

Biomodulation of Inflammatory Cytokines Related to Oral Mucositis by Low-Level Laser Therapy

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ABSTRACT

This study evaluated the effects of LLLT on the expression of inflammatory cytokines related to the development of oral mucositis by gingival fibroblasts. Primary gingival fibroblasts were seeded on 24-well plates (10^5 cells/well) for 24 h. Fresh serum-free culture medium (DMEM) was then added, and cells were placed in contact with LPS (*Escherichia coli*, $1 \mu\text{g mL}^{-1}$), followed by LLLT irradiation (LaserTABLE—InGaAsP diode prototype—780 nm, 25 mW) delivering 0, 0.5, 1.5 or 3 J cm^{-2} . Cells without contact with LPS were also irradiated with the same energy densities. Gene expression of TNF- α , IL-1 β , IL-6 and IL-8 was evaluated by Real-Time PCR, and protein synthesis of these cytokines was determined by enzyme-linked immunosorbent (ELISA) assay. Data were statistically analyzed by the Kruskal–Wallis test, complemented by the Mann–Whitney test ($P < 0.05$). LPS treatment increased the gene expression and protein synthesis of TNF- α , IL-6 and IL-8, while the expression of IL-1 β was not affected. For LPS-treated groups, LLLT promoted significant decreases in the expression of TNF- α , IL-6, and IL-8 at 1.5 J cm^{-2} and 3 J cm^{-2} . These results demonstrate that LLLT promoted a beneficial biomodulatory effect on the expression of inflammatory cytokines related to oral mucositis by human gingival fibroblasts.

INTRODUCTION

Oral mucositis is an adverse effect of oncological treatment that affects nearly 70% of patients receiving this treatment and has a severe impact on the patients' quality of life (1). It is characterized by painful ulcerous and erosive lesions spread over the oral mucosa (2).

Previous studies have demonstrated that inflammatory cytokines such as IL-1 β , TNF- α , IL-6 and IL-8 play an important role in oral mucositis (3–5), since the increased expression of these molecules was directly related to the onset and severity of these lesions (6). In addition, it is already known that persistent inflammatory reaction can impair wound healing (7).

The elucidation of the mechanisms and factors involved in the etiopathogenesis of oral mucositis may also improve treatment strategies. Low-level laser therapy (LLLT) is included in the

armamentarium of treatment strategies for oral mucositis (8–10). It has been demonstrated that LLLT promotes decreased occurrence and severity of these lesions (11). *In vitro* studies also demonstrated that LLLT reduces the expression of inflammatory mediators by gingival fibroblasts (12) and the cytokine concentration of salivary fluid (13).

However, in addition to the already demonstrated effects of LLLT on oral mucosal cells, such as increased cell proliferation and migration (14,15), as well as increased growth factor expression (16), other effects of this therapy on oral mucosal cells have not been completely elucidated. The evaluation of the inflammatory modulation by LLLT on oral mucosal cells may support and improve the use of LLLT for mucositis lesions. Therefore, the aim of this study was to evaluate the expression of inflammatory cytokines by gingival fibroblasts treated with lipopolysaccharides (LPS) and LLLT at specific parameters, for better understanding the cellular and molecular effects of LLLT in situations involving increased inflammatory reaction, such as oral mucositis.

MATERIALS AND METHODS

Cell isolation and culture. Gingival fibroblasts were collected from young (18–24 years of age) and healthy individuals ($n = 3$) who were scheduled to undergo dental extraction (Ethics Committee # 367.654—Araraquara Dental School, UNESP, Brazil). Immediately after each tooth was extracted, the papillary gingival tissue was collected and placed in a flask containing serum-free Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY). The tissue sample was transported to the Experimental Pathology and Biomaterials Laboratory, where cells were isolated by enzymatic digestion within 24 h, in a DMEM solution containing 3 mg mL^{-1} of collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ) for 24 h. After that period, cells were col-

Table 1. Experimental and control groups definition.

Groups	Treatment	
	LPS	LLLT
G1 (negative control)	–	0 J cm^{-2}
G2 (positive control)	+	0 J cm^{-2}
G3	–	0.5 J cm^{-2}
G4	+	0.5 J cm^{-2}
G5	–	1.5 J cm^{-2}
G6	+	1.5 J cm^{-2}
G7	–	3 J cm^{-2}
G8	+	3 J cm^{-2}

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lected and transferred to a new culture flask containing 15 mL of DMEM with 10% fetal bovine serum (FBS) (Gibco).

