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Comparative *in Vitro* Study among the Effects of Different Laser and LED Irradiation Protocols and Conventional Chlorhexidine Treatment for Deactivation of Bacterial Lipopolysaccharide Adherent to Titanium Surface

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Abstract

Objective and background: The present *in vitro* study was designed to evaluate and compare the efficacy of: 1) different dental laser devices used in photoablative (PA) mode, namely commercial CO₂, Er:YAG, and Nd:YAG lasers and a prototype diode laser (wavelength = 810 nm); 2) prototype low-energy laser diode or light-emitting diode (LED) (wavelength = 630 nm), used in photodynamic (PD) mode together with the photoactivated agent methylene blue; and 3) chlorhexidine, used as reference drug, to reduce the activation of macrophages by lipopolysaccharide (LPS), a major pro-inflammatory gram-negative bacterial endotoxin, adherent to titanium surface. **Methods:** RAW 264–7 macrophages were cultured on titanium discs, cut from commercial dental implants and precoated with *Porphyromonas gingivalis* LPS. Before cell seeding, the discs were treated or not with the noted lasers and LED in PA and PD modes, or with chlorhexidine. The release of nitric oxide (NO), assumed to be a marker of macrophage inflammatory activation, in the conditioned medium was related to cell viability, evaluated by the MTS assay and ultrastructural analysis. **Results:** PA laser irradiation of the LPS-coated discs with Er:YAG, Nd:YAG, CO₂, and diode (810 nm) significantly reduced NO production, with a maximal inhibition achieved by Nd:YAG and diode (810 nm). Similar effects were also obtained by PD treatment with diode laser and LED (630 nm) and methylene blue. Notably, both treatments were superior to chlorhexidine in terms of efficiency/toxicity ratio. **Conclusions:** These findings suggest that laser and LED irradiation are capable of effectively reducing the inflammatory response to LPS adherent to titanium surface, a notion that may have clinical relevance.

Introduction

THE EVER-INCREASING CLINICAL EXPERIENCE with endosseous dental implants, which are regarded as the treatment of choice for prosthetic reconstruction of dentition,¹ has provided background to enable understanding of, and to counteract, the main causes of medium- or long-term failure of implant osseointegration. In fact, the inflammatory reaction surrounding an implant may initiate adverse tissue remodeling, eventually resulting in the loss of supporting bone.^{2–5} In this context, contamination of implant surfaces by periodontopathic bacteria plays a key pathogenic role,⁶ therefore posing the indication for an accurate antiseptic treatment of the implants.⁷ However, even if an appropriate

microbial control is achieved, additional factors may contribute to chronicization of periimplant inflammation, such as the bacterial endotoxin lipopolysaccharide (LPS). LPS is released from gram-negative bacteria^{8,9} and is capable of firmly adhering to the titanium (Ti) surface of the implants, where it can persist and act as a chronic inflammatory stimulus.¹⁰ Therefore, the most appropriate approach to favor osseointegration of dental implants should be capable of removing bacteria as well as their pro-inflammatory product, LPS, without damaging the metal contact surface and the cell microenvironment nearby.^{11–13} Recent studies have shown that biocompatible implant surfaces should have absent or minimal endotoxin levels and an intact Ti oxide coating and micro-roughness to allow osseointegration.^{14,15} Several chemical

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and physical methods are commonly used to decontaminate the implant surfaces, including topical citric acid application, abrasive air blast, plastic tool curettage, and ultrasonic scaling in combination with local and systemic antibiotic administration, the results of which, however, do not often meet expectations.^{16,17} Paradoxically, extra release of LPS following bacterial death may even enhance the inflammatory reaction around the implant and hamper its osseointegration.

