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The Influence of Neodymium-Doped Yttrium Aluminum Garnet Laser Pulse Repetition Rate on Cytokine Secretion from Peripheral Blood Mononuclear Cells in Vitro

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Abstract

Objective: Biological effects of infrared laser energy at various exposure parameters have been characterized in previous in vitro and animal studies. However, the impact of pulse repetition rate (PRR) has not been evaluated in this context. The purpose of this investigation was to assess the influence of PRR on cytokine secretion from peripheral blood mononuclear cells (PBMCs) subjected to pulsed neodymium-doped yttrium aluminum garnet (Nd:YAG) laser energy.

Materials and Methods: Rat PBMCs were cultured in vitro then stimulated using a lipopolysaccharide concentration of 0 or 100 ng/ml. Cultures received Nd:YAG laser radiation (1064 nm, 5 W, 30 s) at PRR of 0 (untreated controls), 20, 30, 40, or 60 Hz. Concentrations of tumor necrosis factor-α (TNF-α), macrophage inflammatory protein (MIP)-1α, macrophage inflammatory protein (MIP)-2, monocyte chemoattractant protein-1 (MCP-1), interferon-gamma-induced protein (IP)-10, interleukin (IL)-6, and IL-10 were recorded using a magnetic microsphere immunoassay. The main effects of PRR and LPS stimulation on cytokine concentrations, and the interaction between PRR and LPS stimulation, were assessed using two-way analysis of variance. Bonferroni post hoc tests were used to identify pairwise differences between groups.

Results: The main effect of PRR was statistically significant for MIP-1α ($P = 0.018$), TNF-α ($P = 0.025$), MCP-1 ($P < 0.001$), MIP-2 ($P = 0.013$), and IL-6 ($P = 0.031$). Five of six pro-inflammatory cytokines exhibited significantly lower mean concentrations in laser-exposed compared with control cultures at one or more PRR. However, no statistically significant differences were found between PRR groups.

Conclusions: Under the described conditions, statistically significant differences in cytokine secretion were observed between laser-exposed and control cultures, consistent with prior reports. However, PRR appears to be an irrelevant factor in immunomodulation of PBMCs.

Keywords: Lasers; Inflammation; Monocytes; Cytokines; Low-Level Light Therapy; Lipopolysaccharides

Abbreviations: HMT: Host Modulation Therapy, PBM: Photobiomodulation, PRR: Pulse Repetition Rate, PBMCs: Peripheral Blood Mononuclear Cells, RPMI: Roswell Park Memorial Institute, FBS: Fetal Bovine Serum, LPS: Lipopolysaccharide, LPT: Laser Periodontal Therapy, SRP: Scaling and Root Planning

Introduction

Periodontitis—which represents the most prevalent noncommunicable chronic inflammatory condition affecting humans—is a multifactorial disease resulting in severe periodontal tissue destruction and tooth loss in more than one billion individuals worldwide **[1,2]**.

Putative periodontal pathogens within dental plaque represent the etiology of periodontitis **[3-5]**. However, most of the tissue destruction is not directly attributable to microorganisms or their products. Rather, loss of alveolar bone and clinical attachment derives primarily from the host inflammatory response **[6]**. Complex dental biofilms result in the emergence of a panoply of host-derived cytokines that mediate resorption of alveolar bone and loss of attachment **[6-9]**.

Standard therapy for periodontitis includes professional mechanical plaque removal and establishment of an effective oral hygiene regimen that routinely disrupts biofilm development **[1]**. This approach has served as the mainstay of periodontal therapy for more than a century **[10]**. However, periodontists have also attempted to modify the inflammation and tissue destruction induced by etiological microorganisms—an approach termed "host modulation therapy" (HMT) **[11]**. HMT strategies include use of bone-sparing, antiproteinase, and anti-inflammatory agents **[11,12]**. These pharmacological interventions have had limited clinical applicability due in part to adverse effects of the medications. Electromagnetic radiation may represent a means of therapeutically altering host immunological function without risk of untoward medication-related effects **[13]**.

