Original Research
Infection Control

A single session of antimicrobial photodynamic therapy does not influence the alveolar repair process in rats

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Abstract: The aim of this study was to use microscopic and molecular techniques to evaluate the effects of a single session of antimicrobial photodynamic therapy (aPDT) on the alveolar repair process after tooth extraction in rats. The study sample included 84 rats divided into four groups, as follows: a) Control - untreated socket; b) Laser - socket treated using photobiomodulation; c) TBO - socket treated with topic application of the photosensitizer agent, toluidine blue O (TBO); and d) aPDT - socket treated with TBO and laser irradiation. An additional rat was used for thermal mapping during socket irradiation. The animals were euthanatized at 6, 15, and 28 days after unilateral extraction of the upper incisor. Quantitative and qualitative analyses of the connective and bone tissues, blood clot, blood vessel, and inflammatory infiltrate were performed, and real-time polymerase chain reaction was used to study the expression of genes (collagen type I, osteocalcin, alkaline phosphatase [ALP], runt-related transcription factor 2 [RUNX2], and vascular endothelial growth factor [VEGF]) involved in the bone healing process. No statistically significant differences in microscopic and molecular outcomes were observed between the groups ($p > 0.05$). A positive correlation was seen to exist between blood clot and VEGF ($p = 0.000$), and a negative correlation was observed between bone tissue and ALP ($p = 0.028$) and blood vessel and VEGF ($p = 0.018$). A single session of aPDT in the dental extraction site did not influence the alveolar repair process in rats.

Keywords: Photochemotherapy; Tooth Socket; Rats.

Introduction

Antibiotic resistance is an urgent public health problem[1] that has led the global scientific community to focus on antimicrobial photodynamic therapy (aPDT) as an effective, minimally invasive antimicrobial strategy that decreases the risk of resistance.[2,3]

A wide range of microorganisms, including gram-positive and gram-negative bacteria, viruses, protozoa, and fungi, have exhibited susceptibility to aPDT,[4,5,6] defined as a treatment protocol wherein a combination of visible light and a sensitizing drug causes selective

destruction of microbial cells through generation of reactive oxygen species, such as singlet oxygen.[7] Currently, aPDT is being used in the fields of periodontics,[8,9,10] endodontics,[11,12,13] and oral surgery,[14-19] with two recent clinical studies demonstrating improvement in postoperative wound healing, pain intensity, swelling, halitosis, and temperature following a single session of aPDT after third molar extraction.[16,17] Previous clinical evidence has also found that multiple sessions of aPDT can contribute to the prevention and treatment of osteonecrosis,[18] whereas another study examining rats treated with zoledronate showed that multiple sessions of aPDT improved the alveolar repair process and prevented osteonecrosis.[19]

Despite its clear impact on the viability of microorganisms, there is limited evidence on the outcomes of aPDT therapy in host cells and tissues. Moreover, to the best of our knowledge, there are no studies that evaluate the molecular effects of a single session of aPDT on the alveolar repair process after tooth extraction.

Therefore, the objective of the current study was to use microscopic and molecular techniques to evaluate the effects of a single session of aPDT on alveolar repair after tooth extraction in rats. The hypothesis being tested was that a single session of aPDT would influence the evolution of the alveolar repair process through direct interference with the cells involved in bone healing.

Methodology

Animals

The study sample, consisting of 85 male rats *(Rattus norvegicus, albinus, Wistar)* that were 60 days old, was randomly divided into four experimental groups (Control, Laser, TBO, and aPDT; n = 21 each), and one animal was reserved for use in thermal mapping. Feeding was done using standard rodent food blocks, and water was provided *ad libitum* except for the first 24 hours postoperation when the blocks were triturated. The animals were kept at an average enviromental temperature of 22°C, and artificial ilumination ranging from 5 to 60 LUX was provided in 12 hour cycles. Intramuscular anesthesia was used to sacrifice the animals 6, 15, and 28 days after the experimental surgery, with 28 animals (7 per group) being sacrificed at each stage. Thereafter, four and three animals were randomly selected for microscopic and molecular analysis, respectively. This study received ethical approval from the Ethics Commission of Teaching and Research in animals, University of São Paulo, Bauru, Brazil (CEEPA, process number: 17).