In addition to the refinement of laser technology for biomedical purposes, the use of laser irradiation in the treatment of oral diseases has recently attracted considerable attention. Despite the fact that its advantageousness over the routine dental medicine protocols remains controversial, especially because of the lack of univocal, standardized therapeutic protocols, laser-based therapy is emerging as a promising approach to local bacterial decontamination.^{18–20} When properly used, it has been shown to possess potent bactericidal effects, selective targeting, minimal tissue damage, rapid wound healing, and – indirectly – reduced need for antibiotic and chemotherapeutic drugs.^{21–25} In particular, laser treatment has been found effective for implant surface decontamination.^{26–32} Using an *in vitro* model similar to that reported in the present study, we have previously demonstrated that Nd:YAG laser, the most used instrument for dental purposes, achieved both bacterial ablation and LPS inactivation from Ti implants.^{31,33} However, there are some issues of concern related to the use of Nd:YAG because of its high energy transfer, and to the physical properties of Ti, which absorbs laser energy and may undergo surface damage,³⁴ thus compromising the suitability of the implants for osseointegration.³⁵ In the search for novel light irradiation devices that could override the limitations of Nd:YAG laser, in the present study we used the previously assessed *in vitro* cell culture model³³ to evaluate the ability of novel laser and light-emitting diode (LED) devices to inactivate harmful *Porphyromonas gingivalis* LPS adsorbed to Ti dental implants, and preserve cell viability. Comparison was made with commercially available laser devices (CO₂, Nd:YAG, Er:YAG) and chemical antiseptics (chlorhexidine). The aim of this study was to expand the theoretical basis and provide practical indications for the use of laser and LED sources for the treatment of dental implant surfaces.

Materials and Methods

In vitro cell culture

Mouse RAW 264.7 macrophages were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco Invitrogen, Milan, Italy), 100 U/ml penicillin–streptomycin, 1% L-glutamine 200 mM, (Sigma, St. Louis, MO) and 4.5 g/l glucose, and grown in 5% CO₂ atmosphere at 37 °C. These cells were used to set up an *in vitro* model suited for the study of the effects of different treatments on LPS-induced inflammatory activation, as described.³³ In a typical experiment, macrophages were seeded on Ti discs, 5-mm diameter, 0.5–1 mm thick, cut from the terminal end of commercial dental implants (Bicon Dental implants, Boston, MA) and then cleaned and sterilized. Some of them were used with no further treatment, whereas others were coated with *P. gingivalis* LPS (50 µg/ml, InvivoGen, San Diego, CA). Some of

the LPS-coated discs were subjected to the different irradiation treatments, as described subsequently; others were treated with chlorhexidine 0.3% w/v in H₂O for 1 min. The discs were individually placed into the wells of a 96-well plate: the cut surface was placed downwards and the opposite surface was placed upwards, in contact with the seeded macrophages. Cultures were maintained for 24 h in 250 µl of DMEM, supplemented with 20% fetal bovine serum. Cells seeded into 96-well plate without any treatment were used as basal controls, whereas cells stimulated for 24 h with LPS (50 µg/ml), directly added to the culture medium, were used as positive controls. Additional wells pretreated with methylene blue (MB, Sigma) 0.02% w/v in H₂O were used as controls for the experiments with MB-pretreated Ti discs, as reported in the next section.

Irradiation protocols

The experiments were performed with different light sources—laser and LED—as reported here:

1. Nd:YAG laser (Pulse Master 600 IQ, American Dental Technologies Inc., Corpus Christi, TX)
2. Er:YAG (OpusDuo ECTM, Lumenis, Rome, Italy)
3. CO₂ laser (OpusDuo ECTM)
4. Diode laser (General Project, Montespertoli, Italy)
5. LED source (General Project)

The detailed irradiation parameters are reported in Table 1. For the Nd:YAG laser, these parameters were selected on the basis of those previously shown to completely inactivate LPS.³³ For the other commercial lasers, the irradiation parameters were based on the available information on their use on Ti dental implants in the treatment of periimplantitis. For example, pulsed mode was used for CO₂ laser, as this was shown to cause minimal heating of the metal surface,³⁶ whereas continuous wave mode was adopted for diode laser, as it was reported to offer surface preservation.^{25,37} The diode laser and the LED light sources at the noted wavelengths were prototypes specifically prepared and tuned for the present experiments by the manufacturer.

We tested two different irradiation modes:

1. The photoablative (PA) mode consists of exposure of the disc surface to the light source, which directly interacts with the adherent LPS by microheating/photochemical mechanisms. Laser irradiation was performed for 1 min in non-contact mode, i.e. with the handpiece tip at a 2-mm distance from the disc surface with an angle of 90°. To minimize heating, the laser beam was moved over the disc surface under air flow cooling. By a similar method, we have previously demonstrated that the temperature of the Ti surface remains <25.5 °C³⁸. The tip of the optic fiber (Nd:YAG, diode 810 nm) used to deliver laser beam was cut after each experiment, to prevent decrease of energy output.
2. The photodynamic (PD) mode consists of the immersion of the Ti discs in 30 µl of methylene blue (MB), 0.02 % w/v in H₂O. Upon light irradiation, MB undergoes photoactivation, i.e. excitation to its reactive triplet state, and releases the potent oxidant singlet oxygen,³⁹ which in turn chemically inactivates LPS. The light at 630-nm wavelength was selected as it yields optimal photoactivation of MB. Laser irradiation was performed