It has long been recognized that electromagnetic radiation can interact with living tissues and that it is possible for some of these interactions to produce therapeutic effects **[14-18]**. Laser radiation is unique in that it consists of collimated, monochromatic Gaussian light beams that can be directed toward specific regions on the surfaces of living tissues **[19]**. Reduction in inflammation has been one of the most reproducible effects of laser photobiomodulation (PBM), a term encompassing photophysical and photochemical phenomena unrelated to thermal tissue responses **[13,20-23]**. Wavelengths in the red and near infrared spectral regions (600 through 1200 nm) have shown favorable anti-inflammatory effects **[13]**. Yamaura and colleagues exposed TNF-α-stimulated synoviocytes isolated from rheumatoid arthritis patients to infrared diode laser energy (810 nm), with fluence of 5 or 25 J/cm^2 **[24]**. The authors reported dose-dependent reduction in mRNA and protein levels of TNF-α, IL-1β, and IL-8 **[24]**. Similarly, Hwang et al. compared IL-8 and IL-6 expression in cytokine-stimulated macrophages subjected to laser energy (405, 532, or 650 nm) at doses up to 1.6 J/cm^2 **[25]**. All wavelengths significantly reduced IL-8 expression compared with controls; only the 405 nm wavelength produced statistically significant reduction in IL-6 expression **[25]**. In activated dendritic cells derived from the mouse femur, Chen and colleagues found reduced cell-surface markers of inflammation and IL-12 secretion in response to infrared diode laser irradiation (810 nm, 0.3 or 3 J/cm^2) **[26]**.

Researchers have also assessed the anti-inflammatory effects of lasers emitting in this segment of the electromagnetic spectrum using animal models. Safavi et al. assessed the influence of He-Ne laser irradiation (632.8 nm, 7.5 J/cm^2) on IL-1β, interferon-Y (IFN- Y), and TNF-α expression in wounded gingiva of male Wistar rats **[27]**. The authors noted statistically significant reduction in IL-1β and IFN- Y expression in the laser-irradiated groups compared with controls **[27]**. Aimbire and colleagues evaluated the effect of Ga-AsI-Al diode laser irradiation (650 nm, 5.2 J/cm^2) on TNF-α concentrations in diaphragm muscle tissue from male Wistar rats **[28]**. The authors recorded statistically significant reduction in TNF-α concentrations in the laser-irradiated group compared with untreated controls **[28]**. Although existing evidence from in vitro and animal studies suggest a

possible clinical role for laser irradiation in limiting inflammation, the ideal wavelength remains unidentified, and parameters such as fluence, irradiance, pulse duration, pulse repetition rate (PRR), exposure time, and number of exposures have not been optimized. The purpose of this investigation was to assess the influence of PRR on cytokine secretion levels in stimulated and unstimulated rat PBMC cultures subjected to near-infrared radiation from a neodymium-doped yttrium aluminum garnet (Nd:YAG) laser.

Materials and Methods

Cell Culture

Frozen Rat peripheral blood mononuclear cells (PBMCs) (IQ Biosciences, Berkeley, California, USA) were thawed and suspended in Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% fetal bovine serum (FBS) penstrap. To remove cryoprotectant, cells were centrifuged for 7 minutes at 700 g, then transferred to 96-well plates (2 x 10^4 cells per well). Cells were stimulated with 0 or 100 ng/ml of lipopolysaccharide (LPS), and incubated for 24 hours at 37°C.

Irradiation Parameters

An Nd:YAG laser (1064 nm, Lightwalker AT, Fotona, Dallas, Texas, USA) was used to irradiate cultures, and a 320-micron optical fiber directed the laser beam perpendicularly to the plated cells at a distance of 1.8 mm. A standardized support system ensured a reliable and reproducible laser position. For each group, exposure time and power output remained constant at 30 seconds and 5 W, respectively. PRR was set at 0 (untreated controls), 20, 30, 40, or 60 Hz **(Figure 1, Table 1)**. After irradiation, cells were returned to the incubator for one hour at 37° C.

