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Influence of neodymium-doped yttrium aluminum garnet laser irradiation on pro-inflammatory cytokine concentrations in lipopolysaccharide-challenged peripheral blood mononuclear cells: an in vitro study

by

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ABSTRACT

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Objective: The aim of this study was to assess the influence of neodymium-doped yttrium aluminum garnet (Nd:YAG) laser irradiation on pro-inflammatory cytokine levels in stimulated rat peripheral blood mononuclear cells (PBMCs).

Materials and Methods: We cultured rat PBMCs, then stimulated the cells using various lipopolysaccharide (LPS) concentrations—0, 10, 100, or 1000 ng/ml. Cultures at each LPS stimulation level received Nd:YAG laser irradiation at one of four power values—0 (control), 5, 7.5, or 10 W. Pulse duration, repetition rate, and irradiation duration remained constant at 100 μs, 20 Hz, and 30 s, respectively. Magnetic microsphere immunoassays were completed to

compare levels of six pro-inflammatory cytokines—macrophage inflammatory protein-1α (MIP-1α), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), interferon-γ-inducible protein 10 (IP-10), macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor-α (TNF-α)—in treatment versus control groups.

Results: In LPS-stimulated PBMC cultures irradiated at 5 W, we observed a trend for reduction in the levels of all cytokines assessed. These reductions did not reach statistical significance. The 7.5 and 10 W laser exposures reduced cytokine levels below detection limits.

Conclusions: Nd:YAG laser irradiation appears to reduce levels of pro-inflammatory cytokines in LPS-activated inflammatory cells. Additional research varying laser irradiation parameters, target cell types, and outcome variables may further clarify the influence of Nd:YAG laser energy on the inflammatory response.

KEYWORDS: Lasers; leukocytes, mononuclear; inflammation; cytokines; low-level light therapy; lipopolysaccharides

INTRODUCTION

Dr. Endre Mester, a Hungarian physician, introduced "laser biostimulation" in 1967, just a few years after the appearance of laser technology.[1-3] Mester showed in a murine model that low doses of laser light enhanced excisional wound healing and accelerated hair growth.[2] Since the 1960s, many synonymous terms have been used to describe this phenomenon. Notably, the relevant National Library of Medicine Medical Subject Heading (MeSH) is "low-level light therapy" (LLLT),[4] yet "photobiomodulation" (PBM) has been suggested as the preferred term.[5] PBM is a nonthermal process, relying on red or near infrared (NIR) light to stimulate photochemical and photophysical events within cells and tissues, leading to enhanced healing, pain relief, and reduced inflammation.[5] In general, continuous laser power output of 0.005 to 0.5 W produces PBM, whereas power > 1 W results in concomitant thermal effects.[1] Use of higher power (high-level laser therapy), results in photothermal tissue effects at the site of irradiation, with an adjacent PBM zone, as the light energy penetrates and scatters into surrounding tissues.[6]

Practitioners across a broad spectrum of medical and dental disciplines have applied PBM for a variety of clinical purposes, including treatment of nonhealing skin ulcers,[7-11] cancer therapy-related oral mucositis and acute radiodermatitis,[12-14] paresthesia following orthognathic surgery,[15-18] and androgenetic alopecia.[19] Many authors have reported alleviation of various forms musculoskeletal pain using PBM. PBM appears to have some benefit as a noninvasive treatment for neck pain.[20] A systematic review concluded that, in the setting of acute neck pain, laser therapy significantly reduced pain immediately after treatment, and in

chronic neck pain, LLLT produced sustained benefit for 22 weeks.[21] Compared with placebo, optimal doses of 904 nm and possibly 632 nm wavelengths appear to offer statistically significant reductions in short-term pain and residual disability in lateral elbow tendinopathy (tennis elbow).[22] Considering tendinopathy generally (including radiohumoral epicondylitis, Achilles tendinopathy, rotator cuff tendinitis, and other tendinopathies), a systematic review and meta-analysis identified 12 trials reporting positive LLLT effects and 13 reporting inconclusive results or no effect.[23] The authors noted a high level of heterogeneity among studies and concluded that LLLT was potentially effective in tendinopathy treatment, with available evidence remaining inconclusive.[23] In the last several years, authors have introduced the concept of transcranial PBM to produce central effects.[24] In animal models, promising early research suggests that PBM could offer benefit in treatment of traumatic brain injury, Parkinson's disease, Alzheimer's disease, stroke, and depression.[24]