DENTAL LASER AND LED DEACTIVATE TITANIUM-ADHERENT LPS

3

TABLE 1. IRRADIATION PARAMETERS

<i>Laser/LED beam characteristics</i>						
	<i>Nd:YAG</i>	<i>Er:YAG</i>	<i>CO₂</i>	<i>Diode 810</i>	<i>Diode 630</i>	<i>LED 630</i>
Wavelength (nm)	1064	2940	10600	810	630	630
Irradiation mode	pulsed	pulsed	pulsed	continuous wave	continuous wave	continuous wave
Pulse duration (μ s)	100	400	250	-	-	-
Pulse energy (mJ)	20	15	100	0.5	-	-
Pulse frequency (Hz)	70	100	10	2000	-	-
Pulse power (W)	200	150	250	2	-	-
Beam power (W)	1.4	1.5	1	1	0.150	0.327
Fiber diameter (mm)	0.4	1.3	n.d.	0.4	0.4	11
<i>Laser/LED spot characteristics</i>						
Spot diameter/area at target (mm/mm ²)	0.85/0.57	1.3/1.3	0.3/0.07	0.85/0.57	0.85/0.57	11/95
Fluence (pulse) (J/cm ²)	3.51	2.64	7.53	0.7	-	-
Power density (W/cm ²)	246.7	264.3	75.4	1414.7	175.4	26.3
						0.344

either at a 2-mm distance, with an angle of 90°, or by immersion of the optic fiber tip into the MB solution. LED irradiation was performed at a 2-mm distance from the solution surface.

As a reference standard, the effects of PA and PD irradiation were compared with those of the chemical treatment of the Ti discs with chlorhexidine digluconate (Sigma), 0.3% in H₂O, for 1 min.

Evaluation of inflammatory activation of RAW 264.7 macrophages

The generation of nitric oxide (NO), a typical product of activated macrophages,⁴⁰ was evaluated by measurement of nitrites, stable NO end-metabolites, in the conditioned medium. RAW 264.7 cells (2×10^5) were seeded on the Ti discs, together with proper negative and positive controls.³³ Cell cultures were maintained for 24 h in 250 μ l of DMEM without phenol red, supplemented with 20% fetal bovine serum. The amounts of nitrites in the conditioned media were determined by the Griess reaction, read spectrophotometrically at a 546-nm wavelength, and calculated by comparison with a standard curve of NaNO₂, as described.⁴¹ The reported values are expressed as nmol/ml and are the mean (\pm SEM) of triplicate experiments.

MTS cell viability assay

RAW 264.7 cell viability upon the different treatments was assayed by the MTS CellTiter 96 Aqueous One Solution Assay (Promega, Milan, Italy).⁴² Aliquots of RAW 264.7 cells (2×10^5 cells) were placed in a 96-well cell culture plate in 250 μ l phenol red-free DMEM/well. Some wells contained LPS-coated Ti discs that had been pretreated for 1 min with either 0.02% MB alone or MB photoactivated by the noted light sources. Cells placed into the wells without any treatment, seeded on uncoated Ti discs or stimulated for 24 h with LPS (50 μ g/ml), directly added to the culture medium, were used as controls. After 24 h, 20 μ l MTS solution was added to each well and the plates were incubated at 37°C for 3 h. The absorbance of the product formazan, which is considered to

be directly proportional to the number of living cells in the culture, was measured at 490-nm and 650-nm wavelength using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Transmission electron microscopy

RAW 264.7 cells were examined to highlight the ultrastructural aspects of cell toxicity related to the treatments. The cells (2×10^5) were grown for 24 h in the presence of LPS-coated Ti discs, pretreated with either 0.3% chlorhexidine or 0.02% MB + laser, or LED sources at 630-nm wavelength. The discs were then removed and the cells fixed in 4% cacodylate-buffered glutaraldehyde, post-fixed in 1% phosphate-buffered osmium tetroxide, dehydrated in graded ethanol, and embedded in EPON 812 epoxy resin. Ultra-thin sections stained with uranyl acetate and lead citrate were examined under a JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV.