For the experiment, cells were cultured in complete DMEM containing 10% FBS in 24-well plates (1×10^5 cells/well) for 24 h. Experimental and control groups were defined as demonstrated in Table 1.

Table 2. LLLT selected parameters.

Wavelength (nm)	Power (W)	Distance (cm)	Area of irradiation (cm ²)	Energy densities (J cm ⁻²)	Time of irradiation (s)
780	0.025	2	2	0.5	40
				1.5	120
				3	240

Table 3. Taqman assays selected for gene expression analysis.

Target gene	Taqman assay
hβActin	4333762T
hTNF-α	Hs00174128_m1
hIL-1β	Hs01555410_m1
hIL-6	Hs00985639_m1
hIL-8	Hs00174103_m1

LPS treatment. After 24-h incubation, fresh serum-free DMEM was applied to cultured cells, and $1 \mu\text{g mL}^{-1}$ of LPS (*Escherichia coli*, Sigma-Aldrich, St. Louis, MO) was added to each well. LPS was selected for stimulation of inflammatory cytokine expression (positive control) (17).

In vitro LLLT. After the addition of LPS, cells were irradiated by means of a laser diode device (LaserTABLE—InGaAsP—780 nm; 25 mW) at 0.5, 1.5 and 3 J cm⁻² (16,18,19). LLLT parameters are demonstrated in Table 2.

Gene expression of inflammatory cytokines—real-time PCR. Gene expression of TNF-α, IL-1β, IL-6 and IL-8 after LPS and LLLT was evaluated by Real-Time PCR, as previously described ($n = 4$) (18). RNA was isolated by the RNAqueous kit (Ambion, Grand Island, NY) according to the manufacturer’s instructions. cDNA synthesis was performed with the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Grand Island, NY).

Real-time amplifying reactions were prepared with standardized Taqman reagents (Taqman Gene Expression Master Mix and Taqman Assays), as demonstrated in Table 2. These reactions were detected and analyzed by Step One and Step One Plus Software (Applied Biosystems). Gene expression was analyzed by CT values, normalized by the expression of the selected endogenous gene, βActin (Table 3).

Protein synthesis of inflammatory cytokines—ELISA assay. Protein synthesis of selected cytokines (TNF-α, IL-1β, IL-6, and IL-8) was determined by the enzyme-linked immunosorbent (ELISA) assay (R&D Systems, Minneapolis, MN) in culture medium extracts after LPS and LLLT treatment. Briefly, plates were coated with primary antibodies and incubated overnight at room temperature. Plates were then washed and blocked with BSA solution for 1 h, and samples were added and

