McNutt at all, PNAS February 27, 2018

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- providing transparency on re-use of material and mention any unpublished material (for example manuscripts in press) included in the manuscript in a cover letter to the Editor;
- making sure disclosures, declarations and transparency on data statements from all authors are included in the manuscript as appropriate (see above).

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Examples of such statement(s) are shown below:

• Free text:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Example: CRediT taxonomy:

• Conceptualization: [full name], …; Methodology: [full name], …; Formal analysis and investigation: [full name], …; Writing - original draft preparation: [full name, …]; Writing - review and editing: [full name], …; Funding acquisition: [full name], …; Resources: [full name], …; Supervision: [full name],….

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A Graduate Student's Guide to Determining Authorship Credit and Authorship Order, APA Science Student Council 2006

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To ensure objectivity and transparency in research and to ensure that accepted principles of ethical and professional conduct have been followed, authors should include information regarding sources of funding, potential conflicts of interest (financial or non-financial), informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals.

Authors should include the following statements (if applicable) in a separate section entitled "Compliance with Ethical Standards" when submitting a paper:

- Disclosure of potential conflicts of interest
- Research involving Human Participants and/or Animals
- Informed consent

Please note that standards could vary slightly per journal dependent on their peer review policies (i.e. single or double blind peer review) as well as per journal subject discipline. Before submitting your article check the instructions following this section carefully.

The corresponding author should be prepared to collect documentation of compliance with ethical standards and send if requested during peer review or after publication.

The Editors reserve the right to reject manuscripts that do not comply with the above-mentioned guidelines. The author will be held responsible for false statements or failure to fulfill the above-mentioned guidelines.

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Authors are requested to disclose interests *that are directly or indirectly related to the work submitted for publication*. Interests within the last 3 years of beginning the work (conducting the research and preparing the work for submission) should be reported. Interests outside the 3-year time frame must be disclosed if they could reasonably be perceived as influencing the submitted work. Disclosure of interests provides a complete and transparent process and helps readers form their own judgments of potential bias. This is not meant to imply that a financial relationship with an organization that sponsored the research or compensation received for consultancy work is inappropriate.

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The above should be summarized in a statement and placed in a ‘Declarations’ section before the reference list under a heading of ‘Funding’ and/or ‘Conflicts of

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Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

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Research involving human participants, their data or biological material

Ethics approval

When reporting a study that involved human participants, their data or biological material, authors should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that an independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study. If a study was granted exemption from requiring ethics approval, this should also be detailed in the manuscript (including the reasons for the exemption).

Retrospective ethics approval

If a study has not been granted ethics committee approval prior to commencing, retrospective ethics approval usually cannot be obtained and it may not be possible to consider the manuscript for peer review. The decision on whether to proceed to peer review in such cases is at the Editor's discretion.

Ethics approval for retrospective studies

Although retrospective studies are conducted on already available data or biological material (for which formal consent may not be needed or is difficult to obtain) ethics approval may be required dependent on the law and the national ethical guidelines of a country. Authors should check with their institution to make sure they are complying with the specific requirements of their country.

Ethics approval for case studies

Case reports require ethics approval. Most institutions will have specific policies on this subject. Authors should check with their institution to make sure they are complying with the specific requirements of their institution and seek ethics approval where needed. Authors should be aware to secure informed consent from the individual (or parent or guardian if the participant is a minor or incapable) See also section on **Informed Consent**.

Cell lines

If human cells are used, authors must declare in the manuscript: what cell lines were used by describing the source of the cell line, including when and from where it was obtained, whether the cell line has recently been authenticated and by what method. If cells were bought from a life science company the following need to be given in the manuscript: name of company (that provided the cells), cell type, number of cell line, and batch of cells.

It is recommended that authors check the NCBI database for misidentification and contamination of human cell lines. This step will alert authors to possible problems with the cell line and may save considerable time and effort.

Further information is available from the International Cell Line Authentication Committee (ICLAC).

Authors should include a statement that confirms that an institutional or independent ethics committee (including the name of the ethics committee) approved the study and that informed consent was obtained from the donor or next of kin.

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Research Resource Identifiers (RRID) are persistent unique identifiers (effectively similar to a DOI) for research resources. This journal encourages authors to adopt RRIDs when reporting key biological resources (antibodies, cell lines, model organisms and tools) in their manuscripts.