Statistical analysis

The values are means \pm SEM of three independent experiments, each performed in triplicate ($n = 9$). Statistical analysis of differences among the experimental groups was performed using one-way ANOVA followed by Newman-Keuls multiple comparison test. Calculations were performed using the GraphPad Prism 4.0 statistical software (GraphPad, San Diego, CA).

Results

Effects of laser irradiation in PA mode and LED irradiation on LPS-induced macrophage activation

The ability of the different treatments to reduce the pro-inflammatory effects of LPS on RAW 264.7 macrophages was evaluated by measuring the amount of nitrites, the stable end-products of NO assumed to be a key marker of macrophage activation, in the cells' conditioned medium. After 24 h, the amount of nitrites released by the macrophages increased significantly upon addition of LPS (50 μ g/ml) or seeding on LPS-coated Ti discs, as compared with those

measured in basal conditions or after seeding on uncoated Ti discs (Figure 1). No difference was found between these two latter negative control groups, therefore indicating that Ti per se did not induce macrophage activation. Of note, the LPS-induced increase in nitrites was significantly reduced when the LPS-coated discs were treated with all the tested light sources, the most effective ones being the Nd:YAG laser at both 20 mJ/70 Hz and 15 mJ/100 Hz pulse parameters, and the diode laser at 810 nm wavelength (Figure 1). Statistical analysis of differences among the different light sources is reported in Table 2. Pretreatment of the LPS-coated Ti discs with chlorhexidine also caused a marked nitrite reduction, even though this effect was conceivably related to its marked cell toxicity, as reported subsequently (Figure 1).

Effects of combined laser or LED irradiation and MB in PD mode on LPS-induced macrophage activation

As is shown in Figure 2, the LPS-induced increase in nitrite generation by RAW 264.7 cells caused by added LPS (50 μ g/ml) or LPS-coated Ti discs was slightly, albeit significantly, reduced when the discs were pretreated with non-photoactivated MB. Nd:YAG laser at both 20 mJ/70 Hz and 15 mJ/100 Hz pulse parameters had no PD effects. Conversely, both the diode laser and LED emitting at 630-nm wavelength caused a marked photoactivation of MB, resulting in a striking, significant decrease in nitrites, which appeared greater than that yielded by pretreatment of LPS-coated Ti discs with chlorhexidine. Statistical analysis of differences among the different light sources used in PD

mode is reported in Table 3. On the other hand, the nitrite reduction caused by the diode laser and the LED emitting at 630-nm wavelength in PA mode was lower than that observed after Nd:YAG laser irradiation.

We next investigated whether the PD effects of laser or LED devices + MB on LPS-induced macrophage activation was dependent upon the mode of irradiation. As is shown in Figure 3, PD activation of MB was performed by either maintaining the optical fiber tip 5 mm above the MB solution (dry tip mode) or by immersing the tip directly into the solution (wet tip mode). No statistically significant differences were observed between the two modes, regardless of the use of the diode laser or the LED sources, therefore indicating that effective MB photoactivation occurs independently of the distance between, or the type of, the irradiating device and the target.

Comparison of combined laser or LED irradiation and MB in PD mode vs. chlorhexidine on macrophage viability and ultrastructural morphology

The results of the MTS assay (Figure 4A) showed that the viability of RAW 264.7 cells was slightly impaired when the cells were grown for 24 h in the presence of Ti discs, either uncoated or LPS-coated, whereas LPS per se had no effect. MB alone also slightly reduced the number of viable cells, as did MB-pretreated LPS-coated discs. PD activation of MB with 630-nm-wavelength diode laser or LED had no adverse effects on cell viability, which remained similar to that measured in the presence of non-photoactivated MB. Conversely, pretreatment of LPS-coated discs with 0.3% chlorhexidine caused a dramatic impairment of RAW 264.7 cell viability, despite the fact that the discs had been thoroughly rinsed before being placed in the culture wells. These findings indicated that the ostensible reduction of NO generation by chlorhexidine pretreatment of the LPS-coated Ti discs observed in the previous experiments could be, in fact, attributable to its cytotoxic action and, hence, to massive cell death. To confirm this assumption, normalization of the values of the actual production of NO (as nitrite levels) by the number of viable cells (Figure 4B) showed that the LPS-induced increase in nitrite generation by RAW 264.7 cells stimulated with either added LPS (50 μ g/ml), or LPS-coated Ti discs, was markedly and significantly blunted by MB photoactivated with 630-nm-wavelength diode laser or LED. Conversely, chlorhexidine pretreatment had no significant effects.