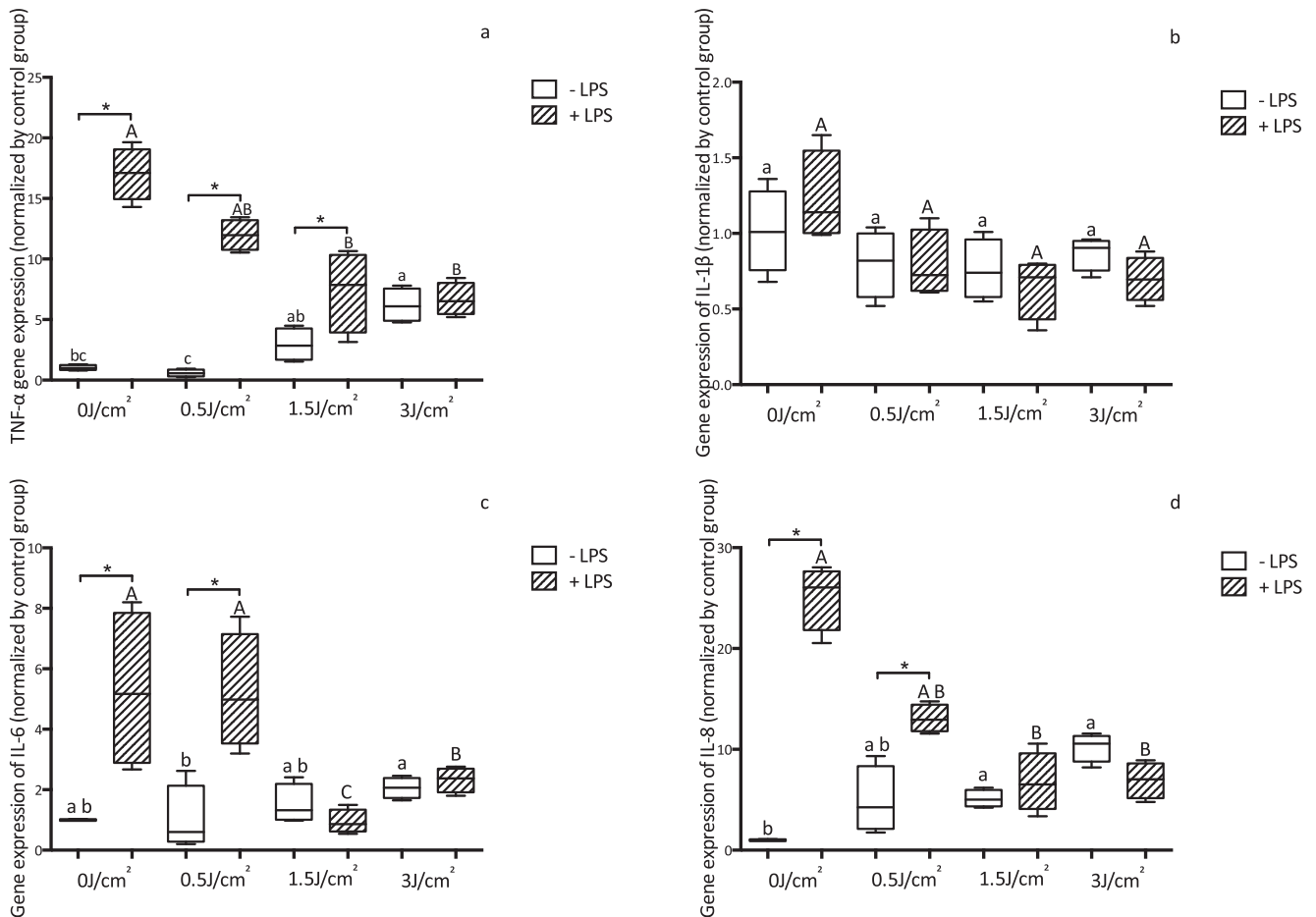


Figure 1. Gene expression of inflammatory cytokines TNF-alpha (a), IL-1beta (b), IL-6 (c), and IL-8 (d) by gingival fibroblasts after LPS and LLLT treatment. *Represents statistically significant difference for each energy density, treated (+) or not (-) with LPS. Different lowercase letters represent statistically significant differences between groups without LPS treatment, while different uppercase letters represent statistically significant differences between groups treated with LPS. Elements of the graph: The bottom side of the box represents the first quartile, and the top side, the third quartile. The horizontal line inside the box is the median. The vertical lines protruding from the box extend to the minimum and the maximum values of the data set.

incubated for 2 h. Secondary antibodies were added, followed by reagent and stop solutions. Protein synthesis was analyzed by spectrophotometry (455 nm) (Synergy H1—Biotek, Winooski, VT) with standard curves containing defined concentrations of each cytokine.

Statistical analysis. Data from gene expression (RT-PCR) and protein synthesis (ELISA) did not adhere to the normal distribution and were analyzed by the Kruskal–Wallis test complemented by the Mann–Whitney test for pairwise comparisons, at a 5% level of significance.

RESULTS

Low level laser therapy alone (-LPS), delivering densities of 1.5 J cm^{-2} (G5) and 3 J cm^{-2} (G7), increased TNF- α and IL-8 gene expression compared with the negative control group (G1). For all other energy densities, no significant difference from G1 was observed.

Non-irradiated gingival fibroblasts in contact with LPS (G2) showed increased expression of all cytokines (Fig. 1a,c,d), except for IL-1 β (Fig. 1b), in comparison with cells not in contact with LPS (G1). Regarding the effects of LLLT on the gene expression of oral-mucositis-related inflammatory cytokines, irradiation after LPS treatment, at energy densities of 1.5 J cm^{-2} and 3 J cm^{-2} (G6 and G8), decreased the expression of TNF- α ,

IL-6 and IL-8, while 0.5 J cm^{-2} (G4) was not effective for the modulation of gene expression of the selected inflammatory cytokines.