Tooth extraction

The animals were anesthized using an intramuscular combination of ketamine chloridrate (Dopalen, Vetbrands, São Paulo, Brazil, 25 mg/kg) and xilazine chloridate (Anasedan, Vetbrands, São Paulo, Brazil 10 mg/kg).[14] Following antisepsis of the surgical area using chlorhexidine gluconate 0.12% (PerioGard, Colgate - Palmolive, Osasco, Brazil), the upper right incisor of each animal was removed using appropriate tools adapted for the procedure.[15] Hemostasis was achieved using a tapered paper cone (Sybron-Kerr, second series, Orange, USA) containing adrenaline at a concentration of 1:1000 (Ariston Indústria Química Farmacêutica Ltda, São Paulo, SP, Brazil) for 1 minute or until clinical confirmation of the absence of bleeding.[14] Upon removal of the cones, the animals were observed for 60–90 seconds in order to confirm absence of blood clots, and the tooth sockets were treated according to the protocol relevant to their specific experimental groups thereafter.

Experimental groups

Control group

After tooth extraction, the socket was irrigated with 1 ml of saline solution, and a dry gauze was placed in the mouth of the animal to prevent aspiration of the solution. Thereafter, bleeding was stimulated through curettage of the socket, and clot formation was achieved by means of digital compression of the socket using a saline-soaked gauze in order to simulate conditions under which normal repair can take place.

Laser group

After tooth extraction, the socket was filled with saline solution using a disposable syringe and

photobiomodulation (PBM) was carried out after 10 minutes. This study used a previously calibrated Gallium–Aluminum–Arsenide (GaAlAs; Twin Laser, MMOptics, São Carlos, Brazil) light source that operates at an optical potency of 40 mW, an exit beam area of 0.04cm^2, maximum irradiance of 1000 mW/cm^2, and has a wavelength belonging to the visible red spectrum (660 nm) due to the absorption of toluidine blue O (TBO; centered at 626 nm). The energy density was calculated using the following formula:

$$ED = (P \times T)/A$$

Where ED = energy density (J/cm^2); P = power (W); T = time (s); and A = area (cm^2).

The laser source was used in punctual mode at maximum potency, resulting in a total density of 50 J/cm^2 and an irradiation time of 600 seconds, and the probe was placed in the center of the socket at 90° in order to allow uniform distribution of energy and minimize energy attenuation through the tissues. Thereafter, the socket was irrigated using 1 ml of saline solution, and the same procedures used in the control group were carried out.

TBO group

Following tooth extraction, the socket was filled with a photosensitizer agent, TBO (Sigma-Aldrich, São Paulo, Brazil), at a concentration of 100 µg/mL using a disposable syringe. Thereafter, the socket was irrigated with 1 ml of saline solution after 10 minutes, and the same procedures used in the control group were carried out.

aPDT group

Following tooth extraction, the socket was filled with TBO at a concentration of 100 µg/mL and a laser was applied after 10 minutes. The light source and other parameters employed were the same as those used in the *Laser* group. This was followed up by the same procedures as those used in the control group.

Thermal mapping

Thermal mapping was performed in order to check for the presence of significant thermal variations (>10°C) in bone tissue.[20]

The main objective of this initial test was to use a thermistor to compare temperature variations inside the socket during laser irradiation in the *Laser* and aPDT groups so that the thermal effects of the laser over the irradiated site could be disregarded.

The thermistor is made up of temperature-sensistive semi-conductors that detect differences in local electric potential by means of the following formula:

$$T = 25.4258 + (2.25065 \times PD)$$

Where PD = potential difference (with the maximum value measured in °C).

A single rat was randomly selected for extraction of the upper right incisor using the protocol described above. Thereafter, the thermistor was placed in the socket at a depth of 2 mm and in contact with the vestibular alveolar wall, and the Laser and aPDT group protocols described above were implemented.

Microscopic analysis

Initially, a qualitative histological analysis was performed. This was followed by a histomorphometric analysis, which was performed as described previously.[14,21]

Briefly, the maxilla was separated from the mandible, and the right portion of the maxilla was recovered for fixation in 10% buffered formalin for 7 days. Thereafter, the samples were decalcified in 4.7% ethylenediaminetetraacetic acid (pH 7.0) for 35 days, histologically processed, and embedded in paraffin wax. Semi-serial 5-µm thick longitudinal sections of the embedded samples were made and stained with hematoxylin-eosin for evaluation by light microscopy. An ocular Zeiss Kpl, 8× (Carl Zeiss MicroImaging Inc., Thornwood, USA), containing an integration reticulum Zeiss II composed of 10 parallel lines with 100 points symmetrically distributed inside the quadrangular area, was used for the quantitative analysis. Forty-five distinct microscopic areas were observed in each socket (magnification 40×), with 15

areas in each section of the socket (apical, medium, and cervical) being selected at regular intervals following randomization or systematic sampling in order to obtain a representative sample of the whole area.[22] The histological structures quantified through morphometric analysis of the alveolar bone healing process included connective and bone tissue, blood clot, blood vessels, and inflammatory infiltrate. The analysis was performed by an observer blinded to the treatment, and the density of each structure was calculated by dividing the total number of computed points by 45 (number of microscopic sites counted per socket).