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Clinical Trial Registration

The World Health Organization (WHO) definition of a clinical trial is "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes". The WHO defines health interventions as “A health intervention is an act performed for, with or on behalf of a person or population whose purpose is to assess, improve, maintain, promote or modify health, functioning or health conditions” and a health-related outcome is generally defined as a change in the health of a person or population as a result of an intervention.

To ensure the integrity of the reporting of patient-centered trials, authors must register prospective clinical trials (phase II to IV trials) in suitable publicly available repositories. For example www.clinicaltrials.gov or any of the primary registries that participate in the WHO International Clinical Trials Registry Platform.

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Summary of requirements

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Ethics approval'.

Examples of statements to be used when ethics approval has been obtained:

• All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of the Medical University of A (No. ...).

• This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No. ...).

• Approval was obtained from the ethics committee of University C. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

• The questionnaire and methodology for this study was approved by the Human Research Ethics committee of the University of D (Ethics approval number: ...).

Examples of statements to be used for a retrospective study:

• Ethical approval was waived by the local Ethics Committee of University A in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

• This research study was conducted retrospectively from data obtained for clinical purposes. We consulted extensively with the IRB of XYZ who determined that our study did not need ethical approval. An IRB official waiver of ethical approval was granted from the IRB of XYZ.

• This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Human Investigation Committee (IRB) of University B approved this study.

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• This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.

• The data reproduced from Article X utilized human tissue that was procured via our Biobank AB, which provides de-identified samples. This study was reviewed and deemed exempt by our XYZ Institutional Review Board. The BioBank protocols are in accordance with the ethical standards of our institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Informed consent

All individuals have individual rights that are not to be infringed. Individual participants in studies have, for example, the right to decide what happens to the (identifiable) personal data gathered, to what they have said during a study or an interview, as well as to any photograph that was taken. This is especially true concerning images of vulnerable people (e.g. minors, patients, refugees, etc) or the use of images in sensitive contexts. In many instances authors will need to secure written consent before including images.

Identifying details (names, dates of birth, identity numbers, biometrical characteristics (such as facial features, fingerprint, writing style, voice pattern, DNA or other distinguishing characteristic) and other information) of the participants that were studied should not be published in written descriptions, photographs, and genetic profiles unless the information is essential for scholarly purposes and the participant (or parent/guardian if the participant is a minor or incapable or legal representative) gave

written informed consent for publication. Complete anonymity is difficult to achieve in some cases. Detailed descriptions of individual participants, whether of their whole bodies or of body sections, may lead to disclosure of their identity. Under certain circumstances consent is not required as long as information is anonymized and the submission does not include images that may identify the person.

Informed consent for publication should be obtained if there is any doubt. For example, masking the eye region in photographs of participants is inadequate protection of anonymity. If identifying characteristics are altered to protect anonymity, such as in genetic profiles, authors should provide assurance that alterations do not distort meaning.

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• Reuse of images: If images are being reused from prior publications, the Publisher will assume that the prior publication obtained the relevant information regarding consent. Authors should provide the appropriate attribution for republished images.

Consent and already available data and/or biologic material

Regardless of whether material is collected from living or dead patients, they (family or guardian if the deceased has not made a pre-mortem decision) must have given prior written consent. The aspect of confidentiality as well as any wishes from the deceased should be respected.

Data protection, confidentiality and privacy

When biological material is donated for or data is generated as part of a research project authors should ensure, as part of the informed consent procedure, that the participants are made aware what kind of (personal) data will be processed, how it will be used and for what purpose. In case of data acquired via a biobank/biorepository, it is possible they apply a broad consent which allows research participants to consent to a broad range of uses of their data and samples which is regarded by research ethics committees as specific enough to be considered “informed”. However, authors should always check the specific biobank/biorepository policies or any other type of data provider policies (in case of non-bio research) to be sure that this is the case.

Consent to Participate

For all research involving human subjects, freely-given, informed consent to participate in the study must be obtained from participants (or their parent or legal guardian in the case of children under 16) and a statement to this effect should appear in the manuscript. In the case of articles describing human transplantation studies, authors must include a statement declaring that no organs/tissues were obtained from prisoners and must also

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Consent to Publish

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Summary of requirements

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Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

Sample statements for **"Consent to participate"**:

Informed consent was obtained from all individual participants included in the study.

Informed consent was obtained from legal guardians.

Written informed consent was obtained from the parents.

Verbal informed consent was obtained prior to the interview.

Sample statements for **"Consent to publish"**:

The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c.

The participant has consented to the submission of the case report to the journal.

Patients signed informed consent regarding publishing their data and photographs.

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