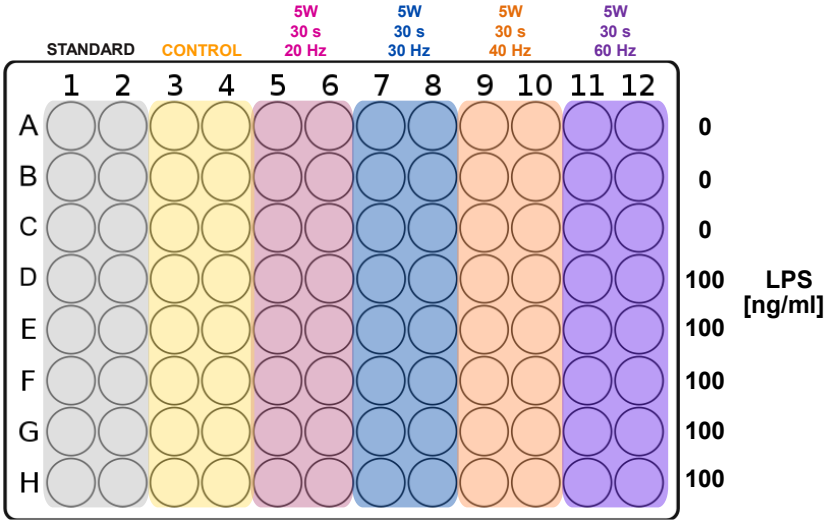

Figure 1. Diagram of 96-well plate depicting the experimental design. Exposure time and average power remained constant at 30 s and 5 W, respectively. For each group, laser parameters differed only in the pulse repetition rate—0 (untreated control cultures), 20, 30, 40, or 60 Hz.

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

Group	Average Power (W)	Peak Power (W)	Pulse Energy (mJ)	Pulse Duration (µs)	Repetition Rate (Hz)	Fiber Diameter (µm)	Irradiance at tip (W/cm^2)	Fluence at tip* (J/cm^2)	Distance to target (mm)	Irradiation time (s)
Control	0	0	0	0	NA	NA	0	0	NA	0
1	5	2500	250	100	20	320	6217	311	1.8	30
2	5	1667	167	100	30	320	6217	207	1.8	30
3	5	1250	125	100	40	320	6217	155	1.8	30
4	5	833	83	100	60	320	6217	103	1.8	30

*Per pulse

Evaluation of Cytokine Concentrations

Supernatant was extracted and analyzed using a magnetic microsphere immunoassay (MAGPIX System, Luminex, Austin, Texas, USA) permitting quantification of IL-6, IL-10, MIP-1α, MIP-2, MCP-1, TNF-α, and IP-10. The sensitivities for these assays were 30.7, 2.7, 0.8, 9.0, 9.0, 1.9, and 1.4 pg/ml, respectively **(Table 2)**. The analysis software (MAGPIX System, Luminex) processed assay images and determined cytokine/chemokine concentrations in pg/ml using standard curves.

Table 2. Inflammatory mediators evaluated by magnetic microsphere immunoassay and the associated minimum detectable concentrations (MDCs)

Analyte		2-Hour Protocol	
		MDC (pg/mL)	MDC + 2 SD
1	Interleukin-6 (IL-6)	30.7	86.2
2	Interleukin-10 (IL-10)	2.7	6.9
3	Macrophage inflammatory protein-1α (MIP-1α)	0.8	2.1
4	Macrophage inflammatory protein-2 (MIP-2)	9.0	21.8
5	Monocyte chemoattractant protein-1 (MCP-1)	9.0	21.8
6	Tumor Necrosis Factor-α (TNF-α)	1.9	7.2
7	Interferon gamma-induced protein 10 (IP-10)	1.4	3.5

Statistical Analysis

For each evaluated cytokine, a two-way analysis of variance was conducted to compare the main effects of LPS stimulation and PRR, and the interaction between these factors, on cytokine concentration. Bonferroni post hoc tests were used to elucidate statistically significant pairwise differences among PRR levels (0, 20, 30, 40, and 60 Hz). Differences were accepted as significant at an alpha level of 0.05.