Molecular analysis

The molecular analysis of the bone repair process was performed as previously described.[23] The maxilla of the three remaining animals in each group were obtained using the same procedures described above (microscopic analysis section), and the area of the right incisor socket was dissected. The samples were stored in microcentrifuge tubes containing 1-mL Trizol (Life Technologies, Rockville, USA), shaken for 30 seconds, and left at ambient temperature for 5 minutes as per the manufacturer's instructions.[24]

Extraction of total RNA from the periodontal tissues was performed using a Trizol reagent as per the manufacturer's protocol, and the complementary DNA was synthesized with 3-µg RNA using a reverse transcription reaction (Superscript III, Invitrogen Corporation, Carlsbad, USA). Real-time polymerase chain reaction (PCR) quantitative mRNA analyses were performed in MiniOpticon (BioRad, Hercules, USA) using the SybrGreen PCR MasterMix (Applied Biosystems, Warrington, UK) and 100-nM specific primers for the gene sequences of integrating factors involved in socket repair (collagen type I [COL-I], osteocalcin [OCN], alkaline phosphatase [ALP], runt-related transcription factor 2 [RUNX2], and vascular endothelial growth factor [VEGF]). The primers were designed using the Primer Express 3.0 software from Applied Biosystems (Foster City, CA, USA), synthesized by Invitrogen (Table 1), and 2.5-ng cDNA (or 5-ng DNA) was used in each reaction. The standard PCR conditions were 95°C (10 min), 40 cycles at 94°C (1 min), 56°C–65°C (1 min), and 72°C (2 min), followed by the standard denaturation curve.

Previously, real-time PCR reactions were optimized by using ideal annealing temperature and

Table 1. Prime sequences and reaction properties.

Target	Sense and anti-sense sequences	t_A (°C)	t_M (°C)	bp
COL-I	AATCACCTGCGTACAGAACGG	62	84	114
	CAGATCACGTCATCGCACAAC			
OCN	TACAAGCGCATCTATGGCACC	61	83	57
	TGTGCCGTCCATACTTTCGAG			
ALP	CGAGCAGGAACAGAAGTTTGC	61	83	57
	TGGCCAAAAGGCAGTGAATAG			
RUNX2	TTCAAGGTTGACCCTCGGA	60	81	172
	AGATCGTTGAACCTGGCCACT			
VEGF	GCCCATGAAGTGGTGAAGTT	61	81	172
	ACTCCAGGGCTTCATCATTG			
b-actin	ATTGAACACGGCATTGTCACC	60	82	150
	GGTCATCTTTTCACGGTTGGC			

t_A: annealing temperature; t_M: melting temperature; bp: base pairs of amplicon size; COL-1: collagen type I; OCN: osteocalcin; ALP: alkaline phosphatase; RUNX2: runt-related transcription factor 2; VEGF: vascular endothelial growth factor.

concentrations of each pair of primers to maximize efficiency and the specificity of amplification. The results were analyzed based on the cycle threshold (Ct) value, which corresponds to the number of cycles in which the amplification reached a certain threshold during the exponential amplification phase of PCR, thereby permitting quantitative analysis of the expression of the evaluated factor in relation to the constitutive gene (b-actin). The specificity of the reactions was confirmed by the dissociation curve t_m (melting temperature), and two samples of water (negative control) were used for each reaction.

Statistical analysis

The normality of the microscopic and molecular outcomes was tested using the Kolmogorov–Smirnov test, and variance analysis was used for analysis. The Pearson's Correlation test was used to examine possible correlations between variables in the same phases of the alveolar repair process. All statistical analyses were performed using Statistica for Windows 7.0 (Statistica for Windows 7.0 Copyright StatSoft, Inc. Tulsa, USA, - http://www.statsoft.com), and statistical significance was set at 5% ($p < 0.05$).

Results

General findings

No postoperative complications were observed, and the rats were able to resume their normal diet with no evidence of weight loss during the experimental period (data not shown). None of the rats died during experimentation, and no infections were observed at the extraction sites.

Thermal mapping

Figure 1 shows the maximum variation in the thermal mapping of the *Laser* (2.2°C) and aPDT (4.2°C) groups.

Microscopic analysis

Prior to morphometric analysis, the histological sections were qualitatively analyzed to allow comparison of the features of the dental sockets undergoing repair within the different control and experimental groups. The results showed that the connective and bone tissue, blood clot, blood vessels, and inflammatory infiltrate were at the same stage of healing in all groups during the same period of evaluation (Figure 2).