Despite many advancements in clinical application of PBM, skepticism surrounding this treatment modality persists, owing to incomplete understanding of the underlying mechanisms and failure to optimize dosimetry for specific clinical purposes.[25] Reduction in inflammation ranks among the most reproducible PBM effects, possibly accounting for many of the observed clinical benefits.[26] In synoviocytes isolated from rheumatoid arthritis patients, PBM (810 nm, 5 or 25 J/cm^2) applied before or after cell stimulation with tumor necrosis factor-α (TNF-α) reduced mRNA and protein levels of TNF-α, interleukin 1β (IL-1β), and interleukin 8 (IL-8) in a dose-dependent fashion.[27] Three wavelengths (405, 532, and 650 nm) reduced IL-8 expression in human annulus fibrosus cells incubated with medium containing the pro-inflammatory cytokines IL-1β, interleukin 6 (IL-6), and IL-8.[28] The 405 nm light energy also reduced IL-6

expression.[28] In lipopolysaccharide (LPS)-stimulated human gingival fibroblasts (HGFs), a 635 nm light emitting diode (LED) reduced multiple inflammatory markers, including cyclooxygenase 2 (COX2), prostaglandin E2 (PGE2), and granulocyte colony stimulating factor (GCSF).[29,30] Sakurai and coworkers irradiated LPS-challenged human gingival fibroblasts using a gallium aluminum arsenide (GaAlAs) diode laser (830 nm, ≤ 6.3 J/cm^2) and noted dose-dependent inhibition of PGE2 as well as reduction of COX2 mRNA.[31] The same group showed reduction of IL-1β under similar experimental conditions.[32] Funk et al. found that helium neon (HeNe) laser irradiation of human peripheral blood mononuclear cells (PBMCs) increased or decreased production IL-1α, TNF-α, IL-2, and interferon-γ (IFN-γ) depending on the dosimetry.[33]

OBJECTIVE

Despite substantial evidence that PBM using light in the red or NIR spectral region can reduce pro-inflammatory cytokine levels in multiple cell types, little is known about the effect of neodymium-doped yttrium aluminum garnet (Nd:YAG) laser energy in this context. The objective of this in vitro study was to assess the influence of Nd:YAG laser irradiation on pro-inflammatory cytokine concentrations in LPS-stimulated rat PBMCs.

MATERIALS AND METHODS

Target Cells

We cultured rat PBMCs (IQ Biosciences, Berkeley, California), then stimulated the cells with varying LPS concentrations—0, 10, 100, or 1000 ng/ml. Cells were then transferred to 96-well plates (2.0×10^4 cells per well).

Irradiation Parameters

An Nd:YAG laser (1064 nm, Lightwalker AT, Fotona, Dallas, Texas) was used to irradiate cultures in treatment groups. We utilized a 300-micron optical fiber and directed the laser beam perpendicularly to the cell level from a distance of 1.8 mm, employing a standardized support to reliably reproduce the laser position. Cultures at each LPS stimulation level received Nd:YAG laser irradiation at one of four power values—0 (control), 5, 7.5, or 10 W (Figure 1). Pulse duration, repetition rate, and irradiation duration remained constant at 100 µs, 20 Hz, and 30 s, respectively (Table 1). After irradiation, we incubated the cells for one hour at 37º C prior to analysis.

Magnetic Microsphere Immunoassay

To analyze PBMC cytokine levels after laser treatment, a magnetic microsphere immunoassay was used (MAGPIX System, Luminex, Austin, Texas). This system provides the capability of multiplexing various individual cytokine immunoassays within a single well, permitting simultaneous assessment of multiple cytokine levels. Six pro-inflammatory cytokines were assessed in this study—macrophage inflammatory protein-1α (MIP-1α),

interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), interferon-γ-inducible protein 10 (IP-10), macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor-α (TNF-α). Color-coded magnetic microspheres tagged with fluorescent dyes were covalently coupled with antibodies specific to the cytokines of interest. After the microspheres were subjected to a test sample, a reporter molecule, labeled with a specific fluorescent dye, was introduced. An LED excited the dyes, and a CCD camera detected the fluorescence from each microsphere. The median fluorescence intensity (MFI) provided the basis for the sample analysis.

Statistical Analysis

We performed each experiment (LPS concentration and laser treatment combination) in duplicate wells per condition and in three independent experiments (n=3). Data are presented as means ± SEM. Mean cytokine concentrations for treatment and control groups were compared using Student's t-tests. Differences were accepted as significant at the $p<0.05$ level.

RESULTS

LPS Controls

LPS caused significant dose dependent increase in all six cytokines (Figure 2). This increase appeared to saturate at the 10 ng/ml LPS concentration for MCP-1 and TNF-α. We observed further increases in the release of MIP1-α, MIP2, IL-6, and IP-10 at 100 and 1000 ng/ml LPS. We noted significant variability in all cytokine concentrations among the different experiments. Thus, to assess effects of laser exposure on cytokine response, we normalized all

cytokine concentrations to the 1000 ng/ml dose in control cultures and expressed all data points as percentages of this control (Figure 3).