By transmission electron microscopy, the RAW 264.7 cells grown for 24 h in the presence of LPS-coated discs pretreated with 0.3% chlorhexidine (Figure 5A) showed well-recognizable signs of cell injury, such as swollen mitochondria, cytoplasmic vacuolization, and nuclear chromatin condensation typical of apoptosis. Conversely, the RAW 264.7 cells grown in the presence of LPS-coated discs pretreated with MB photoactivated with the 630-nm-wavelength laser or LED (Figure 5B) usually showed a normal cytoplasm, organellular complement and nuclear chromatin pattern, the only detectable abnormality being a moderate degree of mitochondrial swelling.

Discussion

There is still controversy in the literature about the usefulness of lasers for the treatment of chronic periodontal and

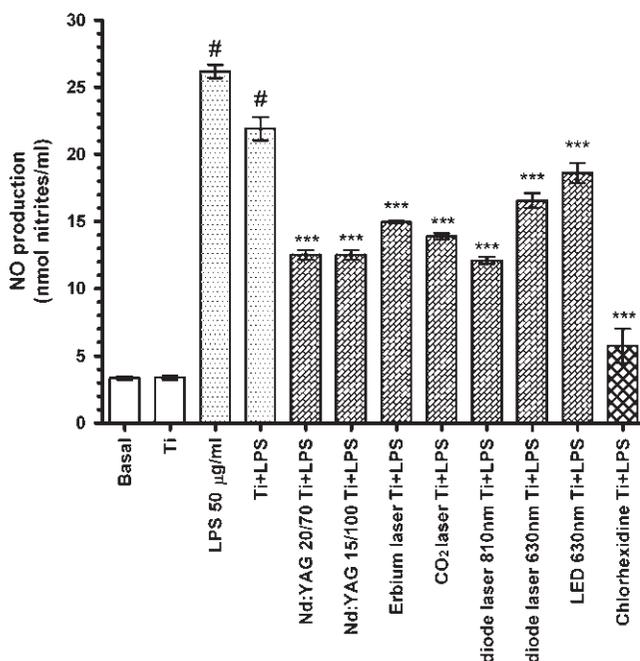


FIG. 1. NO generation by RAW 264.7 macrophages, evaluated by measuring nitrites in the conditioned medium, after 24 h of culture in the different experimental conditions. Lasers were used in PA mode. Significance of differences (one-way ANOVA and Newman-Keuls multiple comparison test; $(n=9)$ #, $p < 0.001$ vs. basal and uncoated Ti discs; *** $p < 0.001$ vs. Ti + LPS.

DENTAL LASER AND LED DEACTIVATE TITANIUM-ADHERENT LPS

TABLE 2. STATISTICAL SIGNIFICANCE OF DIFFERENCES IN NITRITE AMOUNTS AMONG THE LASER AND LED DEVICES USED IN PA MODE

	Nd:YAG 20mJ/70Hz	Nd:YAG 15mJ/100Hz	Er:YAG	CO ₂	Diode 810	Diode 630	LED 630
Nd:YAG 20mJ/70 Hz	-	n.s.	n.s.	n.s.	n.s.	n.s.	<i>p</i> < 0.001
Nd:YAG 20mJ/70 Hz		-	n.s.	n.s.	n.s.	n.s.	<i>p</i> < 0.001
Er:YAG			-	n.s.	n.s.	n.s.	<i>p</i> < 0.05
CO ₂				-	n.s.	n.s.	<i>p</i> < 0.01
Diode 810					-	n.s.	<i>p</i> < 0.001
Diode 630						-	n.s.
LED 630							-

n.s., not significant.
p > 0.05.

periimplant inflammation. Reasons for debate are several and mainly rely on non-univocal standard protocols, such as different laser wavelengths and laser setting parameters, which not allow calculation of energy density.¹⁸⁻²⁰ Despite these limitations, increasing basic and clinical evidence suggests that dental laser treatments can achieve successful antiseptics of the implant surface and periimplant tissues and neutralization of harmful bacterial products, such as LPS.^{7,11,20-32,36,37} In fact, previous studies have shown that Er:YAG is capable of detaching LPS from the dental root⁴³ and CO₂ laser, to cause LPS fragmentation.³⁰ Our recent findings have also demonstrated that Nd:YAG can effectively reduce the pro-inflammatory potential of Ti-adherent LPS.³³ In such a view, the results of the present study provide further evidence for the suitability of laser treatment for the detoxification of Ti dental implants. We have found that,