Figure 3 shows the mean and standard variation of the histomorphometric analysis. No statistically significant differences in connective tissue ($p = 0.614$), bone tissue ($p = 0.480$), blood clot ($p = 0.879$), blood vessels ($p = 0.700$), and inflammatory infiltrate ($p = 0.826$) were observed between the groups.

Molecular analysis

Comparison of the control and experimental groups showed no statistically significant differences in COL-I ($p = 0.551$), OCN ($p = 0.845$), ALP ($p = 0.648$), RUNX2 ($p = 0.486$), and VEGF ($p = 0.500$) (Figure 4).

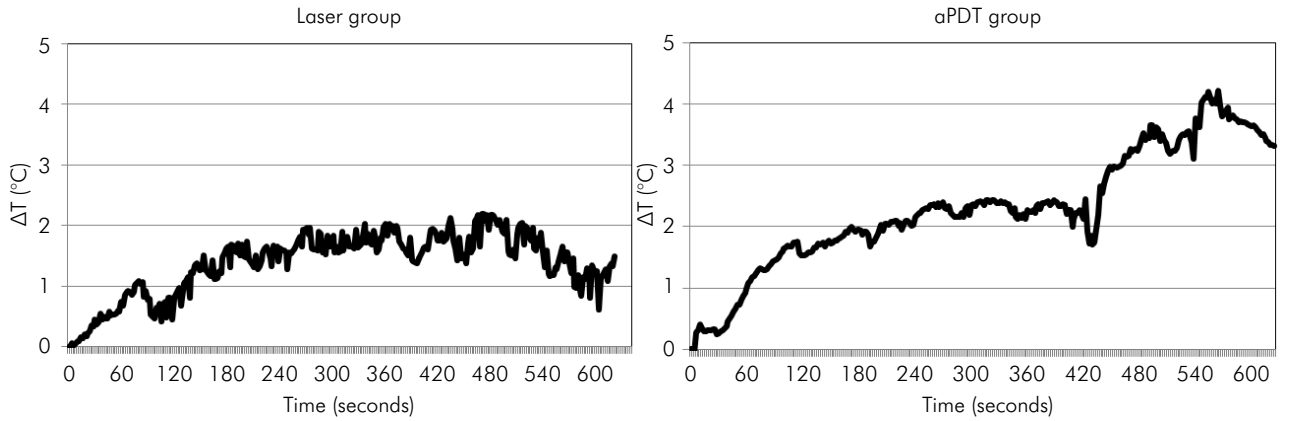
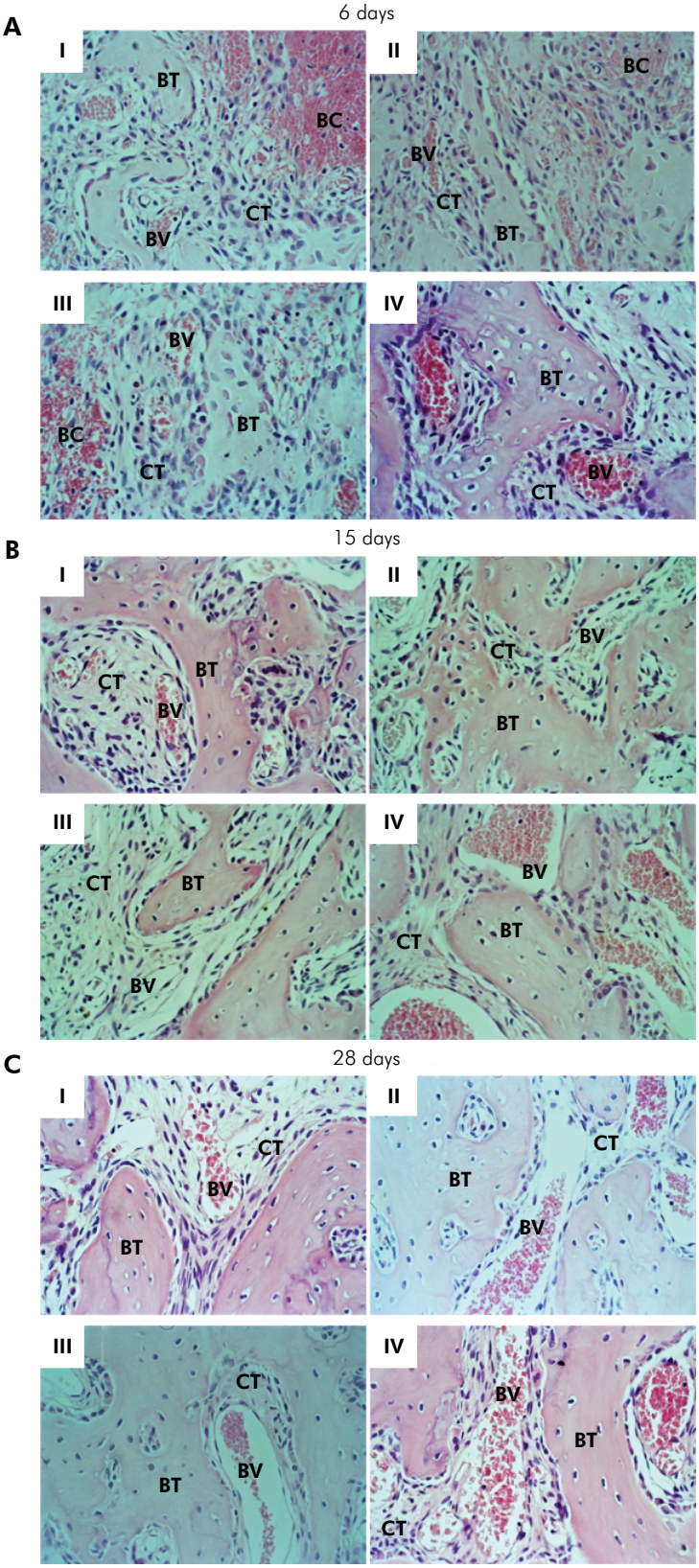