Nd:YAG Laser Irradiation

The plots in Figure 3 illustrate outcomes recorded for cytokines MIP -1α, MCP-1, MIP-2, IL-6, IP-10, and TNF-α. We observed a trend for decreased cytokine levels in cultures receiving irradiation at 5 W. However, this reduction was not significantly different from controls. Cytokine signals were virtually eliminated in the 7.5 and 10 W groups (significantly different from controls at some LPS concentrations).

A Cell Counting Kit (CCK8 test) found equivalent cell counts in the control and irradiated samples. This result indicates that neither cell death nor cell proliferation occurred in response to laser exposure and that the loss of cytokine signal in the 7.5 and 10 W treatment groups was not attributable to cell death.

DISCUSSION

Our purpose was to analyze the effect of Nd:YAG laser irradiation on cytokine levels in LPS-challenged rat PBMCs. Consistent with prior in vitro studies evaluating the impact of red or NIR light on pro-inflammatory cytokine levels, we noted dose-dependent cytokine reduction, with concentrations of all analytes falling below detection limits at the two highest power levels.

As shown in Table 1, the energy density (fluence) utilized in this study appears at least two orders of magnitude higher than a typical PBM dose.[1] Indeed, the laser exposures we applied fall into the high-level laser therapy (HLLT) range. However, use of a very small spot size (300 µm at the fiber tip) results in very high calculated fluence (J/cm^2), even with a modest cumulative dose.[25] It should be noted that the cells in our experiments experienced lower fluence due to beam divergence over the distance between the fiber tip and the 96-well plates. Additionally, at a repetition rate of 20 Hz and a pulse duration of 100 µs, the relaxation time between pulses amounted to 50 milliseconds—500 times longer than the pulse duration. Ultimately, the equivalent cell counts noted in treatment and control groups demonstrate that the high fluence did not destroy the target cells. However, our study was incapable of distinguishing thermal from nonthermal effects. Our observations could relate to unrecorded changes in temperature.

Biphasic dose response is a widely appreciated feature of PBM. For multiple outcome measures—such as collagen synthesis—investigators are able to identify an optimum dose, most often defined by the fluence.[26] Beyond the optimal dose, the biologic response

diminishes, and under some conditions, inhibitory effects predominate. With regard to reduction in pro-inflammatory cytokine levels, we did not observe a biphasic response under the described conditions. Both the low the fluence values in prior studies and the high fluence values we applied appear to produce anti-inflammatory effects.

CONCLUSIONS

The impact of infrared laser energy on cellular inflammatory responses likely depends on 1) target cell type, and 2) irradiation parameters such as wavelength, pulse duration, repetition rate, average power, irradiance (W/cm^2), fluence (J/cm^2), and cumulative dose. Variations among studies in irradiation parameters and target cells may produce strikingly different results. Under the experimental conditions described, Nd:YAG laser irradiation appeared to reduce pro-inflammatory cytokine levels in LPS-activated inflammatory cells. Additional preclinical investigation may clarify inflammatory and other biologic responses to infrared laser irradiation and optimize treatment parameters. Controlled clinical research is necessary to determine if similar responses occur in vivo.

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FIGURES

Figure 1. Experimental design.