Considering the protein synthesis of the same inflammatory cytokines, evaluated by the ELISA method, LLLT irradiation alone (G1) was responsible for a discrete increase of TNF- α and IL-6 synthesis at 0.5 J cm^{-2} and 3 J cm^{-2} (G3 and G7). LPS (G2) was also an effective positive control for TNF- α , IL-6 and IL-8 (Figs. 2a,c,d), while no stimulation was observed for IL-1 β (Fig. 2b), as observed for gene expression.

Low level laser therapy at all energy densities reduced the synthesis of TNF- α , while this effect was observed at 1.5 J cm^{-2} (G6) and 3 J cm^{-2} (G8) for IL-6. However, IL-1 β and IL-8 synthesis was not affected by the phototherapy.

DISCUSSION

The role of inflammatory cytokines in the etiopathogenesis of oral mucositis has been demonstrated by several studies. These studies have shown not only an increase in cytokine expression but also a positive association between the amount of cytokine

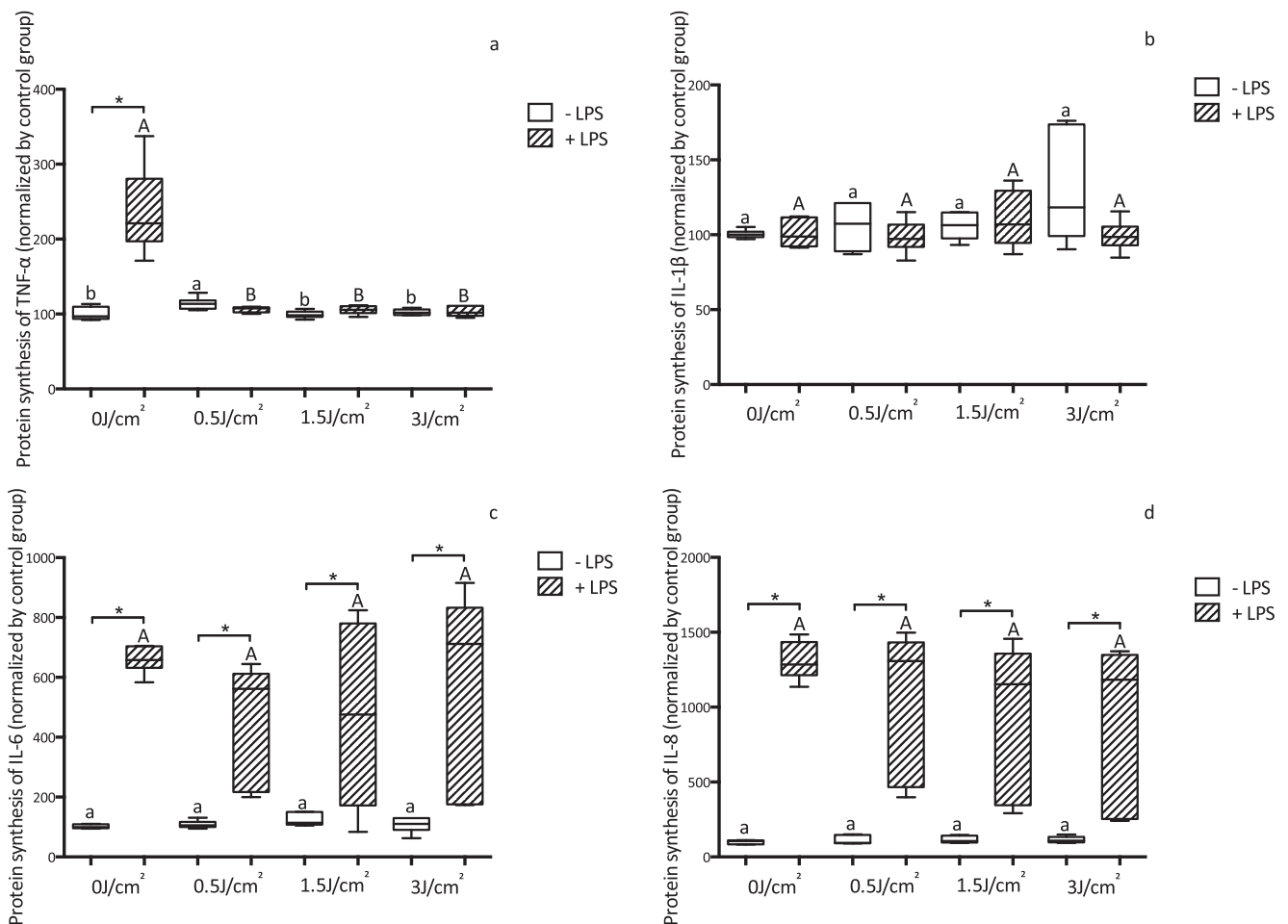


Figure 2. Secretion of inflammatory cytokines TNF- α (a), IL-1 β (b), IL-6 (c), and IL-8 (d) by gingival fibroblasts after LPS and LLLT treatment. *Represents statistically significant difference for each energy density, treated (+) or not (-) with LPS. Different lowercase letters represent statistically significant difference between groups without LPS treatment, while different uppercase letters represent statistically significant difference between groups treated with LPS. Elements of the graph: The bottom side of the box represents the first quartile, and the top side, the third quartile. The horizontal line inside the box is the median. The vertical lines protruding from the box extend to the minimum and the maximum values of the data set.

synthesis and the severity of mucositis (6,20,21). TNF- α , IL-6 and IL-8 are the inflammatory cytokines most commonly described in relation to oral mucositis development (21). In addition, IL-1 β has also been related to this adverse effect (20). These proteins are activated by the NF- κ B signaling pathway, which is also related to several other cell functions, such as cell viability, migration and proliferation (22,23).

The early diagnosis and correct intervention of oral mucositis lesions are of great importance for healing and for improving patients' quality of life (21). LLLT has been shown to improve the healing of oral mucositis lesions (9,24); however, the cellular and molecular mechanisms of this therapy have not been completely elucidated. Previous studies have demonstrated that LLLT affects the secretion of several inflammatory cytokines by cultured cells (12,25–27). In this study, we aimed to evaluate the effect of LLLT at preestablished parameters on the expression of specific inflammatory cytokines related to the etiopathogenesis of oral mucositis (6,20,21).