Figure 1. Thermal mapping of the Laser and antimicrobial photodynamic therapy (aPDT) groups.



BC: blood clot; BT: bone tissue; BV: blood vessel; CT: connective tissue. Original magnification 40×.

Figure 2. Histological sections for examination of alveolar bone healing after (A) 6 days, (B) 15 days, and (C) 28 days.

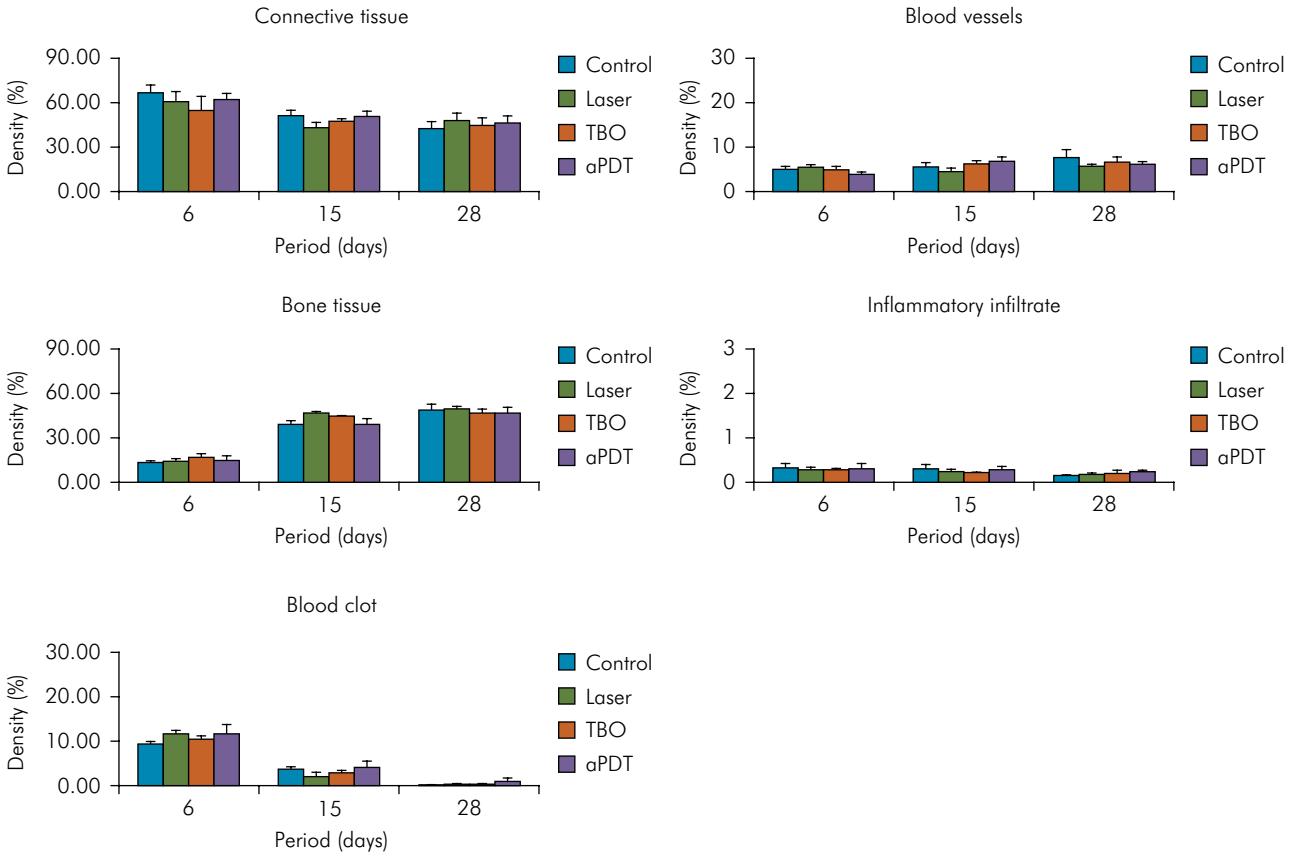


Figure 3. Mean (± Standard Deviation) histomorphometric measurement of alveolar bone healing after 6, 15, and 28 days in the Control, Laser, toluidine blue O (TBO), and antimicrobial photodynamic therapy (aPDT) groups.

A statistically significant positive correlation was observed between blood clot and VEGF, and a negative correlation was seen between bone tissues, ALP, blood vessels, and VEGF (Table 2).

Discussion

This study demonstrated that a single session of aPDT at the dental extraction site did not affect the alveolar repair process in rats. The experimental models used in this study simulated the repair process observed in humans over a short period of time (28 days) and were based on previous studies conducted by our research group.[14,21] This study employed the tooth extraction protocol used by Cardoso et al.[14] and Rodrigues et al.[21] in the control groups of their studies as it effectively prevented development of a dry socket. No postoperative complications were observed in our study.

The inclusion of the Laser and TBO groups allowed elucidation of the individual effects of PBM and TBO on the alveolar repair process and permitted comparison with the *aPDT* group. The selection, preparation, and final concentration (100 μg/mL) of the photosensitizing agent (TBO) used in the TBO and aPDT groups was based on previous evidence demonstrating its antimicrobial action, low toxicity, and availability.[10,25-28] Moreover, the agent was delivered with the help of a discharging syringe that allowed appropriate control over the amount of solution delivered into the alveolar socket, thus contributing to hemostasis and facilitating complete contact of the solution with the entire surface area of the socket. The expected time for the absorption of TBO (10 minutes) was in agreement with previous evidence.[26] In the current study, the authors aimed to determine the effects of TBO-mediated photosensitization and biodistribution of the photosensitizer in the murine buccal mucosa.