when used in PA mode, all the tested laser devices were capable of satisfactorily de-activating *P. gingivalis* LPS adherent to Ti discs cut from implant screws, as judged by the significant reduction of its pro-inflammatory activity *in vitro*. Of note, the diode laser emitting at 810-nm wavelength yielded similar effects as did the Nd:YAG laser, which is currently the most used commercial instrument for dental care purposes. On the other hand, the 630-nm-wavelength diode laser and LED prototypes were also capable of significantly de-activating LPS when used in PA mode, although with lower efficiency than the other laser instruments.

It is known that the PA mode implies a high energy transfer to the irradiated surface, which may cause physicochemical changes of the outer Ti-oxide layer and the micro-ridge framework of the Ti surface,³⁴ designed to optimize the bone-integrating properties of implants.²⁵ To override these limitations, we have performed a comparison between PA and PD modes of irradiation. In fact, PD activation of photosensitive molecules, such as MB, does not require the light to be focused on the Ti surface, thus minimizing the energy transfer required to obtain an effective detoxification. Our results showed that both the 630-nm-wavelength diode laser and LED prototypes, used in combination with MB, were highly efficient de-activators of *P. gingivalis* LPS, as they caused a marked reduction of LPS-induced macrophage activation, which was even more prominent than any of the PA treatments assayed. By contrast, the commercial Nd:YAG laser was substantially ineffective when used in PD mode. These findings highlight the potential usefulness of combined photoactivated agents and low-energy light irradiation with

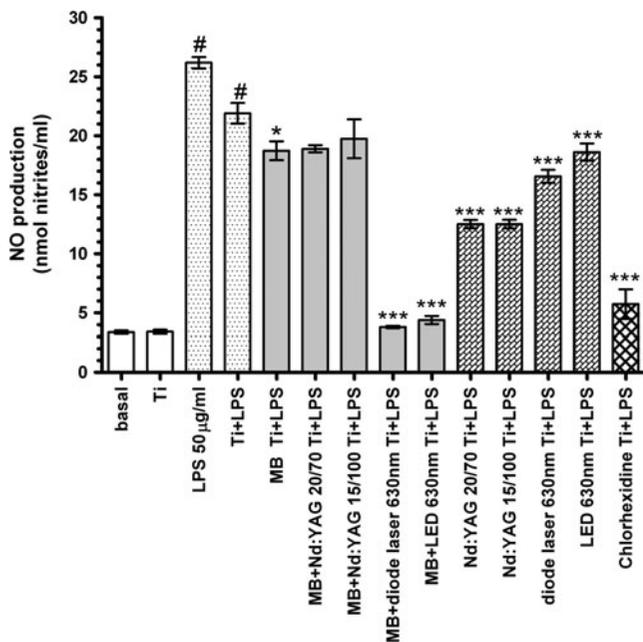


FIG. 2. NO generation by RAW 264.7 macrophages, evaluated by measuring nitrites in the conditioned medium, after 24 h of culture in the different experimental conditions. Grey columns: PD mode; scaled columns, PA mode. Significance of differences (one-way ANOVA and Newman-Keuls multiple comparison test; (n=9) #, *p* < 0.001 vs. basal and uncoated Ti discs; **p* < 0.05; and ****p* < 0.001 vs. Ti + LPS.

TABLE 3. STATISTICAL SIGNIFICANCE OF DIFFERENCES IN NITRITE AMOUNTS AMONG THE LASER AND LED DEVICES USED IN PD MODE IN COMBINATION WITH MB

	Nd:YAG 20mJ/70Hz	Nd:YAG 15mJ/100Hz	Diode 630	LED 630
Nd:YAG 20mJ/70Hz	-	n.s.	<i>p</i> < 0.001	<i>p</i> < 0.001
Nd:YAG 15mJ/100Hz		-	<i>p</i> < 0.001	<i>p</i> < 0.001
Diode 630			-	n.s.
LED 630				-

n.s., not significant.
p > 0.05.