Results

Cytokine secretion by rat PBMCs was evaluated using magnetic microsphere immunoassay following exposure to LPS and treatment with directed Nd:YAG laser energy with PRRs ranging from 20 to 60 Hz (20, 30, 40, and 60 HZ) at constant pulse duration (100 μs), average power (5 W), and irradiation time (30 s). Thus, pulse energy ranged from 83 to 250 mJ, peak power ranged from 833 to 2500 W, and fluence ranged from 103 to 311 J/cm^2 **(Table 1)**. The degree to which LPS increased cytokine concentrations varied by cytokine, with the largest increases noted for IP-10, TNF-α, and MIP-2 **(Figure 2)**. Notably, mean concentrations of five pro-inflammatory cytokines were significantly lower in laser-exposed versus control LPS-stimulated PBMC cultures **(Table 3)**. These were the macrophage/monocyte-produced signaling molecule TNF-α ($F(4, 110) = 2.90$, $P = 0.025$), the chemotactic inflammatory cell recruiting protein MIP-1α ($F(4, 110) = 3.13$, $P = 0.018$), a related protein known as MIP-2 ($F(4, 110) = 3.35$, $P = 0.013$), the monocyte/macrophage chemoattractant protein MCP-1 ($F(4, 110) = 10.51$, $P < 0.001$), and the proinflammatory interleukin IL-6 ($F(4, 110) = 2.76$, $P = 0.031$). The model for IL-10 was not statistically significant ($F(9, 110) = 1.67$, $P = 0.105$), and the main effect of PRR was not statistically significant for IP-10 ($F(4, 110) = 2.01$, $P = 0.098$). The interaction term between LPS stimulation and PRR was significant for the MIP-1α ($F(4, 110) = 3.39$, $P = .012$) and TNF-α ($F(4, 110) = 2.79$, $P = .030$) models.

A trend for peak effect at PRRs of 20 Hz and 40 Hz was noted **(Figure 2)**. Maximal reduction the in mean concentrations of IP-10 and MIP-2 was observed at PRR of 20 Hz, whereas maximal reduction in the mean concentrations of MIP-1α, MCP-1, IL-6, and IL-10 occurred at 40 Hz. It is important to note that these observations include some concentration differences that did not reach statistical significance.