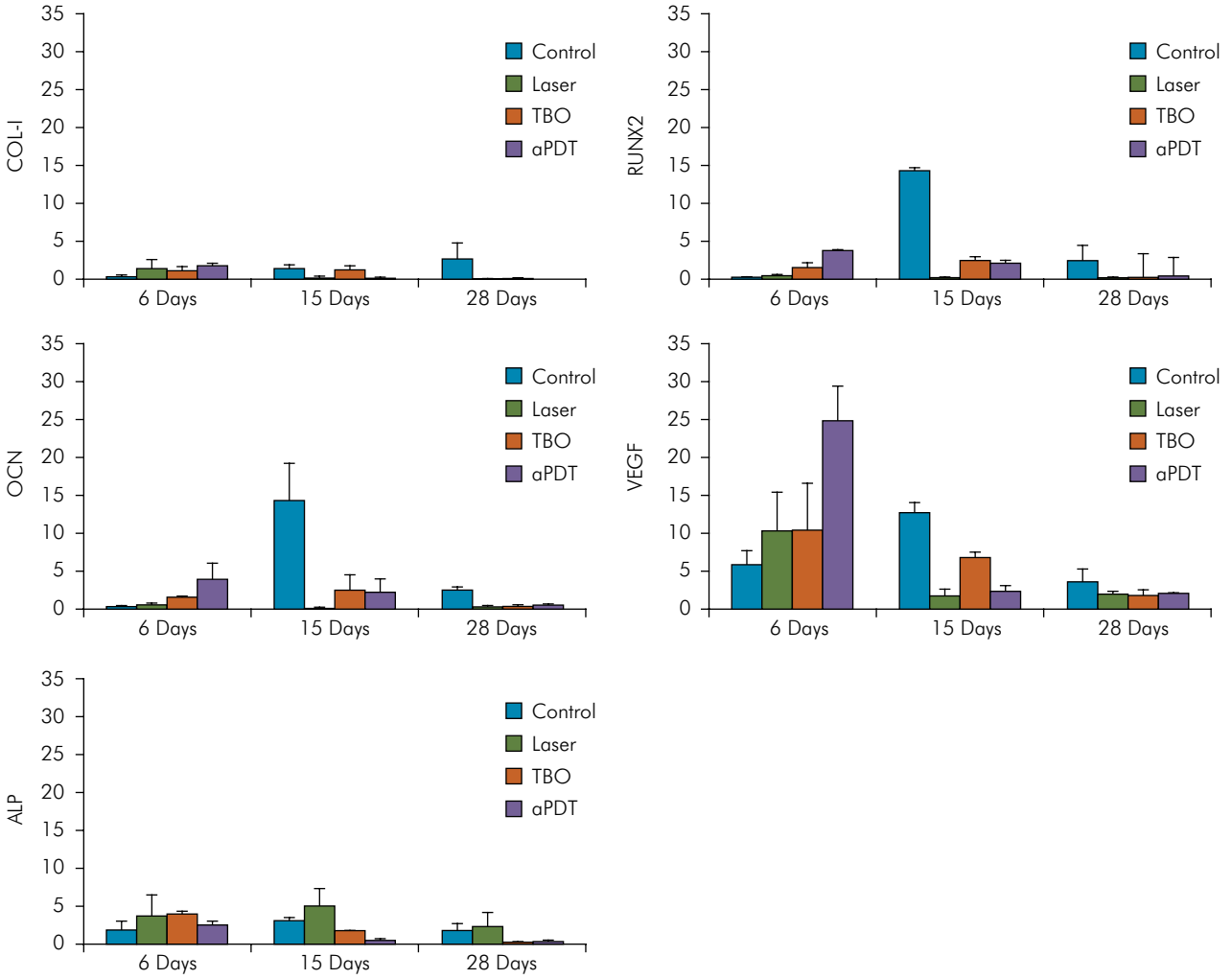


Figure 4. Molecular expression of collagen type I (COL-I), osteocalcin (OCN), alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), and vascular endothelial growth factor (VEGF) after 6, 15, and 28 days of alveolar bone healing; mean values (± Standard Deviation) for the Control, Laser, toluidine blue O (TBO), and aPDT groups.

Table 2. Correlation between the microscopic density of structures and levels of mRNA expression during bone healing.

Variable	COL-I		OCN		ALP		RUNX2		VEGF	
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Connective tissue	-0.063	0.845								
Bone tissue	0.034	0.916	0.162	0.615	−0.631	0.028*	0.465	0.127		
Blood clot									0.867	0.000*
Blood vessel									-0.664	0.018*

*Statistically significant ($p < 0.05$); r = Pearson's test; COL-1: collagen type I; OCN: osteocalcin; ALP: alkaline phosphatase; RUNX2: runt-related transcription factor 2; VEGF: vascular endothelial growth factor.

The biodistribution of TBO was evaluated for a period of 10 minutes, and the results showed no evidence of necrotic or inflammatory changes, suggesting that TBO could be used as a safe antimicrobial strategy for the control of oral infections. However, as the time taken for biodistribution of the photosensitizer and