The increased expression of inflammatory cytokines has been detected in the initiation phase of oral mucositis, and the maintenance of this phase may delay the healing of the oral mucosa (28), as demonstrated by *in vitro* and *in vivo* studies (29–31). Therefore, the biomodulation observed by LLLT could lead to a faster healing process. In addition, LLLT also shows positive effects on the proliferation and migration of oral mucosal cells that could also collaborate with oral mucosal healing (15,16,32).

The results obtained in the present study corroborate with previous clinical findings, since LLLT at selected parameters reduced the gene expression and protein synthesis of evaluated inflammatory cytokines, except for IL-1 β (12). These results suggest that LLLT can exert biomodulatory effects on the expression of these cytokines by oral mucosal cells, which could accelerate oral wound-healing and improve pain symptomatology.

For cells treated only with LLLT at 1.5 J cm⁻² and 3 J cm⁻², a discrete increase in TNF- α and IL-8 gene expression was observed, and for the former, protein synthesis was also enhanced at 0.5 J cm⁻². This effect may be related to the wound-healing improvement shown by LLLT, since it is already known that these cytokines, mainly TNF- α , play an important role in tissue repair (7). In low concentrations, these inflammatory cytokines stimulate cell proliferation and migration during the initial phases of wound-healing (31,33). The lack of significant stimulation of IL-1 β expression by LPS may be related to the posttreatment period evaluated (24 h), as also demonstrated in a previous study (34).

An interesting finding of the study was a high standard deviation in the results, mainly for protein synthesis, due to the different results obtained for the three patients, as demonstrated by other authors (22,34). In addition to the fact that the behavior of the cells after LPS treatment and LLLT was similar for all patients, the magnitude of the results was quite different for each patient. However, the selection of different individuals is relevant, considering the external validity of the results, despite negatively influencing data dispersion. The results obtained demonstrated that LLLT can promote the biomodulation of inflammatory cytokine expression and synthesis by gingival fibroblasts, and that this effect could be related to the enhancement and acceleration of oral wound-healing.

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