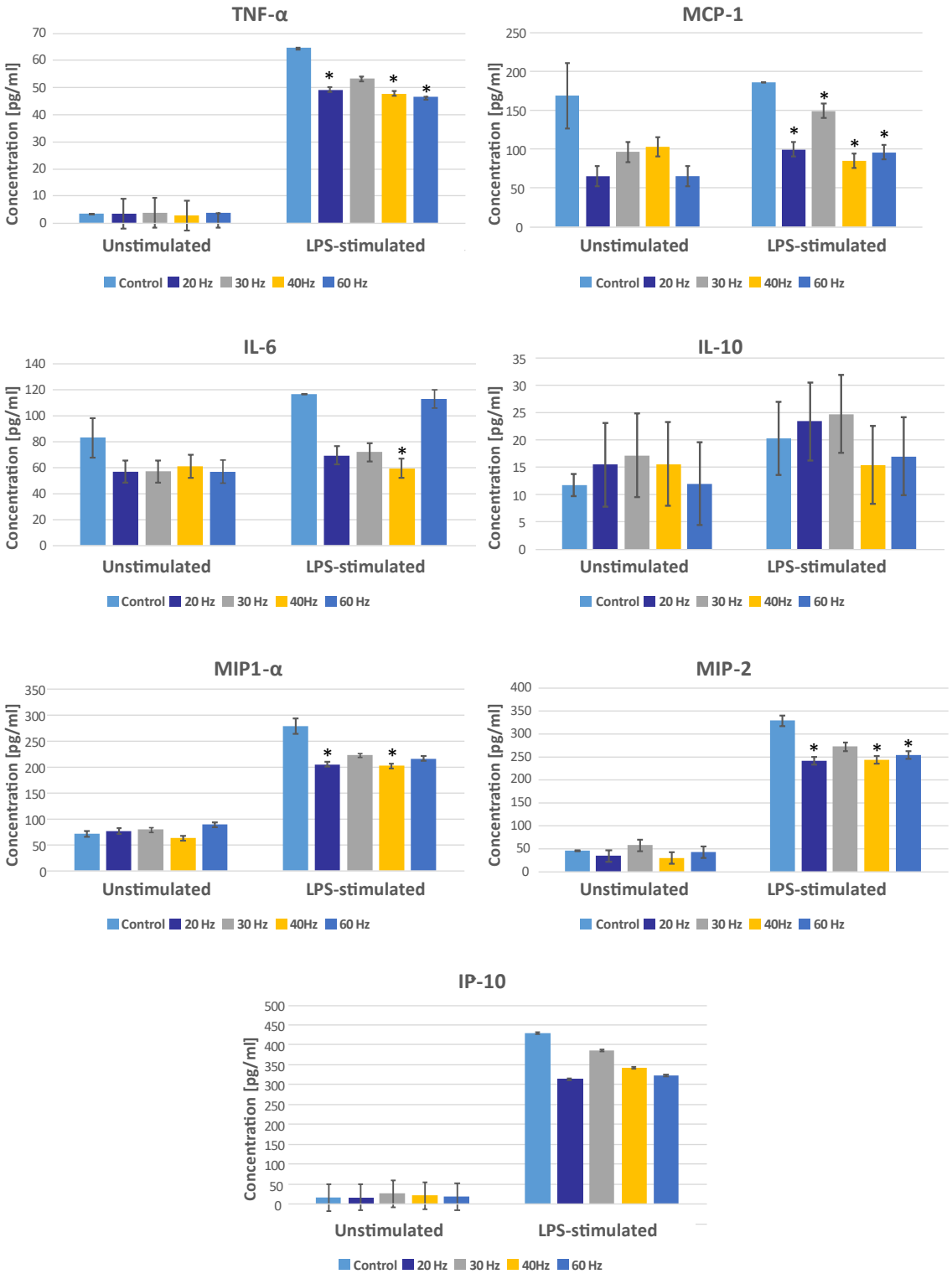

Figure 2. Cytokine secretion in response to pulsed neodymium-doped yttrium aluminum garnet laser irradiation at various pulse repetition rates in lipopolysaccharide-stimulated and unstimulated peripheral blood mononuclear cell cultures. Statistically significant decreases in concentration compared with controls (no laser irradiation) are identified by asterisks.

All cytokines evaluated except IL-10 exhibited a trend for concentration reduction in laser-irradiated cultures compared with controls, with MIP1-α, TNF-α, MCP-1, MIP-2, and IL-6 each exhibiting a statistically significant decrease in concentration at one or more PRRs. Bonferroni post hoc tests identified the specific PRRs that resulted in statistically significant concentration differences compared with controls **(Table 4)**. No statistically significant differences were noted between laser-irradiated cultures. Thus, PRR had no detectable influence on any cytokine concentration in this

investigation.