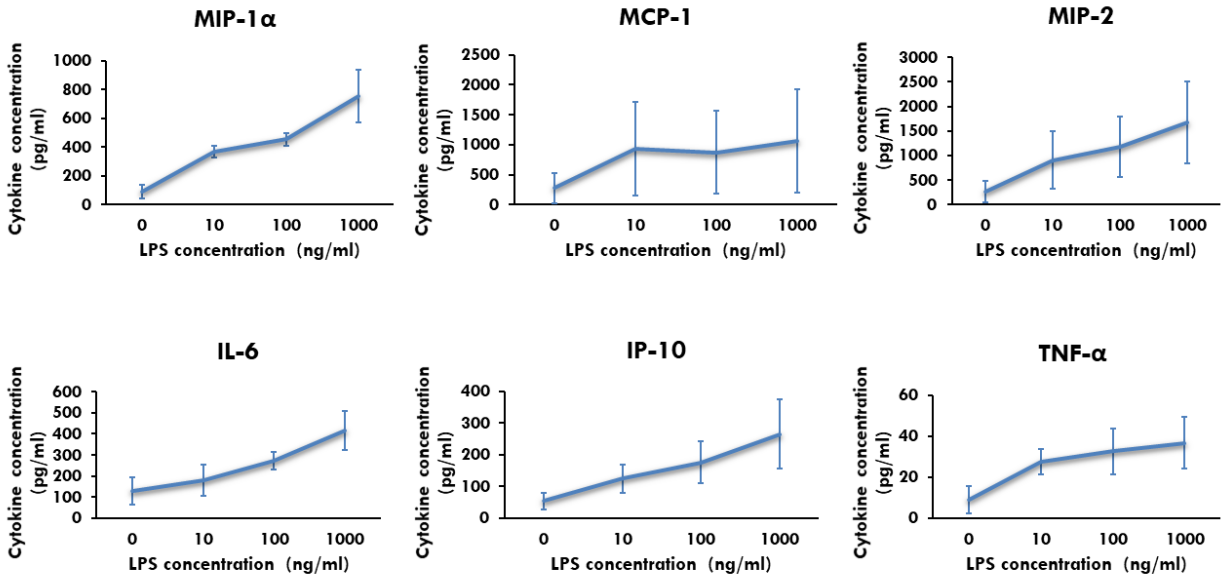

Figure 2. Dose response effects of LPS on cytokine release from PBMC. Average cytokine concentrations expressed in pg/ml from 3 independent experiments are shown with SEM.

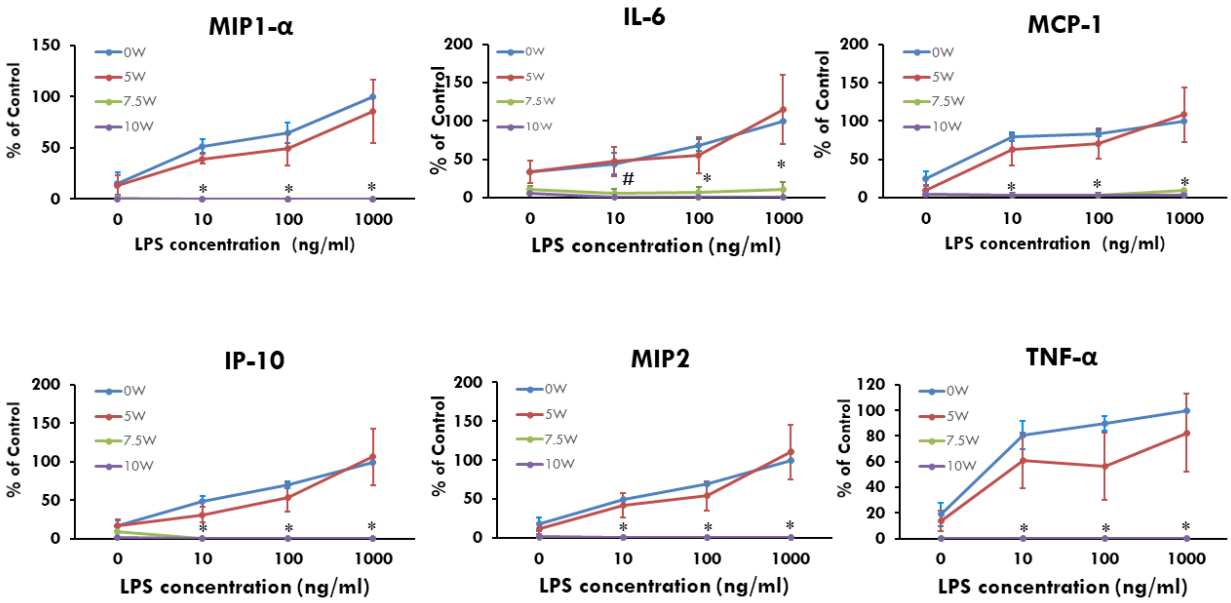

Figure 3. Effect of Nd:YAG laser exposure on cytokine response to LPS stimulation. Cytokine concentrations from three independent experiments are shown as % of control. Within each experiment the values of control cultures at 1000 ng/ml LPS concentration are shown as 100%.

*Significant difference in 7.5W and 10W groups compared to control cultures ($P < 0.05$).

#Significant difference between 10W and control groups ($P < 0.05$).

TABLES

Table 1. Nd:YAG laser irradiation parameters by treatment group.

Group	Average Power (W)	Pulse Energy (mJ)	Pulse Duration (μs)	Repetition Rate (Hz)	Peak Power (W)	Fiber Diameter (μm)	Irradiance at tip (W/cm^2)	Fluence at tip (J/cm^2)	Distance to target (mm)	Irradiation time (s)
1*	0	0	0	20	0	NA	0	0	NA	0
2	5	250	100	20	2500	300	7074	354	1.8	30
3	7.5	375	100	20	3750	300	10,610	531	1.8	30
4	10	500	100	20	5000	300	14,147	707	1.8	30

*Control