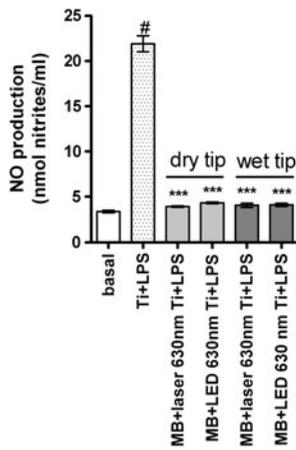


FIG 3. NO generation by RAW 264.7 macrophages, evaluated by measuring nitrites in the conditioned medium, after 24 h of culture in the different experimental conditions. Laser and LED were used in PD mode. Significance of differences (one-way ANOVA and Newman-Keuls multiple comparison test; $(n = 9)$ #, $p < 0.001$ vs. basal and uncoated Ti discs; *** $p < 0.001$ vs. Ti + LPS.

appropriate wavelength for the treatment of implant surface. In this context, the observation that the efficacy of the 630-nm-wavelength diode laser and LED prototypes as PD activators of MB is not dependent upon the distance between the optic fiber tip and the target, further accounts for the simple and versatile use of such methods in dental practice. For example, this property may allow for MB to penetrate and undergo effective photoactivation deeply in the periimplant zone. In a practical perspective, it is likely that PD disinfection/detoxification may be easily used at sites where bacteria and their by-products cannot be removed by mechanical instruments, because of complex implant surface geometry. Of note, recent clinical studies have shown that MB and low-energy laser irradiation in periodontitis and periimplantitis is markedly effective in reducing the clinical signs of periodontitis, such as blushing and bleeding on probing, and the local levels of pro-inflammatory cytokines.⁴⁴⁻⁴⁶

Conclusions

The present findings provide experimental background to the concept that low-energy PD irradiation can be more effective than the conventional chlorhexidine treatment, which is currently viewed as the treatment of choice for implant surface antisepsis.⁴⁷⁻⁴⁹ In fact, both the 630-nm-wavelength diode laser and LED prototypes used in PD mode caused only a modest decrease of RAW 264.7 cell viability. This is at variance with chlorhexidine (0.3%), which rather caused severe cell injury and massive death. By electron microscopic examination, the RAW 264.7 cells exposed to chlorhexidine showed cytoplasmic and nuclear signs of severe injury and apoptosis, which are consistent with the known toxic effects of chlorhexidine, mainly dependent upon increased oxidative stress, intracellular Ca^{2+} imbalance, and mitochondrial dysfunction.⁵⁰ Of note, when referring the actual NO production by activated RAW 264.7 macrophages to the amount of viable cells, the pretreatment with photoactivated MB proved to be the most potent in reducing the pro-inflammatory effects of Ti-adherent LPS, an effect achieved in the absence of substantial cytotoxic effects, which could impair the viability of periimplant cells involved in osseointegration.

The present study confirms and extends the notion that low-energy laser irradiation, for example by CO₂ and diode laser devices, is a suitable therapeutic approach to periim-