Table 3. Results of two-way factorial ANOVA for each cytokine evaluated

MIP-1α					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	686106.37[a]	9	76234.04	40.93	<.001
Intercept	2534268.15	1	2534268.15	1360.71	<.001
LPS	623689.25	1	623689.25	334.87	<.001
PRR	23326.62	4	5831.66	3.13	.018
LPS * PRR	25245.42	4	6311.36	3.39	.012
Error	204870.87	110	1862.46		
Total	4306291.87	120			
Corrected Total	890977.24	119			
a. R Squared = .770 (Adjusted R Squared = .751)					
TNF-α					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	69762.81[a]	9	7751.42	73.27	<.001
Intercept	85882.54	1	85882.54	811.81	<.001
LPS	66559.77	1	66559.77	629.16	<.001
PRR	1225.05	4	306.26	2.90	.025
LPS * PRR	1180.24	4	295.06	2.79	.030
Error	11637.04	110	105.79		
Total	217768.59	120			
Corrected Total	81399.85	119			
a. R Squared = .857 (Adjusted R Squared = .845)					
MCP-1					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	188278.65[a]	9	20919.850	6.02	<.001
Intercept	1385150.30	1	1385150.297	398.78	<.001
LPS	15047.14	1	15047.135	4.33	.040
PRR	146017.24	4	36504.311	10.51	<.001
LPS * PRR	15607.98	4	3901.995	1.12	.349
Error	382083.34	110	3473.485		
Total	2125855.83	120			
Corrected Total	570361.99	119			
a. R Squared = .330 (Adjusted R Squared = .275)					
MIP-2					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1516999.412[a]	9	168555.490	54.953	<.001
Intercept	2696576.963	1	2696576.963	879.152	<.001
LPS	1434227.702	1	1434227.702	467.594	<.001
PRR	41048.325	4	10262.081	3.346	.013
LPS * PRR	23176.701	4	5794.175	1.889	.117
Error	337397.158	110	3067.247		
Total	5875212.463	120			
Corrected Total	1854396.570	119			
a. R Squared = .818 (Adjusted R Squared = .803)					
IP-10					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3365757.27[a]	9	373973.03	57.01	<.001
Intercept	3998789.17	1	3998789.17	609.62	<.001
LPS	3228033.05	1	3228033.05	492.11	<.001
PRR	52799.34	4	13199.84	2.01	.098
LPS * PRR	50828.43	4	12707.11	1.94	.109
Error	721549.12	110	6559.54		
Total	10484044.58	120			
Corrected Total	4087306.39	119			
a. R Squared = .823 (Adjusted R Squared = .809)					
IL-6					

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	62224.02[a]	9	6913.78	2.88	.004
Intercept	616962.20	1	616962.20	256.66	<.001
LPS	14936.23	1	14936.23	6.21	.014
PRR	26526.82	4	6631.71	2.759	.031
LPS * PRR	11256.24	4	2814.06	1.171	.328
Error	264422.37	110	2403.84		
Total	1036932.62	120			
Corrected Total	326646.39	119			
a. R Squared = .190 (Adjusted R Squared = .124)					
IL-10					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2089.38[a]	9	232.15	1.67	.105
Intercept	33253.80	1	33253.80	238.80	<.001
LPS	935.60	1	935.60	6.72	.011
PRR	682.54	4	170.64	1.23	.304
LPS * PRR	285.59	4	71.40	.51	.726
Error	15317.97	110	139.25		
Total	55915.29	120			
Corrected Total	17407.35	119			

Table 4. Bonferroni post hoc tests

MIP-1α					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	20 Hz	44.25*	12.46	.006	8.56	79.94
	40 Hz	51.35*	12.46	<.001	15.66	87.03
TNF-α					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	20 Hz	9.46*	2.97	.019	.96	17.97
	40 Hz	10.77*	2.97	.004	2.26	19.28
	60 Hz	11.14*	2.97	.003	2.63	19.65
MCP-1					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	20 Hz	92.10*	17.01	<.001	43.36	140.84
	30 Hz	49.64*	17.01	.043	.90	98.38
	40 Hz	87.47*	17.01	<.001	38.73	136.21
	60 Hz	94.45*	17.01	<.001	45.71	143.19
MIP-2					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	20 Hz	58.96*	15.99	.004	13.16	104.76
	40 Hz	59.30*	15.99	.003	13.55	105.15
	60 Hz	48.07*	15.99	.033	2.27	93.87
IL-6					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	40 Hz	43.76*	14.15	.025	3.21	84.30

Based on observed means.