the associated risk of infection and tissue (periodontal ligament and alveolar bone) damage is a limiting factor for the success of aPDT, this study used a longer TBO biodistribution time (10 minutes), which has been shown to be safe by a previous *in vivo* study in rats.[26] The source of light (GaAlA lasers) used in this study was selected based on the TBO absorption wavelength, which is in the visible red spectrum (centered at λ= 626 nm) making it suitable for use in aPDT associated with PBM.[28]

Thermal mapping of the laser in biological tissues is especially important for the establishment of safety parameters for clinical application.[20] However, the thermal effect in the bone tissues was not considered clinically important in the current study as the aPDT group exhibited increased hyperthermia, which was expected since the TBO present on the socket walls absorbs light resulting in a greater increase in temperature (4.2°C) when compared to the non-photosensitized socket (2.2°C). The results showed that the laser parameters used induced local hyperthermia (up to 4.2°C), but this did not alter the bone healing process as the maximum temperature reached was below 10°C.[20] These results were in accordance with Eriksson et al.[29] who found that bone tissue was sensitive to increases in temperatures up to 47°C.

Similar to previous evidence,[14,21] the present study aimed to evaluate the repair process in all extensions of the dental socket, regardless of the region, as the combination of the photosensitizing agent and photobiomodulation was expected to produce effects on all surfaces of the dental socket.