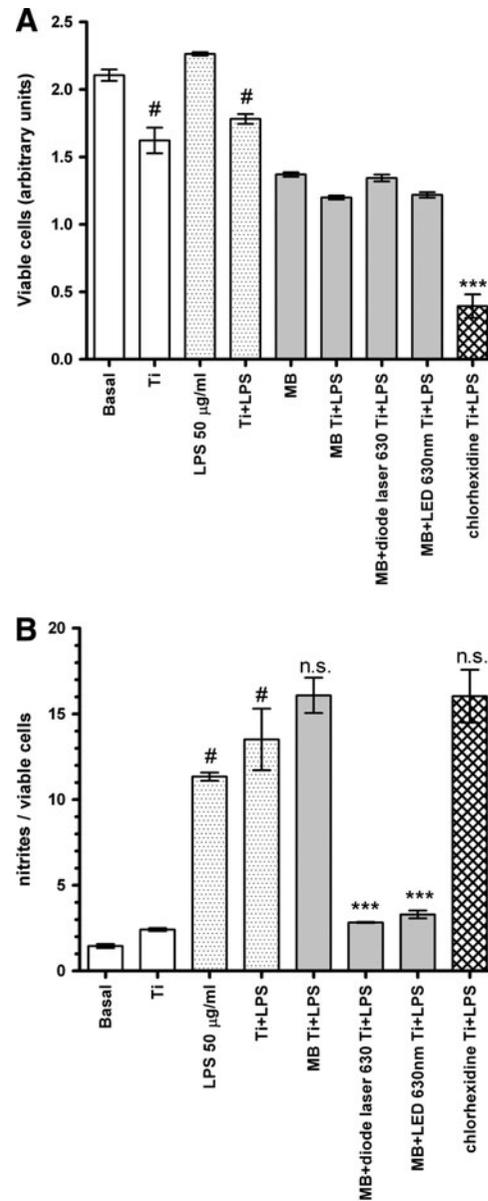


FIG. 4. **A.** Viability of RAW 264.7 macrophages, evaluated by the MTS assay after 24h of culture in the noted experimental conditions. **B.** NO generation by RAW 264.7 macrophages after 24h of culture in the noted experimental conditions, normalized by the number of viable cells. Significance of differences (one-way ANOVA and Newman-Keuls multiple comparison test; $(n = 9)$ #, $p < 0.001$ vs. basal; *** $p < 0.001$ vs. the other groups; *** $p < 0.001$ vs. Ti + LPS; n.s., not significant vs. Ti + LPS.

plantitis, as it can yield optimal decontamination and detoxification of the metal surface in the absence of substantial heat-induced alterations, which may then hamper osseointegration.^{23-27,30-33,51} Compared with the commercially available Nd:YAG, Er:YAG, and CO₂ lasers, the diode laser and LED prototypes studied here possess the advantage of a simpler mechanical and optical structure, allowing for a diode laser and an LED to be easily combined into a single instrument suitable for both PA and PD applications. This could favor the diffusion of such instruments among dental

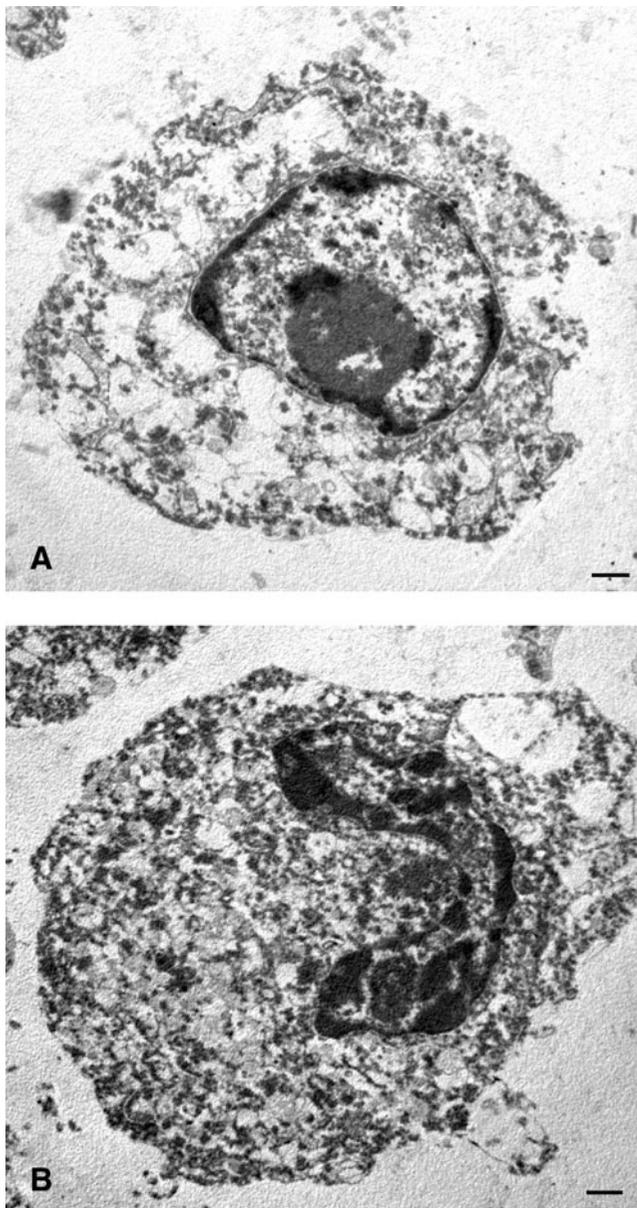


FIG. 5. Representative ultrastructural features of RAW 264.7 macrophages after 24 h of culture on LPS-coated Ti discs pretreated with: **A)** 0.3% chlorhexidine; and **B)** 0.02% MB photoactivated with 630-nm-wavelength LED. Bar = 1 μ m.

practitioners to expand their therapeutic repertoire. Obviously, additional data from experimental studies with clinically relevant animal models, as well as from clinical trials, are required to validate this assumption.

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Author Disclosure Statement

No conflicting financial interests exist.

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