*Mean difference is significant at the .05 level.

Discussion

Despite clinical advantages associated with laser periodontal therapy (LPT), the therapeutic use of pulsed Nd:YAG laser energy in the treatment of periodontitis remains a controversial topic within the field of periodontology **[29-33]**. Currently available evidence supports only modest clinical benefit beyond scaling and root planing (SRP) alone when Nd:YAG lasers are used adjunctively with SRP **[33]**. High heterogeneity among studies and lack of controlled clinical research has hampered the ability of clinicians to draw conclusions regarding LPT efficacy and predictability **[32,33]**. It has been suggested that PBM-induced modulation of inflammation is a unique benefit of LPT among available periodontitis treatments **[34]**. However, it will be necessary

to define specific laser energy parameters (pulse duration, PRR, average power, spot size, fluence, cumulative energy delivered) that produce immune modulation in a predictable and governable manner. Toward that objective, this study evaluated the impact of Nd:YAG laser PRR variation on the secretion of cytokines in unstimulated and LPS-stimulated rat PBMC cultures.

Prior studies have consistently reported statistically significant reductions in various inflammatory markers in response to PBM using devices that emit in the red and infrared portions of the electromagnetic spectrum **[13,20-28]**. Observations in the present study are consistent with these prior investigations. Only two evaluated cytokines did not exhibit a statistically significant change in concentration in any laser-exposed group—IP-10 and the potent anti-inflammatory cytokine IL-10. No previous investigation using a near infrared laser has directly assessed the influence of PRR on cytokine secretion. Under the described conditions, PRR exerted no statistically significant influence on levels of the evaluated mediators of inflammation.

The high number of technical parameters with potential to influence outcome measures represents a major challenge in conducting and interpreting research into the biological effects of lasers. It has been suggested that fluence (also called energy density) may be the parameter most appropriate for defining the "dose" applied **[13]**. For multiple outcome measures, biphasic dose responses have been reported. Over a range of PBM exposure levels, a response maximum is reached at some value. When the exposure is increased beyond that threshold, the observed positive response diminishes or vanishes. At even higher fluence values, the investigator may find a negative or inhibitory result **[13]**. It is likely that the results recorded in the present study were highly dependent upon the specific irradiation parameters applied and the target cell type. Although PRR had no apparent effect on cytokine concentrations in the present study, repetition rate could influence cytokine secretion when lower or higher fluence values are applied.

LPS, which is a constituent of gram-negative bacterial cell walls, was used to stimulate PBMC cell cultures in this in vitro study. The bacterial species that are frequently isolated together at sites exhibiting bone and attachment loss—*Porphyrmonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*—are facultative gram-negative anaerobes **[3-5]**. Thus, the LPS used in this study represented an attempt to mimic a stimulant that mononuclear cells encounter at periodontitis-affected sites. However, the model used in this study in no way replicates the complexity of the in vivo microenvironment. At periodontitis sites receiving LPT, laser energy interacts with a diverse set of bacterial species within the biofilm, host cells of the innate and adaptive immune systems, and numerous cell types within the sulcular epithelium, gingival connective tissue, alveolar bone, and periodontal ligament. Thus, the complex cascade of gene expression and intercellular signaling likely induced by LPT will be challenging to fully characterize **[35-41]**.

Conclusions

Under the described conditions, statistically significant differences in cytokine secretion were observed between laser-exposed and control cultures. However, findings of the present study do not support a correlation between PRR and cytokine concentrations. These observations may be highly specific for the target cell type utilized and the radiation parameters applied.

Author contributions: All authors have contributed substantially to conceptualization of this investigation, drafting the article, critical review, and editing. All authors have approved the final version of the manuscript.

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