Microscopic analysis (Figures 2 and 3) showed that the aPDT group did not exhibit interference of the alveolar repair process (connective tissue, bone tissue, blood clot, blood vessels, and inflammatory infiltrate) under the tested conditions, and the same healing stage was observed in all groups examined during the same period. These results were in accordance with the control groups of previous studies that evaluated the alveolar repair process in rats using the same methodology for tooth extraction and microscopic analysis.[14,21]

The alveolar repair process is a fibroproliferative response mediated by growth factors and cytokines. Its chronology starts with vascular and cellular inflammatory events, blood clot formation, and tissue development after granulation tissue replacement (6 days). In the current study, a qualitative (Figure 2) and quantitative (Figure 3) reduction in blood clot density and an increase in well-organized connective and bone tissues with thin trabeculae were seen to occur over a period of 15 days, and the alveolar repair process was consolidated with the formation of bone tissue with thick trabeculae and defined medullar spaces after 28 days.[14,15,21]

In addition to the well-established traditional method of morphometric analysis, real-time PCR also allowed molecular exploration of the alveolar repair process after a single session of aPDT in rats.[14,21] This is a practical resource as it permits rapid validation of the tooth socket repair process when compared to the time taken for histochemical and morphometric processing necessary for microscopic analysis.[14] Moreover, it allows quantification of the expression of genes directly involved in the formation, maintenance, and/or renovation of the structures observed microscopically, thus allowing identification of a correlation between gene expressions and the presence of these structures. The results showed that the expression of COL-I, OCN, ALP, RUNX2, and VEGF in the control group was similar to that observed during alveolar repair process after incisor extraction, as reported previously.[14] Comparison of the control and experimental groups showed no statistically significant differences in the alveolar repair markers (Figure 4), suggesting that a single session of aPDT did not interfere with the expression of alveolar repair marker mRNA. As this was the only study of its kind, comparison of the molecular test results with previous evidence was not possible.

Comparison of the histomorphometric and molecular analyses outcomes showed a positive correlation between the blood clot density and VEGF expression, and a negative correlation between the blood vessel density and VEGF expression (Table 2). Although these results were expected, they have not been demonstrated previously due to decreased VEGF expression associated with substitution of the blood clot by other tissues (bone, connective, and vessels) during the repair process, as reported by Cardoso et al.[14] and Rodrigues et al.[21] The absence of a correlation

between blood vessels and VEGF expression in the study conducted by Cardoso et al.[14] could likely be attributed to the effects of infection that caused delays in the alveolar repair process. VEGF can be produced by various cells (macrophages, lymphocytes, or resident cells of the connective tissue), and its production may increase during the initial stages of inflammation[14] and under some conditions (hypoxia, wound healing, and during the repair process) associated with an increase in vascular proliferation, as observed in this study.

A negative correlation between bone tissue density and ALP expression (Table 2) was observed in the current study. ALP, a bone neo-formation marker of osteoblastic activity related to osseous mineralization in the initial phases of the repair process, is considered to be the most common indicator of bone formation and,[11,14] although the decreased expression during the normal alveolar repair process observed in the current study was expected, these results have not been reported previously in the literature. Cardoso et al.[14] found no difference in ALP bone density and VEGF expression, and this could possibly be attributed to the presence of local infection.

Therefore, the findings of this study suggest that the marker used for analyzing VEGF expression could be used as a marker for angiogenesis in the alveolar repair process and that used for analyzing ALP expression could be used as a marker in the initial stages of osseous metabolism in rats with no evidence of infection and for the specific time periods studied.

Finally, similar to findings reported by Rodrigues et al.,[21] several important events were seen to occur 15 days after tooth extraction in the current study. Microscopically, these included formation of a well-organized connective tissue, numerous thin newly formed bony trabeculae, and a small number of blood clots, whereas molecular examination showed peak gene expression of bone markers (OCN, ALP, and RUNX2) in the control group. These findings were in agreement with those reported by Cardoso et al.[14]

It is possible that the presence of infection may affect the interaction between aPDT and the tissues, which in turn could alter the molecular response. Two *in vitro* studies previously reported that preirradiation for a period of 1 min in aPDT was sufficient to have an effect on biofilms[30] and the antimicrobial rate of *Streptococcus mutans*.[31]

Future studies with single and multiple sessions of aPDT in different clinical conditions (with and without infection) and different preirradiation times are necessary in order to better understand the molecular mechanisms involved in the alveolar repair process. These studies are particularly important as aPDT has emerged as an efficient therapeutic approach for the treatment and prevention of osteonecrotic lesions formed due to tooth extraction as well as periodontic and endodontic lesions.

Conclusion

Within the limitations of the current study, it can be concluded that a single session of aPDT at the dental extraction site does not influence the alveolar repair process in rats.

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